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Reduced Regulatory T Cell Diversity in NOD Mice Is Linked to Early Events in the Thymus

Cristina Ferreira,* Donald Palmer,† Kenneth Blake,† Oliver A. Garden,‡ and Julian Dyson*

The thymic natural regulatory T cell (Treg) compartment of NOD mice is unusual in having reduced TCR diversity despite normal cellularity. In this study, we show that this phenotype is attributable to perturbations in early and late stages of thymocyte development and is controlled, at least in part, by the NOD Idd9 region on chromosome 4. Progression from double negative 1 to double negative 2 stage thymocytes in NOD mice is inefficient; however, this defect is compensated by increased proliferation of natural Tregs (nTregs) within the single positive CD4 thymocyte compartment, accounting for recovery of cellularity accompanied by loss of TCR diversity. This region also underlies the known attenuation of ERK-MAPK signaling, which may preferentially disadvantage nTreg selection. Interestingly, the same genetic region also regulates the rate of thymic involution that is accelerated in NOD mice. These findings highlight further complexity in the control of nTreg repertoire diversity.

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In this study we carried out a detailed analysis of NOD thymic development, using different approaches to investigate the mechanisms responsible for the restriction of TCR repertoire diversity within the nTreg subset. Our findings demonstrate that the deficiency in the Treg population results from an intrinsic defect within the T cell lineage and is regulated by genes on chromosome 4. The same genetic interval is also associated with an early developmental defect at the DN1 to DN2 transition and accelerated, age-related thymic involution, suggesting that the Treg phenotype...
might be a consequence of abnormalities in T cell precursors. Furthermore, we show that genes in this locus regulate the ERK1/2–MAPK pathway known to play a key role in thymocyte differentiation.

**Materials and Methods**

**Mice**

NOD, NOD Idd9, C57BL/6 (B6), (NOD × B6)F1, and B6Rag−/− mice were housed and bred under specific pathogen-free conditions. Procedures were approved by local ethical review process and performed under Home Office License.

**Cell sorting**

Thymocytes from two females aged 5–6 wk were pooled and sorted into CD4+CD8+CD25+ or CD4+CD8+CD25−CD45RA− populations. Approximately 100,000–150,000 cells were collected for each population. Sorting was performed on a FACSAria or a FACSDiva (BD Biosciences).

**Flow cytometry**

All Abs were purchased from eBioscience, BioLegend, or BD Biosciences with the exception of phospho-ERK (p-ERK Thr202/Tyr204), which was obtained from Cell Signaling Technology. DN thymocytes were identified by staining with anti-CD25– and anti-CD4–specific Abs after exclusion of CD3, CD4, CD8α, B220, CD11c, GR1, Ter119, TCRαβ, and DX5–positive cells. For p-ERK staining, total thymocytes were incubated with 5 μg/ml biotinylated anti-CD3 and anti-CD28 Abs at 37°C for 2 min. Streptavidin at a concentration of 20 μg/ml was then added and 1 min later cells were fixed and permeabilized as previously described (17) and stained with anti-CD4, anti-CD8, and anti-p-ERK Abs.

**RT-PCR and sequence analysis**

RNA was extracted using Trizol (Invitrogen, Paisley, U.K.). cDNA was synthesized with SuperScript II RNase H− reverse transcriptase (Invitrogen) and random hexamers (Amersham Biosciences, Chalfont St. Giles, U.K.). For TCRαα analysis, cDNA was amplified by PCR using the forward AV9 and reverse ACα primers. PCR products were cloned into the pCR2.1 vector using the TOPO TA cloning kit (Invitrogen). PCR was then performed on individual colonies using the same primers and sequenced using the internal α C region ACseq. To quantify diversity (D), the Shannon entropies (H) were calculated resulting in values ranging from 0 to 1, expressed in terms of percentage. Primer sequences were (5′ to 3′); AV9-ACACCGTTGTTAAGGCACC, ACα-CTTTCTACGCAAGGAGTGTCG, ACseq-CATAAGCTTCTACGCTTCCAG.

**BM chimeras**

B6Rag−/− mice were irradiated with 400 rad and injected iv with a total of 5–10 × 10^8 CD4+CD8−depleted BM cells. Animals were analyzed 6–8 wk after reconstitution. For mixed chimeras, (NODxB6)F1 recipients were irradiated with 1000 rad, cells mixed 9:1 (NOD:B6) and animals assayed 12–13 wk after reconstitution.

