Lineage Divergence at the First TCR-Dependent Checkpoint: Preferential \( \gamma \delta \) and Impaired \( \alpha \beta \) T Cell Development in Nonobese Diabetic Mice

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Lineage Divergence at the First TCR-Dependent Checkpoint: Preferential γδ and Impaired αβ T Cell Development in Nonobese Diabetic Mice

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The first TCR-dependent checkpoint in the thymus determines αβ versus γδ T lineage fate and sets the stage for later T cell differentiation decisions. We previously showed that early T cells in NOD mice that are unable to rearrange a TCR exhibit a defect in checkpoint enforcement at this stage. To determine if T cell progenitors from wild-type NOD mice also exhibit cell-autonomous defects in development, we investigated their differentiation in the Notch-ligand–presenting OP9-DL1 coculture system, as well as by analysis of T cell development in vivo. Cultured CD4 and CD8 double-negative cells from NOD mice exhibited major defects in the generation of CD4 and CD8 double-positive αβ T cells, whereas γδ T cell development from bipotent precursors was enhanced. Limiting dilution and single-cell experiments show that the divergent effects on αβ and γδ T cell development did not spring from biased lineage choice but from increased proliferation of γδ T cells and impaired accumulation of αβ T lineage double-positive cells. In vivo, NOD early T cell subsets in the thymus also show characteristics indicative of defective β-selection, and peripheral αβ T cells are poorly established in mixed bone marrow chimeras, contrasting with strong γδ T as well as B cell repopulation. Thus, NOD T cell precursors reveal divergent, lineage-specific differentiation abnormalities in vitro and in vivo from the first TCR-dependent developmental choice point, which may have consequences for subsequent lineage decisions and effector functions. The Journal of Immunology, 2011, 186: 000–000.
istics of the cell’s TCR and associated signaling apparatus. Many T cell identity genes, including those needed for TCR signaling, are turned on in the DN2–DN3 stages for first use at β- or γδ-selection (1). For this reason, any fundamental genetically determined defect in T cells affecting signaling, survival, or proliferation could alter fate choices at this stage while also having an effect on, without being complicated by, subsequent lineage choices, positive and negative selection, and peripheral activation events.

Autoimmune type 1 diabetes results from abnormal responses to self-antigens by peripheral T cells, due to both a failure of T cell self-tolerance mechanisms and breakdown of active T cell suppression of these autoreactive cells (reviewed in Ref. 11). A number of studies have reported that different T lineages from autoimmune diabetes-prone NOD mice have defective numbers and/or responses to various stimuli relative to cells from other nonautoimmune mouse strains as possible factors contributing to disease. The T cell lineages implicated in type 1 diabetes include CD4, CD8, NKT, and regulatory αβ T cells, as well as γδ T intraepithelial lymphocytes (11–16). However, by the time mature T cells initiate and propagate an autoimmune response to peripheral tissues, they have already undergone a sequence of prior developmental choices and signaling encounters with APCs that may have contributed cumulatively to their abnormalities. Therefore, to shed light on the possible origins of inappropriate responses of T cells in NOD mice, we have turned to the earliest stage when the TCR response machinery is first used to dictate cell fate. This is during intrathymic development, at the checkpoint where commitment to the αβ and γδ T cell lineages is established. Although T cell populations in the thymi of NOD mice appear to be generally similar to those of other mouse strains in steady state (17, 18), the early DN populations have not been investigated in great detail. Several studies have reported abnormalities in NOD thymic development. We previously reported that thymocytes from NOD Prkdcscid and NOD-Rag1−/− mice appear to break through the β-selection checkpoint, resulting in the aberrant development of DP cells (19). NOD thymic also have been shown to accumulate newly differentiated DP cells more slowly than conventional strains after dexamethasone-induced depletion of the DP compartment (20). Furthermore, a number of studies show defects in NOD DP cell responses to various stimuli, including responsiveness to Con A (21), apoptosis induced by glucocorticoids and gamma-irradiation (20) or anti-CD3ε (22), and responses to thymic selection signals (23–26). NOD mice also have defects in development of NKT cells from DP (17, 27). All of these traits point to potential alterations in TCR signaling in NOD thymic T cells, which may have consequences for mature T cell activity (12). The nature of early T cell responses is set by the cellular signaling components, which in turn have effects on the thresholds for distinct responses including positive and negative selection of αβ TCR+ DP cells (reviewed in Refs. 7, 28).

The earliest stages of T cell development are difficult to assess in the presence of ongoing TCR rearrangements because they represent only a small fraction of total thymocytes and because of the tremendous proliferation that occurs during differentiation from DN stages to DP, which can mask underlying developmental defects. In addition, steady-state proportions of thymic populations are affected by lineage decisions, cell death, and emigration (7). We have therefore used several approaches to compare the earliest stages of T cell development and lineage decisions in NOD to nonautoimmune C57BL/6 (B6) mice. To investigate the intrinsic developmental potential of NOD T cell precursors, we took advantage of the Notch-ligand–expressing OP9-DL1 coculture system, which has been used extensively to elucidate the roles of Notch and other factors on the T cell developmental process in mice and humans (29–32). We also carried out detailed flow cytometric analyses of early T cell developmental stages as well as tracking of population dynamics by BrdU labeling in vivo and in mixed bone marrow chimeras. Our findings demonstrate that NOD early T cells exhibit impaired β-selection, whereas γδ-selection is strongly enhanced.

