CD45 Regulates Migration, Proliferation, and Progression of Double Negative 1 Thymocytes

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CD45 is a protein tyrosine phosphatase that is expressed on all nucleated hematopoietic cells, from stem cells to memory cells. Although its function in regulating the threshold of Ag receptor signaling is well established, its role in other leukocytes, particularly progenitor cells, is not well defined. In this study, we find CD45 affects early thymocyte development. Examination of the CD4<sup>−</sup>CD8<sup>−</sup> double negative (DN) populations revealed a significant reduction in the DN1 population, in both the numbers of CD117<sup>+</sup> DN1 cells (the early thymocyte progenitors) and the CD117<sup>−</sup> DN1 cells in the thymus of CD45<sup>−/−</sup> mice. There was also a reduced frequency of CCR9<sup>+</sup> Lin<sup>−</sup>Sca-1<sup>−</sup>c-Kit<sup>+</sup> cells and common lymphoid progenitors in the CD45<sup>−/−</sup> bone marrow. Competitive bone marrow reconstitution showed a reduced contribution of DN1 cells from CD45<sup>−/−</sup> cells, consistent with an intrinsic defect in these cells. CD45<sup>−/−</sup> DN1 cells exhibited reduced proliferation in vivo and reduced CXCL12-mediated migration in vitro. The loss of CD45 led to the accumulation of an intermediate DN1.5 thymocyte population in vivo that was dependent on Notch for progression. In vivo, CD117<sup>−</sup> DN1 cells gave rise to γδ T cells. In vitro, CD117<sup>−</sup> DN1 cells progressed to DN4 on OP9-DL1 cells but CD117<sup>−</sup> DN1 cells lacking CD45 did not. CD45<sup>−/−</sup> CD117<sup>−</sup> DN1 cells were also deficient in TCRβ expression. Thus, CD45 deficiency affects the development and progression of DN1 thymocytes. The Journal of Immunology, 2010, 185: 2059–2070.

Development of T cells in adult mice starts with hematopoietic progenitor cells from the bone marrow traveling through the blood to seed the thymus. These self-renewing and multipotent progenitors in the bone marrow are characterized by the lack of expression of lineage markers (Lin<sup>−</sup>) and expression of Sca-1 and c-Kit (CD117) and are thus termed LSK cells (reviewed in Ref. 1). More restricted common lymphoid progenitors (CLP) that are Lin<sup>−</sup>Sca-1<sup>−</sup>c-Kit<sup>+</sup> are also present in the bone marrow (2). However, the identity of the blood progenitor cell that seeds the thymus from the blood and the mechanism of progenitor cell mobilization is not known, although it has been shown that chemokines, such as CCL25, are involved in controlling thymic homing (2, 3).

In the thymus, the early thymic progenitor cells are found within the CD4<sup>−</sup>CD8<sup>−</sup> double negative (DN) population. Four differentiation stages of DN thymocytes (DN1–4) have been defined based on CD25 and CD44 expression. The earliest thymocyte progenitors (ETP) reside in the DN1 (CD44<sup>+</sup>CD25<sup>−</sup>) population at the corticomedullary junction. Their differentiation into DN2 (CD44<sup>+</sup>CD25<sup>−</sup>) and DN3 (CD44<sup>+</sup>CD25<sup>+</sup>) cells is accompanied by their outward migration to the cortex (4) in response to specific chemokines, such as CXCL12 (reviewed in Ref. 5). Differentiation into DN4 (CD44<sup>+</sup>CD25<sup>+</sup>) and subsequently to CD4 and CD8 double positive (DP) cells is accompanied by the inward migration of the cells from the cortex to the medulla, where selection and maturation into single positive (SP) CD4 or CD8 T cells occurs. Positively selected thymocytes that escape negative selection mature into naïve T cells and exit the thymus into the peripheral circulation (reviewed in Ref. 6).

The DN1 population is multipotent and has the potential to differentiate into B cells, NK cells, T cells, macrophages, and dendritic cells. Notch signals drive these progenitors toward the T cell lineage and limit their differentiation toward other lineages, including the NK cell lineage (7). Notch signals also promote the commitment toward the αβ T cell lineage and away from the γδ T cell lineage (reviewed in Ref. 8). During early thymocyte development, there is interplay between Notch and IL-7 signals that together regulate proliferation, survival, and progression (9). Specifically, Notch and IL-7 induce the proliferation of DN1 cells (10) and their progression to DN2 (11).