**Results**

**The restricted TCR repertoire of NOD nTregs is cell autonomous**

We previously reported that NOD mice select an nTreg population with a highly restricted TCR repertoire (3). In this study, we have investigated the mechanisms underlying this defect. First, to determine whether the defect was intrinsic to the T cell lineage and independent of the origin of the thymic stroma, we generated BM chimeras in which T cell–depleted BM from NOD or B6 mice was injected into irradiated B6 Rag−/− deficient recipients. Six to 8 wk after reconstitution, thymic conventional T cell (Tconv) (CD4+CD8−CD25−) and nTreg (CD4+CD8−CD25+ or CD4+CD8−CD25−) thymocytes were FACs sorted. Using our previously described approach, the TCR repertoire was analyzed by amplifying AV9 rearrangements by RT-PCR, cloning, and sequencing to determine their predicted CDR3 composition. As shown in Fig. 1 the AV9 TCR repertoires of thymus sourced nTregs and Tconvs are comparably diverse in the B6 to B6 chimeras. In contrast, in the NOD to B6 chimeras, although the thymus-sourced Tconvs display normal TCR diversity, their nTreg repertoire is severely restricted compared with the corresponding Tconv population as well as the B6 nTreg population. These results were similar to those obtained from direct analysis of NOD and B6 mice (3) and indicate that the mechanisms responsible for the NOD nTreg repertoire restriction is inherent to the T cell lineage and not dependent on the origin of the supporting thymic stroma.

**NOD thymocytes show a partial block at the DN1 to DN2 transition**

Although the process through which nTregs are selected in the thymus is still a subject of discussion, there is evidence that commitment is dependent on both early, TCR-independent events (18) and later, TCR-dependent (19) signaling. To assess and compare NOD and B6 thymocytes developing in the same thymic environment, we generated mixed BM chimeras. NOD and B6 BM cells were mixed and injected into irradiated (NOD × B6)F1 mice and analyzed after reconstitution using the Thy1.1/Thy1.2 congenic marker to distinguish the donor origin. We observed a developmental disadvantage of NOD thymocytes when in competition with B6 thymocytes, which hindered this analysis, especially at the low cellularity of the DN stages (Supplemental Fig. 1). To overcome this, we increased the representation of NOD over B6 to 9:1. Analyses of the resulting chimeric thymi showed that the relative proportions of thymocytes deriving from NOD and B6 varied greatly through early development (Fig. 2A, 2B) Thus, although as expected there was a clear overrepresentation of NOD-derived cells at the DN1 stage reflecting their input ratio, this dominance was soon lost with NOD thymocytes accounting for 77–85% of the T cell population. Furthermore, we show that genes in this locus regulate the ERK1/2–MAPK pathway known to play a key role in thymocyte differentiation. Therefore, we generated BM chimeras in which T cell–depleted BM from NOD or B6 mice was injected into irradiated B6 Rag−/− deficient recipients. Six to 8 wk after reconstitution, thymic conventional T cell (Tconv) (CD4+CD8−CD25−) and nTreg (CD4+CD8−CD25+) thymocytes were FACs sorted. Using our previously described approach, the TCR repertoire was analyzed by amplifying AV9 rearrangements by RT-PCR, cloning, and sequencing to determine their predicted CDR3 composition. As shown in Fig. 1 the AV9 TCR repertoires of thymus sourced nTregs and Tconvs are comparably diverse in the B6 to B6 chimeras. In contrast, in the NOD to B6 chimeras, although the thymus-sourced Tconvs display normal TCR diversity, their nTreg repertoire is severely restricted compared with the corresponding Tconv population as well as the B6 nTreg population. These results were similar to those obtained from direct analysis of NOD and B6 mice (3) and indicate that the mechanisms responsible for the NOD nTreg repertoire restriction is inherent to the T cell lineage and not dependent on the origin of the supporting thymic stroma.