Materials and Methods

Mice

NOD/ShiLtJ, C57BL/6J, BALB/cJ, NOD.Rag1−/−, and B6.Rag1−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and then bred and maintained in microisolator cages in our specific pathogen-free mouse colony in the Caltech Animal Resources facility (Pasadena, CA). The (NOD × B6)F1.Rag1−/− mice used for bone marrow chimeras were bred in our facility from the parent strains. Mice were used for these studies at 4–7 wk of age. Euthanasia and animal care followed National Institutes of Health guidelines under protocols approved by the institutional animal care and use committee.

Ab staining, cell sorting, and flow cytometric analysis

To isolate DN populations, mice were sacrificed, their thymus were removed, and single-cell suspensions were made. Mature cells were depleted as previously described (33) by staining with biotinylated Abs to CD8a, TCRγδ, TCRβ, Gr1, Ter119, CD122, NK1.1, D5x, and CD11c, after which the cells were incubated with streptavidin-coated magnetic beads and then passed through a magnetic column (Miltenyi Biotec, Auburn, CA). Eluted DN cells were either stained for direct flow cytometric analysis or for the sorting of specific DN populations into OP9 cultures. ETP (CD25−CD44hi−ε−γδ−TCR−), DN2 (CD25−CD44hi−ε−γδ−TCR+), DN3a (CD25−CD44hi−ε−γδ−TCR+CD27hi−FSCm), and DN3b (CD25−CD44hi−ε−γδ−TCR+CD27hi−FSCm) precursor cells, as well as γδ T cells, were sorted from DN cells using a FACSAria with Diva software (Becton Dickinson Immunocytometry Systems, Mountain View, CA). For analysis of cytokines, cells were forcefully pipetted, filtered through nylon mesh to remove stromal cell clumps, and stained for CD45 to identify input cells plus various combinations of lineage identifying Abs. Cells were analyzed using a FACScalibur (Becton Dickinson Immunocytometry Systems) or MACSQuant (Miltenyi) and FlowJo software (Tree Star, Ashland, OR). Abs were purchased from eBioscience (San Diego, CA). For intracellular staining, DN thymocytes were stained with surface markers, fixed and permeabilized using Cytofix/CytoPerm Kit (BD), and stained with conjugated Abs to TCRβ or TCRγδ.

Cell culture

OP9-DL1 cocultures were carried out as previously described (33, 34). For T cell development, thymic DN cells were placed on monolayers of OP9-DL1, supplemented with 2.5 ng/ml IL-7 and 5 ng/ml Flt3L. After 7 d, cultures were transferred to new plates with fresh OP9-DL1 cells and the medium replaced, supplemented with 1 ng/ml IL-7. Human forms of the cytokines were used and obtained from Peprotech (Rocky Hill, NJ). Tissue culture media and FCS were obtained from Invitrogen/Life Technologies (Carlsbad, CA). For bone marrow cultures, cells were column-depleted of mature cells using biotinylated Abs to CD3ε, CD4, CD8, CD19, Gr-1, Ter119, CD11b, NK1.1 as described earlier. Cells (107) were plated on OP9-DL1 monolayers in 10-cm tissue culture flasks (Corning) with the addition of 10 ng/ml IL-7 and 5 ng/ml Flt3L. After 6 d, the bone marrow-derived cells, which are predominately DN1 and DN2 cells, were removed from the monolayers and replated on OP9-DL4 cells with 2 ng/ml IL-7. For proliferation assays, DN cell subsets were FACS sorted and then stained for 8 min at 37°C with 5 μM CFSE (Cell Trace CFSE Proliferation Kit, Invitrogen), in accordance with the manufacturer’s instructions, before they were placed in culture.

In vivo assays

For BrdU pulse-labeling of thymocytes, B6 and NOD mice were injected i.p. with 1 mg BrdU in 0.2 ml PBS twice 4 h apart. Mice were sacrificed after 1, 2, and 3 d, and their thymocytes were stained with surface Abs followed by intracellular staining for BrdU–FITC in accordance with BrdU Flow Kit instructions (BD Biosciences). For mixed bone marrow chimeras, female 8- to 16-wk-old (NOD × B6)F1.Rag1−/− mice were irradiated with 400 rad (±13Cs source) or retroperitoneally injected with 5 × 106 bone marrow cells (BMcs) from each of NOD and B6 mice, mixed 1:1, and assayed between 5 and 9 wk after cell injection. To reduce the risk of infection, mice were housed using autoclaved cages, bedding, and food and...
were treated with an antibiotic, Baytril (Bayer), in drinking water for several days before and 2 wk after irradiation.

RNA extraction and real-time quantitative PCR analysis

RNA extraction and real-time quantitative PCR analysis
RNA was isolated from sorted TCRγδ+ cells from B6 and NOD mouse thymi and real-time quantitative PCR carried out as previously described (19). Briefly, cells were lysed in Qiagen (Qiagen, Valencia, CA), RNA was isolated using RNAeasy extraction kits (Qiagen), and cDNA reverse transcribed using random hexamers and SuperscriptIII (Invitrogen) following the manufacturer’s instructions. cDNA samples were diluted and mixed with gene-specific primers and SYBR GreenER (Invitrogen) and run on an ABI Prism 7900HT Sequence Detector (ABI, Mountain View, CA). Primers used for this study were synthesized by Eurofins MWG Operon (Huntsville, AL) and have been published previously (19, 35, 36) except for those for Sox13: forward, 5'-GAGAACTCTGGCTCAGTGAC-3'; reverse, 5'-GACCAAAAGCTGGATTCCCT-3'. Vγ1.1 and 3γ4 primer sequences were obtained from Verykakis et al. (37). Results were calculated using the ΔΔCt method, normalizing all samples to Actb expression.