The DN1 population is a heterogeneous population that can be divided into two major subsets depending on the expression of CD117, distinguishing a major CD117<sup>+</sup> population and a minor CD117<sup>−</sup> population capable of repopulating the thymus, referred to as ETP (12). These two populations can be further divided into five subsets (DN1a–e) based on CD24 expression (13). The CD117<sup>+</sup> DN1a–b subsets have high-proliferative capacity and show typical progression from DN1 sequentially to DN4 when cocultured in vitro with OP9-DL1 stromal cells and develop into T cells in vivo. The CD117<sup>−</sup> DN1c subset has a lower pro-

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Received for publication August 17, 2009. Accepted for publication June 7, 2010.

This work was supported by Grant 77712 from the Canadian Institutes of Health Research.

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Abbreviations used in this paper: 7AAD, 7-aminoactinomycin-D; CLP, common lymphoid progenitor; DLL1, Delta-like–1; DN, double negative; DP, double positive; EGFP, enhanced GFP; ETP, early thymocyte progenitor; Lin, lineage; LSK, Lin<sup>−</sup>Sca-1<sup>−</sup>c-Kit<sup>+</sup>; FPA, paraformaldehyde; SP, single positive; UBC, University of British Columbia.

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The Journal of Immunology
liferative capacity but also progresses sequentially from DN1 to DN4. The CD117 DN1d–e subsets progress through a noncanonical pathway from DN1 to DN4 and have been shown to develop into SP T cells in vitro (13).

The differentiation of thymocytes is accompanied by the migration of cells to specific niches within the thymus to receive the appropriate signals for development (reviewed in Ref. 5). This migration is driven by various chemokines produced by thymic stromal cells. CXCL12 is predominantly expressed in the cortex of the thymus where it is important for the recruitment of thymic progenitors into the cortex (14). This is important for thymocyte development as thymus-specific deletion of the CXCL12 receptor, CXCR4, leads to a block in development and the accumulation of DN1 thymocytes at the corticomedullary junction (15). In contrast, CCL19 and CCL21 chemokines, which bind to CCR7, are predominantly expressed in the medulla yet deletion of CCR7 leads to the accumulation of a DN1, CD44+CD25lo population at the corticomedullary junction (15). These data indicate a close relationship between localization and development of cells in the thymus.

CD45 is a transmembrane protein tyrosine phosphatase that is specifically expressed on all leukocytes and hematopoietic progenitor cells. The best established function for CD45 is its ability to regulate the threshold of Ag receptor signaling by dephosphorylating Src family kinases (reviewed in Ref. 16). The major substrates of CD45 in thymocytes and T cells are Lck and to a lesser extent, Fyn. In CD45-deficient mice, there is a partial block in DN3 progression and almost a complete block in the development of DP to SP cells, attributed to an increased threshold of pre-TCR and TCR signaling, respectively. This leads to a major reduction in mature SP T cells in the periphery of CD45-deficient mice (17, 18). CD45 has been implicated as a positive regulator of chemokine-induced migration in Jurkat T cells (19) and a negative regulator of IL-3 mediated mast cell proliferation (20). Because migration and proliferation are two key processes in thymocyte development, this suggested that CD45 may affect early T cell development. In this study, we find that CD45 is a positive regulator of proliferation and migration of early thymocytes and is required to maintain normal ETP numbers in the thymus. We identify intermediate DN1.5 stages in the progression of both CD117+ and CD117−DN1 populations that require Notch signals for progression. CD45 is not required for the progression of CD117+DN1 cells to DN2 and DN3, but is required for the noncanonical progression of the CD117+DN1 population to DN4 and for the expression of TCRβ in this population.

Materials and Methods

Mice

C57BL/6 (CD45+/−), exon 9 CD45 null (17), and IL-7Ra−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The CD45 null mice were backcrossed onto the C57BL/6 background three more times to generate a ninth generation backcross (CD45−/−). Mice that ubiquitously express an enhanced GFP (EGFP) transgene under the control of a β-actin promoter (21) on the C57BL/6 background were kindly provided by F. Rossi (University of British Columbia [UBC]). The CD45−/−EGFP+ mice were then crossed with CD45−/− mice to generate the EGFP-expressing CD45 null mice. Mice heterozygous for EGFP expression (EGFP+/−) were used in experiments. Mice were housed and maintained by homozygous matings in the Wesbrook Animal Unit at the UBC. Animal experimentation was conducted in accordance with protocols approved by the University Animal Care Committee and Canadian Council of Animal Care guidelines. All mice were sex and age matched and used between 6–12 wk of age.