**FIGURE 1.** Restricted TCR repertoire diversity in NOD nTregs is a cell-autonomous defect. Thymic nTregs (CD4+CD25+) and Tconvs (CD4+CD25−) were sorted from B6 Rag−/− reconstituted with BM from B6 (A, B) or NOD (C, D) origin and frequency of individual AV9 TCR sequences was determined. Each unique CDR3 sequence is represented by a segment proportional in size to its frequency. Total numbers of sequences collected are: B6 nTregs (n = 85), B6 Tconvs (n = 75), NOD Tregs (n = 82), NOD Tconvs (n = 77). Repertoire diversity (D value) is shown for each population. Color is used for clarity only and does not represent a particular sequence. The data shown are representative of two independent experiments.

Although the process through which nTregs are selected in the thymus is still a subject of discussion, there is evidence that commitment is dependent on both early, TCR-independent events (18) and later, TCR-dependent (19) signaling. To assess and compare NOD and B6 thymocytes developing in the same thymic environment, we generated mixed BM chimeras. NOD and B6 BM cells were mixed and injected into irradiated (NOD × B6)F1 mice and analyzed after reconstitution using the Thy1.1/Thy1.2 congenic marker to distinguish the donor origin. We observed a developmental disadvantage of NOD thymocytes when in competition with B6 thymocytes, which hindered this analysis, especially at the low cellularity of the DN stages (Supplemental Fig. 1). To overcome this, we increased the representation of NOD over B6 to 9:1. Analyses of the resulting chimeric thymi showed that the relative proportions of thymocytes deriving from NOD and B6 varied greatly through early development (Fig. 2A, 2B) Thus, although as expected there was a clear overrepresentation of NOD-derived cells at the DN1 stage reflecting their input ratio, this dominance was soon lost with NOD thymocytes accounting for <10% of DN2 thymocytes (Fig. 2A, 2B). To exclude the possibility of the defect being caused by an artifact of the system used, we investigated DN thymocyte development directly in untreated NOD and B6 mice. Our results revealed a partial block at the DN1 to DN2 transition in NOD mice compared with B6 mice (Fig. 2C), confirming observations obtained in the mixed BM chimeras (Fig. 2A, 2B). Interestingly, in the mixed BM chimeras, the representation of NOD progeny in subsequent thymocyte subsets shows a marked recovery, particularly within the nTreg subset where...
NOD frequencies increase to $\sim 50\%$ (Fig. 2B). We previously suggested that the reduced TCR diversity seen within the NOD nTreg population may be associated with a defect in positive selection (3). The enhanced recovery of these cells in a competitive setting appears at odds with this hypothesis. However, we speculated that increased proliferation of NOD nTregs from the DP stage might account for their normal cellularity but reduced TCR diversity. To determine whether this was the case, we compared the proportion of nTregs expressing the Ki-67 marker of proliferation in NOD- and B6-derived thymocytes of the NOD plus B6 BM to (NOD x B6) F$_1$ chimeras. As shown in Fig. 3, the proportion of proliferating NOD-derived nTregs is indeed augmented in comparison with those of B6 origin. This suggests that following clonal selection, proliferation acts as a compensatory mechanism, particularly favoring nTregs, which might contribute to limiting TCR diversity while maintaining normal Treg cellularity.

Restricted TCR diversity in NOD nTregs is associated with a locus on chromosome 4

In a parallel approach to understand the cause of the NOD nTreg defect, we analyzed the TCR repertoires of two NOD congenic strains, NOD Idd3/10/18 and NOD Idd9. In these strains, genomic segments from the type I diabetes–resistant strains B6 and C57BL/10 have been introgressed into chromosomes 3 and 4 of NOD mice, respectively. Congenic mice have been instrumental in identifying genetic loci associated with disease susceptibility, and these two strains are largely protected from disease (20, 21).

Because it is known that Tregs play an important role in disease protection, we investigated the role of these loci in TCR repertoire selection. The Tconv A V9 TCR repertoires were of comparable diversity between all mouse strains analyzed and similar to B6 as we have shown before (3). When we assessed NOD Idd3/10/18 thymic nTreg AV9 TCR repertoires, we observed a reduction in TCR diversity comparable to that seen in NOD nTregs (Fig. 4A, 4E). This suggests that the introgressed alleles in the Idd3, Idd10, and Idd18 regions of chromosome 3 are not responsible for the restriction of the NOD nTreg repertoire. Interestingly, when we did a similar study comparing NOD Idd9 with NOD thymic nTregs, the NOD Idd9 population was found to have a highly diverse TCR repertoire comparable to the diversity of the Tconv populations (Fig. 4C–F) and nTregs in B6 mice (3). These results indicate that nTreg TCR repertoire diversity is, at least in part, regulated by a gene on chromosome 4.