Results

NOD DN cells are impaired in the generation and maintenance of αβ T lineage DP cells, but not γδ T cells, in OP9-DL1 cocultures

Because steady-state thymic T cell populations reflect a combination of intrinsic and extrinsic factors, proliferation, cell death, and emigration, which can mask developmental differences, we wished to assess the intrinsic developmental potential of T cell precursors from B6, NOD, and BALB/c mice under the same environmental conditions using the OP9-DL1 coculture system (30, 34). Immature DN thymocytes were purified by depletion of mature cells and placed in OP9-DL1 coculture with IL-7 and Flt3L. When analyzed on day 12 of culture, B6 and BALB/c DN cells were found to generate and maintain high percentages of DP cells as expected, whereas NOD DN cells produced a much lower proportion of DP cells under the same conditions (Fig. 1A, top panels). In the same cultures, NOD DN cells produced a higher proportion of γδ T cells relative to B6 and BALB/c DN cells (Fig. 1A, bottom panels).

To assess the kinetics of stage-specific αβ versus γδ lineage development of NOD and B6 DN cells, we tracked the development of purified intrathymic DN precursor subsets, including DN1/ETP (c-Ki67−CD25−CD44hi), DN2 (c-Ki67−CD25−CD44hi), and DN3a (c-Ki67−CD25−CD44hi) populations that retain both γδ and αβ T cell potential, as well as DN3b cells (c-Ki67−CD25−CD44hi) cells, which, as previously shown for B6 mice, have already completed TCRβ rearrangement and pre-TCR signaling (34). Although NOD thymocytes also upregulate CD27 at β-selection, they do so to a lesser extent than B6 cells (see later), so NOD and B6 DN3b cells were enriched by sorting based on combined expression of relatively high levels of CD27 and large size (high forward scatter [FSC]), whereas DN3a cells were sorted based upon lower CD27 and lower FSC (38).

When precursor DN subsets from NOD and B6 thymi were placed in OP9-DL1 cocultures, neither B6 nor NOD DN1/ETP and DN2 cells generated DP cells by day 7 (Fig. 1B). However, by day 14, B6 DN1/ETP and DN2 cells cultured cells contained abundant cells that had differentiated to the DP stage, whereas NOD DN1 and DN2 cells produced few if any DP cells. This is not due simply to a delay in differentiation, as NOD DP cell production did not increase with time (data not shown).

In contrast, DN3 cultures from both B6 and NOD thymi began to express CD4 and/or CD8 by day 7 of OP9-DL1 coculture (Fig. 1B), a time point when few DN1 or DN2 cells from either strain had turned on CD4 or CD8. Confirming the αβ T cell lineage choice of the accumulated DP cells, all cultures that contained abundant DP cells, including B6 and NOD DN3a and DN3b at day 7, as well as all B6 DN subsets at day 14, clearly expressed surface TCRβ, whereas those NOD and B6 cultures with few DP cells express very little TCRβ (Fig. 1C). At day 7, the percentage of DP from NOD DN3a cells was moderately lower in comparison with that of B6 DN3a cells, and NOD DN3b cells appeared to produce fewer cells overall and a lower proportion of DP cells. By day 14, the differences were even more pronounced, with a very low proportion of DP cells in NOD DN3a cultures and very few surviving cells in the DN3b cultures. This latter result was confirmed in further experiments when the ability of individual post-β-selection DN3b cells to generate DP cells was tested. Single DN3b cells were sorted from NOD and B6 thymi and placed in OP9-DL1 coculture for 7 d. Although all individual NOD and B6 DN3b cells proliferated and differentiated, fewer DP cells were generated in the NOD cultures (35.2 ± 8.9, mean ± SEM) compared with that in B6 cultures (179.5 ± 53.5) (p < 0.005, Mann–Whitney U test). These results suggest a possible loss of differentiation potential, proliferation, and/or viability in the generation of DP cells from DN cells sorted both before and after β-selection in the NOD thymus.

To identify the stages at which the initial differentiation of the NOD DN cells might be blocked, the same cultures were stained with CD44 and CD25 at day 7 (Fig. 1D). Most of the cells from cultures seeded with DN3a and DN3b cells from either B6 or NOD mice had progressed to DN4, or later stages, as shown by the downregulation of CD25 and CD44 by day 7, in agreement with their predominately DP phenotype. Although both B6 and the NOD DN1 and DN2 cells differentiated successfully to the DN3 stage, the NOD DN1 and DN2 cell cultures produced a much lower proportion of DN4 and later cells (Fig. 1D), suggestive of a partial block at the β-selection checkpoint from NOD DN1 and DN2 cells.

In marked contrast to their poor production of DP cells, the DN1, DN2, and DN3a cultures from NOD thymocytes generated γδ T cells very successfully (Fig. 1E). All of the NOD populations produced a higher proportion of γδ T progeny than that of the corresponding B6 precursors, with DN2 cells producing the greatest differences: 2.8% from B6 and 21% from NOD DN2 cultures on day 14. As expected, very few γδ T cells were generated from sorted DN3b cells from either strain, in agreement with our previously published results for B6 mice (34). Thus, even though NOD DN1 and DN2 cells are extremely poor at passing through the β-selection checkpoint to generate DP αβ T lineage cells in vitro, they are normal or enhanced in their ability to produce γδ T lineage cells.

These results show that NOD precursors exhibit a lineage-specific intrinsic defect in development when placed in OP9-DL1 cultures. DN3 cells from NOD mice, even those that had undergone β-selection in the thymus, generate fewer DP cells than do B6 DN3 cells, and the earliest DN1 and DN2 T cell precursors sorted from NOD thymi are profoundly defective in their ability to go through β-selection in vitro. However, these cells retain the ability to produce abundant TCRγδ+ cells.

Prior thymic contact is not required for skewing of αβ versus γδ T cell fate from NOD cells

Although the highly skewed production of αβ versus γδ lineage cells from NOD DN cells appears intrinsic to these thymic-derived cells, two concerns could be raised. First, these cells might have already encountered biasing interactions in the thymus from which we isolated them. Second, the OP9-DL1 cultures present the Delta-like 1 (DL1) Notch ligand, whereas the Notch ligand expressed in the normal thymus is Delta-like 4 (DL4), which pro-
vides a somewhat different Notch signal to these cells (39–41). Therefore, we also tested the T lineage development of pre-thymically derived BMC progenitors in vitro, providing DL4 as the source of Notch ligands rather than DL1. As shown in Fig. 2A, cells derived from precursor-enriched B6 and NOD BMCs generate both DP and γδ T cells on OP9-DL4 after 19–22 d of culture or were maintained at 2 ng/ml throughout the culture period (data not shown). B–E, Development of FACS sorted DN1, DN2, DN3a, and DN3b (post β-selection) cells from B6 and NOD thymi cultured in 2.5 ng/ml IL-7 and 5 ng/ml Flt3L. B, Flow cytometric plots showing CD4 and CD8 DP cell development at days 7 and 14 from each progenitor population. C, Histograms showing levels of surface TCRβ expression in the same cultures for B6 and NOD cell populations at days 7 and 14 of culture. No data are shown for NOD DN3b cells on day 14 because very few cells are remaining. D, Flow cytometric plots of CD44 versus CD25 showing differences in initial DN differentiation in the same cultures at day 7. E, γδ T cell development in the same cultures at day 14. Percentages of cells in each gate are indicated. These results are representative of at least three independent experiments.

NOD DN2 cells generate a higher absolute number of γδ T cells and fewer DP cells than those generated by B6 DN2 cells in vitro

Skewed production of γδ T cells relative to DP cells could result from lineage-specific alterations in proliferation or survival as well as lineage choice itself. To distinguish between these possibilities, we focused on the DN2 cells, which particularly differed between NOD and B6 in terms of their abilities to generate DP versus γδ T cells in OP9-DL1 cocultures. First, we assessed the total cell numbers of DP and γδ T cells generated from limited numbers of precursor cells in these cultures. Ten DN2 cells from B6 and NOD thymi were sorted directly into each of 24 individual
OP9-DL1 cultures and analyzed by flow cytometry on day 13. The percentage of γδ T cells out of the total number of differentiated cells (calculated as 100 × number of γδ T/[number of γδ T + number of DP]) for each well is shown in Fig. 3A (left panel), and a highly significant difference was found between cultured B6 and NOD DN2 cells. Although DN2 cells from NOD and B6 thymi yielded comparable total numbers of CD45+ cells per well, NOD cells produced significantly higher absolute numbers of γδ T cells, as well as significantly fewer DP cells (Fig. 3A, right panels). Thus, both poor DP cell differentiation and enhanced γδ T cell development contribute to the NOD phenotype.

To determine whether the differences in αβ versus γδ T differentiation are the result of alterations in lineage choice, single-cell differentiation analyses were performed. Because commitment to the γδ T cell lineage may occur as early as the DN2 stage (42), we sought to determine if DN2 cells from either mouse strain start with a biased frequency of cells precommitted to either lineage. For this experiment, single cells from two subsets of the DN2 CD44+CD25+c-Kit population were tested separately based on their percentage of γδ T cell lineages. γδ T cell production and reduced DP cell generation after γδ T lineage commitment of the precursors but rather the result of enhanced γδ T cell production and reduced DP cell generation after the lineage choice is made.

FIGURE 2. NOD bone marrow progenitor cells also show attenuated in vitro development of DP αβ T cells and enhanced γδ T cell development. BMCs enriched for progenitors were isolated from NOD and B6 mice and cultured on OP9-DL1 and DL4 cells as described in Materials and Methods.

Flow cytometry plots show the generation of CD4/CD8 DP cells and γδ T cells from B6 and NOD bone marrow cultures analyzed after 19 and 22 d (A) and two independent cultures established from mixing equal numbers of NOD and B6 BMCs, analyzed after 20 d (B). Results are representative of at least three replicate wells from one of two independent experiments with similar results.