The Idd9 congenic chromosome 4 region regulates the DN1 to DN2 transition and the rate of thymic involution

Because our observations highlighted the existence of a partial block in the DN1 to DN2 transition of NOD mice (Fig. 2) and the possibility that such an early thymic defect impacts on later selection events, we next analyzed T cell development in NOD Idd9 mice. As described above, we generated BM chimeras, where a 9:1 mix of NOD Idd9 and B6 BM cells was transferred into irradiated (NOD x B6) F$_1$ mice. Assessment of relative frequencies in the reconstituted thymic subsets revealed that in contrast to NOD-derived cells, the proportion of NOD Idd9 thymocytes was relatively stable throughout all the developmental stages, although DN1 representation within the DN stage was still higher in NOD Idd9 than in B6 (Fig. 5A, 5B). This suggests that the DN1 to DN2 block seen in the NOD strain was partially corrected by the introduction of the congenic region. Analysis of populations of DN thymocytes in untreated animals supports this conclusion. As shown in Fig. 5C, NOD Idd9 displays a DN1 to DN2 transition phenotype intermediate between those of NOD and B6. Interestingly, we observed that this intermediate phenotype only becomes apparent in aged mice. Young mice from both
Statistical significance was calculated using the paired t test. Proliferation is assessed in FOXP3+ (Treg) and FOXP3− and summary graph (n = 3) (B) mice and frequency of individual Aβ9 TCR sequences was determined. Each unique CDR3 sequence is represented by a segment proportional in size to its frequency. Total numbers of sequences collected are: NOD Idd3/10/18 nTregs (n = 85), NOD Idd3/10/18 Tconvs (n = 88), NOD Idd9 nTregs (n = 81), NOD Idd9 Tconvs (n = 78), NOD Tconvs (n = 93), and NOD Tconvs (n = 84). Repertoire diversity (D value) is shown for each population. Color is used for clarity only and does not represent a particular sequence. The data shown are representative of n = 3 (NOD Idd3/10/18), n = 3 (NOD Idd9), and n = 2 (NOD) independent experiments.

The ERK–MAPK signaling cascade is well known to play a critical role during TCR-mediated positive selection from the DP stage (25). We have previously proposed the hypothesis that the restricted TCR repertoire seen in NOD thymic nTregs might be a consequence of signaling defects (3). Interestingly, a recent study showed that ERK phosphorylation is dampened in NOD T cells, both in thymus and spleen, highlighting a potential fault in this pathway (15). We reasoned that if attenuated ERK–MAPK activation was linked to the restricted TCR repertoire observed in the NOD and NOD Idd9 strains show increased accumulation of DN1 thymocytes relative to control B6 mice. However, from 3 mo of age onward, a divergence also becomes apparent between NOD and NOD Idd9 (Fig. 5D). Indeed from 5 mo of age, NOD DN1 frequencies are significantly higher than those found in NOD Idd9. A developmental block from the DN1 to DN2 transition stage has been reported previously in aged mice as well as in strains exhibiting rapid thymic involution (22, 23). To investigate whether the observed increase in DN1 frequencies in NOD mice was associated with an accelerated rate of thymic involution, we determined total thymocyte numbers at different ages. Analysis of the three mouse strains revealed that NOD and NOD Idd9 (n = 3) (B) are shown, *p < 0.05 between NOD and B6. Statistical significance was calculated using the paired t test.

Once again the NOD Idd9 phenotype is intermediate between the other two strains (NOD Idd9/B6 p = 0.0001, NOD Idd9/NOD p = 0.0002) with around a third of the nTreg population staining positively for the Ki-67 proliferation marker (Fig. 7A). The increase in proliferating nTregs, but not CD4+ Tconvs, is significantly higher in NOD mice compared with B6 controls. Because an increased proportion of thymic NOD nTregs is proliferating in comparison with CD4+ Tconvs, we wanted to determine whether this characteristic was associated with the NOD Idd9 locus and linked with the early thymic defects described above. Therefore, we next compared Ki-67 expression in thymus sourced nTregs from the three mouse strains. As seen in Fig. 7A, the proportion of proliferating nTregs, but not CD4+ Tconvs, is significantly higher in NOD mice compared with B6 controls. Once again the NOD Idd9 phenotype is intermediate between the other two strains (NOD Idd9/B6 p = 0.0001, NOD Idd9/NOD p = 0.0002) with around a third of the nTreg population staining positively for the Ki-67 proliferation marker (Fig. 7A). The increased proportion of proliferating nTregs in NOD and NOD Idd9 may contribute to the higher proportion of nTregs within the thymic CD4+ single-positive subset (Fig. 7B). However, the observation that NOD and NOD Idd9 have similar proportions of nTregs despite the lower proliferation rate seen in the latter strain suggests positive selection might be more efficient in this strain contributing to the increased TCR diversity seen in NOD Idd9.