NOD γδ T cells proliferate more rapidly than do B6 γδ T cells in vitro

The normal preponderance of αβ-lineage DP cells over γδ T cells in the thymus depends not only on the frequency of different selection events but also on the greater extent of net proliferation that is normally associated with the αβ-lineage program after β-selection. To address the question of whether some of the differences in αβT versus γδ T cell development are attributable to a NOD defect in proliferation, unsorted thymocytes (“All”) as well as sorted DN1/ETP, DN2, DN3a, and DN3b populations were stained with CFSE and placed in OP9-DL1 culture. NOD DN subsets showed similar or slightly faster proliferation rates than B6 cells over the first 4 d of culture (Fig. 4A), ruling out a generalized proliferation defect. Most prominently, the γδ TCR-positive cells generated in the first 4 d from NOD DN3a cultures underwent more rapid and extensive proliferation than that of γδ TCR-positive cells from B6 cell cultures, whereas the γδ TCR-negative cells from NOD and B6 cells proliferated more similarly (Fig. 4B). Thus, the high numbers of γδ T cells generated from NOD DN cells result, at least in part, from their greater proliferation after surface TCRγδ expression in comparison with B6 γδ T cells. Furthermore, the lower numbers of DP cells generated from NOD DN3 cells is not due to an initial defect in proliferation after β-selection.
NOD and B6 thymic γδ T cells exhibit similar patterns of gene expression

We recently reported the expression of many key T cell genes in DN populations and γδ T cells from NOD and B6 mice and found no major differences in expression of Ptcra, Notch1, Hes1, Tcf7, Runx1, and Bcl11b among others (33). However, recent evidence suggests that γδ T cells may be composed of multiple lineages, distinguished by their different dependence on specific transcription factors: Sox13, Id3, or Zbtb16/Plzf (37, 43–45). To determine whether NOD thymocytes preferentially generate all classes of γδ T cells or just one particular subtype, cells were analyzed for expression of these transcription factor genes as well as for diagnostic TCR-Vγ rearrangements. We sampled Vγ rearrangements representing members of the TCR-Cγ1 cluster, which includes adult, fetal, and tissue-specific receptors (reviewed in Ref. 8). We also assayed for Vγ1.1-Jγ4 rearrangements, which mark a recently characterized population of innate γδ T cells with Vγ1.1-Vδ6.3 TCRγδ receptors. These cells rapidly produce IL-4 and IFN-γ, upregulate Zbtb16/PLZF, a critical transcription factor in NKT differentiation (45), and are Id3 independent (37). NOD and B6 γδ T cells expressed fairly similar levels of transcripts from Vγ3, Vγ5, Vγ4, and Vγ2 to Cγ1 rearrangements, as well as Vγ1.1-Jγ4 rearrangements (Fig. 5). No differences were observed in expression of Id3 or of the CD3e, CD247 (CD3ζ), and Sox13
FIGURE 5. Expression of TCR-Vγ rearrangements and other genes in sorted thymic NOD and B6 γδ T cells. Quantitative PCR results from TCRγδ+ cells sorted from NOD and B6 thymi using primers to detect subsets of specific TCR-Vγ rearrangements and other T cell genes. Data are shown as the geometric mean ± SD, n = 2–5. For additional gene expression comparisons, see Ref. 33.

genes. However, Zbtb16/Plzf expression was somewhat higher in NOD γδ T cells in comparison with B6 cells. Given the similarity in TCR Vγ usage, this could reflect a generally elevated state of activation (45). Thus, there appear to be few if any major differences in the types of γδ T cells produced in NOD and B6 mouse thymi, suggesting that the differences we observe in development and rates of proliferation might be generalized to multiple γδ T cell subsets.

NOD thymocytes exhibit multiple abnormalities around the β-selection checkpoint

A number of costimulatory and other cell surface receptors, with known effects on TCR signals, survival, and/or proliferation, are induced at β- and γδ-selection in response to TCR signaling via the newly produced pre-TCR and γδ TCR, similar to events after the activation of peripheral T cells. The combinations and levels of these molecules can not only indicate the nature and intensity of the TCR signals but also influence further response thresholds through their modulating and enhancing roles. The degree of CD5 upregulation, for example, has been a useful indicator of the intensity of signals driving γδ-selection versus β-selection (10), and once induced, CD5 can affect further development (10) and signal thresholds for positive and negative selection (46, 47).

We have previously shown that upregulation of the costimulatory receptor, CD27, involved in mature T cell survival and proliferation, can be used to distinguish DN3 cells that have rearranged and transcribed an intracellular (ic) TCRβ (DN3b) from those that are still TCR-negative in B6 mice (34). However, NOD DN3 cells express lower amounts of CD27 after β-selection (icTCRβ+) (Fig. 6A, top row, left panel). The costimulatory receptor CD28, which is not expressed until after β- (or γδ-) selection (48), and CD5 are also induced to a lower level in TCRβ+ NOD DN cells than that in B6 cells (Fig. 6A, top row, second and third panels). These observed differences are not due to variations in the total amount of TCRβ expressed nor to cell size, as the icTCRβ staining intensity and FSC histograms are similar between the two strains (Fig. 6A, top row, fourth and fifth panels).

In contrast to the results for αβ T cell precursors, icTCRγδ+ cells from B6 and NOD thymi express similar levels of surface CD27 and CD28 (Fig. 6A, bottom row, first and second panels). Furthermore, NOD γδ T cells express a broader range of surface CD5 levels, both higher and lower, and a higher total amount of TCRγδ than that expressed by B6 γδ T cells, despite comparable cell sizes as determined by FSC profile (Fig. 6A, bottom row, fourth and fifth panels). The higher levels of CD27 and CD5 attained by thymic γδ T cells from both NOD and B6 mice reflect the higher intensity of signals generated at γδ-selection compared with those generated at β-selection (9, 10). Likewise, the lower induction of CD27, CD28, and CD5 in icTCRβ+ cells from NOD mice compared with that in icTCRβ+ cells from B6 mice suggests that NOD cells may experience a weaker or otherwise altered β-selection signal, whereas γδ T cell signals are more similar between the two strains.

Depletion of subsets after β-selection in the normal NOD thymus

B6 and NOD thymi exhibit few obvious differences in their proportions of major populations in steady state (17, 18). However, because the OP9-DL1 coculture system revealed major intrinsic defects in NOD differentiation at the earliest TCR-dependent checkpoint, we performed a series of detailed flow cytometric analyses of freshly purified NOD and B6 thymocytes to evaluate more closely if DN cells within the NOD thymus exhibit any comparable abnormalities in vivo. We found that whereas all DN populations are present, as measured by CD44 and CD25 profiles, the percentages and shapes of the differentiation plots differ (Fig. 6B, left panels). Most prominently, the levels of surface CD25 are much higher in CD25+ cells from NOD than in B6 cells, as seen in the density plots, as well as in histograms of all CD44+ cells (including DN3 to DN4 cells) (Fig. 6B, right panel). Furthermore, NOD thymi consistently have a lower proportion of DN4 cells relative to DN3 cells (Fig. 6B, left panels, lower left versus lower right quadrants). Elevated CD25 in DN3 cells and lower proportions of DN4 cells can be indicative of a partial block in β-selection as reported for thymi from mice with mutations in the pre-TCR or Notch signaling pathways (49–52).

The generally accepted route of differentiation for B6 mouse αβ T cells, after β-selection and the downregulation of CD25 leading to the DN4 stage, is the upregulation of CD8 into immature single-positive (ISP) cells prior to the expression of surface CD4 in the DP stage (7). A previous study reported low numbers of CD8+ ISP cells in NOD thymi (17). To confirm this finding and to determine the route of differentiation from DN4 to DP in NOD thymi, CD8 ISP cells were distinguished from mature CD8 single-positive (SP) cells by their retention of high levels of surface CD24 (heat shock Ag; HSA), which then decline during differentiation. B6 thymocytes follow this pattern, expressing a high percentage of total gated CD8+ cells that are HSAhi and CD2int (ISP) cells compared with that of the mature HSA intermediate and CD2int SP CD8+ cells (Fig. 6C). However, NOD thymocytes have a much
lower percentage of CD8 ISP cells that is only partially offset by a corresponding HSA hi CD2int CD4+ ISP population. Furthermore, none of the populations from NOD thymi have a major subset of cells with the same level of HSA that is expressed by the B6 ISP cells, as indicated by the red boxes (Fig. 6C, right panels), suggesting a paucity of cells transiting through this stage of development.

The interpretation that there might be a reduced influx into the ISP compartment from previous stages was further supported by the kinetics of proliferation and turnover of DN and DP subsets in B6 and NOD thymi in vivo, as determined by the fate of a cohort of BrdU pulse-labeled cells. A previous study reported an anomalous dip in BrdU+ DP cells in NOD mice after 2–3 d (18). We wished to confirm and extend this finding in the context of a β-selection defect by additional DN cell analysis. Mice were injected i.p. with BrdU, sacrificed after 1, 2, or 3 d, and the thymocytes were stained for surface receptors and intracellular BrdU. One day after BrdU injection, the proportions of labeled cells within the DN2/DN3 (CD25hi), DN4 (CD25lo CD44lo), and DP cell populations 1–3 d after in vivo BrdU pulse-labeling, showing a major difference in accumulation of BrdU+ DP cells on day 2 between B6 and NOD mice. Mean ± SD values over time are plotted for B6 and NOD thymocytes.

FIGURE 6. NOD thymocytes show evidence of a partial block at β-selection and abnormalities in differentiation to DP cells in vivo. A, Histograms showing levels of activation-induced surface receptors CD27, CD28, and CD5 on freshly isolated DN thymocytes from NOD (black lines) and B6 (solid gray) mice, using intracellular (ic) staining for TCRβ (top row) or TCRγδ (bottom row) to distinguish cells that have passed through β-selection or γδ-selection, respectively. Data are representative of three to four independent experiments. B, Flow cytometric analysis showing CD44 versus CD25 expression in DN subsets from freshly isolated NOD and B6 thymi (left panels). Percentages of cells in each quadrant are as indicated. The histogram compares CD25 surface expression levels between gated NOD and B6 CD44hi DN cells (DN3 and DN4 cells) from two mice of each strain (right panel). Data are representative of >10 mice of each strain. C, Flow cytometric analyses showing CD4 versus CD8 plots for total thymocytes from NOD and B6 mice (left panels), CD8 and CD4 gated cells, stained to distinguish immature (ISP) cells from mature SP cells (middle panels), and histograms showing HSA levels for DN, DP, CD8+, and CD4+ cells (right panels), which distinguish less mature cells (HSA hi) from more mature cells (HSA lo). The red boxes indicate the intermediate HSA level for B6 CD8 ISP cells (right top panel, arrow) with no corresponding major population in the NOD thymus (right bottom panel). Data are representative of three independent experiments. D, Percentages of BrdU+ cells among DN2/3 (CD25hi), DN4 (CD25lo CD44lo), and DP cell populations 1–3 d after in vivo BrdU pulse-labeling, showing a major difference in accumulation of BrdU+ DP cells on day 2 between B6 and NOD mice.
as expected due to turnover and differentiation from nonlabeled DN3 precursors, confirming the pulse nature of the labeling. The B6 DP compartment showed an almost 2-fold increase in the proportion of BrdU" cells over the proportion at day 1, again as expected due to continuing differentiation of the BrdU"-labeled DN4 cells from the preceding days. However, the NOD DP compartment did not show a corresponding increase in the percentage of BrdU" cells at day 2, but rather showed a decline. The failure of BrdU-labeled NOD cells to become DP was not due to their retention in the DN4 compartment. The rapid loss of BrdU" cells from all phenotypic compartments suggests that failure to accumulate ISP cells in NOD thymi is not due to poor proliferation or rapid modulation of surface CD4, CD8, and HSA, but instead due to a survival failure. Taken together, our results support the notion that NOD early T cells in vitro and in vivo have a partial block to a survival failure. Taken together, our results support the notion that NOD early T cells in vitro and in vivo have a partial block to a survival failure. Taken together, our results support the notion that NOD early T cells in vitro and in vivo have a partial block to a survival failure.