The ERK–MAPK pathway is regulated by a locus in chromosome 4

Because an increased proportion of thymic NOD nTregs is proliferating in comparison with CD4+ Tconvs, we wanted to determine whether this characteristic was associated with the NOD Idd9 locus and linked with the early thymic defects described above. Therefore, we next compared Ki-67 expression in thymus sourced nTregs from the three mouse strains. As seen in Fig. 7A, the proportion of proliferating nTregs, but not CD4+ Tconvs, is significantly higher in NOD mice compared with B6 controls. Once again the NOD Idd9 phenotype is intermediate between the other two strains (NOD Idd9/B6 p = 0.0001, NOD Idd9/NOD p = 0.0002) with around a third of the nTreg population staining positively for the Ki-67 proliferation marker (Fig. 7A). The increased proportion of proliferating nTregs in NOD and NOD Idd9 may contribute to the higher proportion of nTregs within the thymic CD4+ single-positive subset (Fig. 7B). However, the observation that NOD and NOD Idd9 have similar proportions of nTregs despite the lower proliferation rate seen in the latter strain suggests positive selection might be more efficient in this strain contributing to the increased TCR diversity seen in NOD Idd9.

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the NOD strain, then ERK signaling in the NOD Idd9 strain, which shows high nTreg TCR diversity, should be increased. We measured the degree of ERK activation in CD4 T cells through intracellular staining with an Ab specific for the phosphorylated form of ERK (Thr 202/Tyr204). This method is incompatible with Foxp3 staining and negatively affects the staining intensity of surface markers (26), which is especially disadvantageous when studying low-density molecules or small populations, as is the case of CD25hi Tregs. For this reason we analyzed ERK activation in total single positive CD4 (SP4) thymocytes and peripheral CD4 T cells. In agreement with Mingueneau et al. (15), the proportion of CD4+ T cells showing ERK phosphorylation was reduced in the NOD strain compared with B6. When we compared levels of ERK phosphorylation in CD4+ T cells from NOD Idd9, these were increased compared with the NOD strain and in line with the level observed in B6 controls (Fig. 8), in both thymic and splenic cells. This finding is consistent with our hypothesis suggesting that genes encoded by the congenic region in chromosome 4 are involved in the TCR selection process through regulation of the ERK–MAPK pathway.

However, note that the association between attenuated ERK phosphorylation in Idd9 SP4/CD4 and increased nTreg diversity is a correlation. Because ERK–MAPK inhibitors have pleiotropic effects as they also operate in thymic epithelium (27), establishing a functional link would require a different approach such as the introduction of a hemizygous conditional knockout (CD4-cre) onto the Idd9 background to reduce ERK expression.

Discussion
Tregs have been extensively studied in the context of type I diabetes. However, not much is known about how the diversity of the nTreg TCR repertoire affects the progression of this disease or about the genetic factors controlling nTreg development and repertoire selection. Our analyses of NOD and the congenic strain
NOD Idd9 demonstrate that TCR diversity within the nTreg population is controlled by a gene encoded within a 38-Mbp region of chromosome 4. The Idd9 congenic region offers significant protection from disease and contains at least three subregions that independently influence type I diabetes (Idd9.1, Idd9.2, and Idd9.3), which include the candidate genes \(Lck\) and \(4-1\text{BB}\) (20). Studies using the NOD Idd9 congenic strain have shown that the Idd9 interval is involved in maintaining tolerance within the islet-specific pancreatic CD8\(^+\) T cell population (28) and that expression of Idd9 alleles in CD4\(^+\) T cells prevents the expansion of pathogenic CD8\(^+\) T cells (29). These observations and the recent study by Yamanouchi et al. (30) showing that the suppressive activity of Tregs is controlled by the Idd9.1 locus support a model by which genes in the Idd9 region control disease progression by influencing nTreg efficacy, possibly through regulating their TCR repertoire diversity. Although this study does not identify a specific gene responsible for the reduced complexity of the NOD nTreg TCR repertoire, we identify several features of early T cell development in NOD mice that may contribute to this phenotype.