**Lineage-specific differences in the establishment of αβ T versus γδ T cell and B cell populations in mixed bone marrow chimeras**

To determine whether preferential development of γδ T cells, as well as reduced production of αβ T cells, from NOD precursors occur in vivo as well as in vitro, we used mixed bone marrow chimeras to compare the kinetics of T cell differentiation and export between NOD and B6 cells in the same animal. An equal number of NOD and B6 BMCs were injected into irradiated (NOD × B6) F1.Rag1−/− mice, which were assayed for reconstitution at 5–9 wk of age. Five weeks postinjection, both NOD (CD45.1+) and B6 (CD45.2+) cells were found in the thymus (Fig. 7A, left panels) with relatively normal steady-state proportions of DN, DP, SP, and γδ T populations established at this time. However, a lower proportion of CD45.1" NOD cells was found in the spleen compared with that of CD45.2" B6 cells (Fig. 7A, right panels). The NOD cells were predominately CD19" B cells with a T/B cell ratio of 2.2:85 compared with the B6 T/B ratio of 35:56 (Fig. 7A, right top panels). This T cell repopulation defect was primarily due to disproportionately fewer TCRαβ" T cells. In contrast, relatively normal percentages and total yields of NOD TCRγδ cells were found. Thus, in vivo as well as in vitro, there is an NOD defect in production of αβ T cells, and this is lineage specific.

A summary of results from all six mixed bone marrow chimeric mice analyzed at 5–6 and 8–9 wk of age is shown in Fig. 7B and 7C. In 5 of 6 thymi and 6 of 6 spleens from chimeric mice, NOD cells exhibited a lower ratio of TCRαβ"/TCRγδ" cells compared with that of B6 cells in the same mouse. In agreement with the steady-state phenotype of NOD mice, this effect was kinetic, not absolute. At the later time point, by 8–9 wk postinjection (Fig. 7B, 4 and 5), the relative proportions of TCRαβ"/TCRγδ" cells in the thymus and spleen became more similar between NOD and B6 than those observed at 5–6 wk (Fig. 7B, 1–3), indicating that NOD αβ T cells eventually accumulate. The defect in NOD αβ T production was again shown to be lineage specific, as relatively high numbers of B cells were produced in these animals (Fig. 7C). Thus, NOD BMCs were much less efficient than B6 BMCs at producing mature αβ T cells in chimeric hosts, even though they were capable of rapidly generating γδ T and B cells. These results

**FIGURE 7.** NOD cells exhibit differences in establishment of γδ T versus αβ T cells in mixed bone marrow chimeras. BMCs isolated from NOD and B6 mice and 5 × 10⁶ cells from each strain were mixed and injected into irradiated (NOD × B6) F1.Rag1−/− recipients. Mice were sacrificed after 5–6 or 8–9 wk and analyzed by flow cytometry for lymphocyte populations in thymus and spleen using CD45.1 (NOD) and CD45.2 (B6) to distinguish donor strains. A, Flow cytometric analysis of a representative chimeric mouse (No. 1) analyzed at 5 wk postinjection. Plots on the left show thymocytes were stained for CD4 and CD8 (upper panels) and CD3ε and TCRγδ (lower panels). Splenocytes, shown on the right, were stained for αβ TCR and CD19, to distinguish B cells (upper panels), and CD3ε and TCRγδ (lower panels). B and C, Summary of FACS staining results from six mixed bone marrow chimeric mice, three analyzed after 5–6 wk (No. 1–3) and three after 8–9 wk (No. 4–6). B, Graphs showing the ratio of TCRαβ"/TCRγδ" cells in thymus (left) and spleen (right) generated from B6 (black bars) and NOD (white bars) donors. C, Graph showing the relative percentages of B, αβ T, and γδ T lymphocytes derived from B6 and NOD donors in spleens from the same individual chimeric mice.
show that the NOD genotype confers divergent effects on αβ and γδ lineage differentiation in vivo as well as in vitro.

**Discussion**

The first TCR-dependent checkpoint is the first of many stages in which the TCR signaling apparatus of the cell is used for survival, lineage choice, and differentiation functions. The earliest T lineage decision, which is made at this point, is the fundamental choice between αβ T and γδ T cell fates, which is determined by differences in TCR signal strength and results in initiation of very different developmental programs. Furthermore, recent evidence suggests that different γδ T cell lineages may be selected at this point depending on the characteristics of the rearranged TCR and transduced signal at this stage (reviewed in Ref. 43).