The current prevailing view regarding nTreg development proposes that the nTreg population contains a moderately self-reactive TCR repertoire, which is selected through TCR/MHC–peptide interactions of higher affinity than the conventional CD4\(^+\) T cell population (31). Based on this model, the intensity of the TCR signal is a crucial factor in determining T cell fate and the TCR repertoire within the different thymic T cell subsets. In this study we observed that the decreased ERK phosphorylation reported previously in NOD mice (15) was corrected in the NOD Idd9 strain. The ERK–MAPK signaling cascade is induced by TCR engagement and is thought to play an important role in T cell development, especially during the positive selection stage (32). Taking into account that nTregs require higher affinity TCR/MHC interactions for their selection, which is likely to be translated into increased signal intensity, it is likely that a defect in the ERK–MAPK pathway would have a particularly strong impact on TCR selection within this population. The requirement for higher affinity TCRs as compensation for weaker intracellular signaling could lead to a more stringent selection and result in fewer TCR specificities entering the nTreg repertoire. Alternatively, CD4\(^+\) Tcrons are selected through lower affinity TCR/MHC interaction, and thus dampening of the ERK signal may have little or no effect on the TCR repertoire diversity of this subset. Consistent with this view, Maine et al. (33) have shown that a deficiency in \(PTPN22\), a gene encoding an inhibitor of TCR signal transduction, specifically impacts nTreg selection, altering the CD4/Treg ratio in the thymus. Our results suggest that the existence of a functional link between ERK signal and nTreg TCR diversity is a possibility that merits further investigation.

**FIGURE 7.** Decreased proliferation but similar frequency of NOD Idd9 nTregs. (A) Percentage of proliferating cells within thymic nTreg (CD4\(^+\)CD8\(^−\)FOXP3\(^+\)) and Tconv (CD4\(^+\)CD8\(^−\)FOXP3\(^−\)) subsets assessed by staining with the Ki-67 marker. (B) Percentage of FOXP3\(^+\) cells within CD4\(^+\)CD8\(^−\) thymocytes. Experimental numbers are \(n = 12\) (NOD), \(n = 11\) (NOD Idd9), and \(n = 4\) (B6). **\(p < 0.001\).

**FIGURE 8.** Idd9 locus regulates level of ERK phosphorylation downstream of TCR signaling. Thymocytes were activated through TCR/CD28 stimulation and level of ERK phosphorylation was accessed after 1 min in CD4\(^+\)CD8\(^−\) cells. Gray histograms show unstimulated cells. Data shown are representative of two independent experiments.
Our analysis highlighted a second NOD thymic defect of impaired DN1 to DN2 transition, which is associated with an accelerated rate of thymic involution. Interestingly, both phenotypes are features of aged, normal mice and are both partially corrected in the NOD Idd9 congenic, suggesting a functional link. Although this early thymic defect and the defect in nTreg repertoire diversity may be controlled by different genes integrated within independent pathways, the observation that both are associated with the same genetic region suggests the possibility of functionally linked phenotypes. Indeed, it is not uncommon, and is more genetically resourceful, for the same molecule to be involved in multiple pathways. The impaired DN1 to DN2 transition in NOD mice may alter the composition of the DN2 compartment, which has been shown to be heterogeneous with regard to potential for differentiation into the nTreg lineage (18). Potentially related to this observation, subsequent development of NOD thymocytes after the DN stage is advantaged over B6 and NOD Idd9 particularly into the nTreg lineage. This effect appears to be, at least in part, due to increased proliferation of NOD thymocytes. We show this phenotype is cell intrinsic, occurring in mixed BM chimeras in which NOD and B6 thymocytes develop in F1 thymi. This observation suggests that differences in extrinsic factors such as thymic Treg niche size (34, 35) do not play a major role in regulating the proportion of proliferating Tregs. In B6, thymic dwell time of Tregs is approximately 1 d longer than for the Tconv SP4s (36), although both subsets undergo around one cycle of division before exit (37). The dwell time of NOD nTregs is not known but is a potential factor contributing to the increased nTreg proportion. The increased size of the NOD nTreg compartment (Figs. 2A, 7) was also recently described by Tellier et al. (38) who showed it was controlled by the Idd16 locus on chromosome 17, further highlighting the complexity of nTreg selection.