The OP9-DL1 coculture system provides the Notch ligands and other factors required by early T cells for passage through the first TCR-dependent checkpoint and differentiation into γδT or αβ T cells, and it is a powerful tool allowing detailed study of the T cell developmental program and lineage choice in both mice and humans (29–32, 53, 54). Using this in vitro culture system, we show that NOD early DN cells are very poor at generating DP αβ T cells, but the same cells under the same conditions exhibit enhanced γδ T cell differentiation and proliferation. This was true of purified DN thymocytes as well as precursor enriched BMCs and in the presence of DL4, the normal thymic Notch ligand, rather than DL1. Because the DN thymocytes are the progenitors of all T cell lineages, defects expressed at these earliest stages may also be present in all subsequent stages and may have a major impact on later thymic selection events, lineage choices, and peripheral developmental choices and responsiveness.

Although the OP9-DL1 cell culture system may exacerbate the relatively poor DP differentiation by NOD early T cell progenitors, we have several additional lines of evidence that indicate that β-selection may also be defective in the NOD thymus. First, the heightened CD25 expression in NOD DN3 cells is similar to pre-TCR and Notch mutations that give a partial block at heightened CD25 expression in NOD DN3 cells (42, 57), before cells reach the conventional β-selection checkpoint. That does not appear to be the case here, as single DN2 cells sorted from NOD thymi were predominately bipotent just as in B6 thymi, despite producing relatively higher numbers of γδ T cells compared with that of DP cells. Thus, the skewing of NOD differentiation toward the γδ T lineage is more likely to be due to survival and proliferation than to lineage choice. The major differences in αβ T versus γδ T cell development in vitro may result from alterations in intrinsic signaling pathways in NOD early T cells with differential effects on the two lineages. For example, stronger intrinsic IL-7 signals, lower Notch signals, weaker β-catenin/TCF-1 activity, and stronger TCR signals have all been shown to favor the differentiation, survival, and/or proliferation of γδ T cells over αβ T cells (58–62). Although lineage choice may be normal in NOD DN cells, their ability to survive, proliferate, and differentiate in the chosen lineage may differ between β- and γδ-selected T cells. Normally, in B6 and most strains examined, the β-selection program dictates much greater proliferative expansion than that of γδ-selection. Our results imply that this difference is greatly reduced or reversed in NOD mice.

In addition to their preferential development from NOD DN cells in OP9-DL1 culture, we found that γδ T cells generated in vivo in NOD thymi proliferate more, express a wider range of surface CD5, and upregulate all assayed activation-induced receptors very well. This suggests that NOD γδ T cells may be produced as a result of unusually robust or variable signaling responses in the thymus. Thus, it is possible to interpret the preferential development of γδ T cells from NOD precursors as an outcome from a stronger or altered TCR signal, such that γδ T cell differentiation, proliferation, and survival is actively promoted (9, 10, 63, 64) whereas DP differentiation and survival are disfavored (54). Alternatively, the TCR signal may not be stronger in NOD cells, but they may interpret any viability-promoting signal as a γδ T cell selection signal, thereby leaving many cells that undergo the β-selection program starved of viability support. Although further work is needed to resolve which intrinsic mechanisms are affecting αβ versus γδ T cell differentiation in NOD DN cells, there is evidence that immature and mature NOD T cells may transduce abnormal TCR signals during activation (reviewed in Ref. 12). In addition, NOD.Prdc Δcdt1 Δnotch3 and NOD.Rag2−/− thymocytes fail to arrest at the β-selection checkpoint, undergoing spontaneous differentiation to DP cells without restoration of thymic cellularity (19), suggesting that NOD DN cells, even in the absence of a rearranged TCR, may undergo a spontaneous, but only partial, TCR signal. A number of factors can cause this type of breakthrough phenotype including enhanced TCR and Notch signals (6, 65). Notably, we previously reported data showing a higher level of Notch3 and Dtx1 expression, but not Notch1 or Hex1, in freshly isolated NOD DN3a cells in comparison with that of B6 DN3a cells (33), although the significance of this difference is not yet clear.

Recent studies have demonstrated that events at β-selection or even earlier can have a major effect on the characteristics and further lineage choices of αβ TCR+ DP cells (66–68). Loss of a subset of differentiating cells after β-selection might result in a differentially selected DP population that, for example, transduces a stronger than average TCR signal or is more resistant to certain types of apoptosis. If aberrant NOD β-selection events alter the signaling characteristics of the final DP population, this could have major ramifications for negative and positive selection of conventional CD4 and CD8 T cells. The same could be true for the selection of γδ T cells as well as “unconventional” αβ T cells with self-reactive TCRs, such as NKT, CD8αα, and regulatory T cells (69). In addition, although little is known about possible roles of γδ T cells in autoimmune, γδ T intraepithelial lym-
phocytes may play an important role in peripheral tolerance and have been found to be able to control diabetes progression in NOD mice (14). γδ T cells reactive to insulin peptide B:9-23 have been reported to be generated in NOD but not B6 mice (70), which provides further evidence of their potential role in autoimmune diabetes. The diversity of γδ T lineages, their peripheral immune responses, and their roles in disease have been a subject of great interest very recently, especially in light of the ability of particular subsets to rapidly secrete IL-17, IFN-γ, and IL-4 (reviewed in Refs. 43, 71).

The results of this study show that NOD lineage T cells have fundamental and intrinsic abnormalities from the earliest stages in their responses to activating signals, either via TCR or other environmental triggers, even in the absence of MHC/peptide/APC interactions. These defects may affect lineage choices and TCR repertoires via alterations in DP selection thresholds or alter peripheral immune responses to specific stimuli, or both. Finally, whether or not these traits found in NOD early T development directly contribute to autoimmunity, these studies demonstrate that different mouse strains can show major variations in β- and γδ-selection developmental programming, which may deviate significantly from the currently accepted pathways.

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