Lck and the TNFR superfamily member 4-1BB have previously been suggested as Idd9 candidate genes (20). The role of Lck in disease development is controversial. Although this molecule is crucial for T cell development and function, and NOD polymorphisms have been reported (39), human studies have concluded that a major role for Lck polymorphisms in type I diabetes is unlikely (40, 41). Additionally, Lck is a signal-transducing element associated with TCR activity and thus not likely to be implicated in the very early, TCR-independent stages of thymic development. Considering the defective NOD nTreg TCR repertoire as an independent phenotype could be consistent with involvement of Lck. Indeed, Lck is a key component of TCR signal transduction in contrast with ERK, which is not required for T cell activation (42). However, because proliferation in CD3/CD28-stimulated NOD T cells is not decreased (7), we consider that alterations in this gene are unlikely to underlie any of the defects reported in the present study.

Alternatively, there are indications that 4-1BB plays a role in thymocyte differentiation. Kim et al. (43) have reported that 4-1BB and its ligand 4-1BBL are preferentially expressed among DP thymocytes and suggested that 4-1BBL signaling via 4-1BB is involved in the process of positive selection. They proposed a costimulatory function for these molecules, which, in conjunction with the signal received through the TCR, induces maturation of DP thymocytes. Sequencing of the 4-1BB gene identified three coding differences between NOD and NOD Idd9 (20). These changes do not influence the surface expression level of 4-1BB, but they affect T cell activation, with NOD T cells showing reduced proliferation in response to simultaneous stimulation through TCR and 4-1BB (44). This indicates that a weaker signal is transduced by NOD 4-1BB and, if indeed this molecule plays a role in positive selection, it might result in generation of a skewed TCR repertoire. Interestingly, it was recently reported that the Idd9 locus mediates the age-dependent accumulation of a subset of 4-1BB+ Tregs, which show increased functional suppression (45).

A function for 4-1BB at earlier thymic differentiation stages has not been reported; however, some studies have shown that 4-1BB can regulate TNF-α expression (46, 47) and vice versa (48), suggesting a possible link between the two. Overexpression of TNF-α under control of its natural promoter has been reported to block early thymocyte development at the DN1 to DN2 stage, promoting accelerated thymic atrophy (49) and mirroring our own observations in the NOD thymus. This effect, similar to the phenotypes we describe, is intrinsic to the hematopoietic cell compartment. Altogether, this makes 4-1BB a promising candidate for further investigation in relation to the thymic phenotypes highlighted in this study.

The Idd9 interval also includes other genes of interest such as p110δ and mTOR, known to be involved in Treg differentiation and function (50–52), as well as several zinc finger proteins containing Krüppel-associated box domains, which can be involved in cell differentiation and development. Therefore, careful dissection of the Idd9 region is essential to pinpoint the genes of interest. Analysis of Idd9 subcongenic mouse strains will allow exclusion of unrelated genes and focus on the more promising candidates.

Considering the potential role of the thymic NOD phenotypes identified in this study as influenced by the Idd9 congenic region (accelerated involution, retarded DN to DN2 transition, and subsequent advantaged thymocyte development and increased Treg proliferation), we propose that the size and composition of the NOD nTreg compartment can be rationalized as follows. Attenuated ERK–MAPK activation imposes a higher stringency for permissible TCRs to enter the nTreg lineage, resulting in restricted TCR repertoire diversity. Subsequently, enhanced proliferation leads to accumulation of a larger proportion of nTregs within the NOD SP4 compartment in comparison with B6.

The thymic defects reported in this study are of interest, not only in the context of type I diabetes, but also to the wider understanding of T cell development and of the mechanisms regulating thymic atrophy. In this study we uncover a possible link between early T cell development abnormalities and selection of a Treg population bearing a defective TCR repertoire, and we demonstrate that both defects can be linked to a genetic interval in chromosome 4.

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Disclosures
The authors have no financial conflicts of interest.

References
NOD Treg DIVERSITY LINKED TO EARLY THYMIC DEFECT