Abs and reagents

Anti-CD3e (145-2C11), CD4 (GK1.5), CD8α (53-6.7), CD11b (M1/70), CD19 (MB19-1), CD24 (M1/69), CD25 (PC61.5), CD117 (ACK2), CD127 (73R34), γc TCR (UC7-13D5), Gr-1 (RB6-8C5), NK1.1 (PK136), Sca-1 (D7), TCRβ (H57-597), and Ter-119 mAb conjugated to FITC, PE, PE-Cy5, PE-Cy7, allophycocyanin, Alexa Fluor 647, allophycocyanin-Cy7, or Pacific Blue were from BD Pharmingen (Mississauga, Ontario, Canada), eBioscience (San Diego, CA) or the Biomedical Research Centre Ab Facility (Vancouver, British Columbia, Canada). Annexin V-FTTC was from SouthernBiotech (Birmingham, AL). Bcl-2 and isotype control-FTTC were from BD Pharmingen. Anti-CD45 (E3/2) and CD44 (IM7) mAbs were conjugated to Alexa Fluor 488, Alexa Fluor 647 or Pacific Blue (Invitrogen, San Diego, CA) as per manufacturer’s instructions. Biotinylated anti-CD3e, CD4, and CD8α mAbs (American Type Culture Collection, Manassas, VA) were purchased from EZ-Link Sulfo-NHS-Biotin Reagents (Pierce, Rockford, IL). Anti-biotin MicroBeads were from Miltenyi Biotec (Auburn, CA). Polyclonal anti-CCXR4 (G-19) Ab was from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 488-conjugated donkey-anti-goat IgG was from Invitrogen. CD11b, CD19, γc TCR, Gr-1, and NK1.1 were used to deplete Lin+ thymocytes. CD19 was used as the B cell marker as the conventional B220 mAb cell marker, B220, is an isoform of CD45 and is not expressed in CD45−/− mice. CD3e, CD4, CD8α, CD11b, CD19, γc TCR, Gr-1, NK1.1, and Ter-119 mAbs were used to deplete Lin+ cells from the bone marrow. Recombinant mouse CXCL12, IL-7, and Flt3L were from PeproTech (Rocky Hill, NJ).

Cell isolation and enrichment

Thymi were passed through a strainer in FACS buffer (PBS, 4% v/v FCS, 2 mM EDTA) or MACS buffer (PBS, 0.5% w/v BSA, 2 mM EDTA) to generate a single-cell suspension. To enrich for DN thymocytes, cells were labeled with biotinylated anti-CD4 and CD8α mAbs, washed once with FACS buffer and then labeled with anti-biotin MicroBeads before passing through LS columns or the AutoMACS (Miltenyi Biotec) according to manufacturer’s instructions. Total bone marrow cells were prepared by flushing the tibiae and femurs with wash buffer (HBSS, 5% v/v FCS, 2 mM EDTA). Bone marrow cells were treated with RBC lysis buffer (0.83% w/v NH4Cl, 10 mM Tris-HCl pH 7.25) for 10 min, washed twice with wash buffer.

Flow cytometry and fluorescence activated cell sorting

Cells (1 × 106–3 × 107) were incubated with 50–300 μM BrdU diluted in FACS buffer on ice for 20 min. Bone marrow cells were first blocked with 2.4G2 tissue culture supernatant for 15 min prior to incubation with Ab for flow cytometry. In some cases, cells were stained with 1 μg/ml 7-aminoactinomycintin-D (7AAD) for 5 min to exclude nonviable cells. Cells were washed twice before being resuspended in FACS buffer or fixed with 1% w/v paraformaldehyde (PFA) in PBS and analyzed on an LSRII flow cytometer using FACS Diva software (BD Biosciences). Data were analyzed on FlowJo (TreeStar, Ashland, OR).

For intracellular staining of Bc2 expression, the DN-enriched thymocytes were first labeled for surface Ags, then fixed and permeabilized with 1% w/v PFA/0.1% v/v Tween-20/PBS for 1 h at room temperature, prior to labeling with anti-Bc2 or isotype control-FTTC for 30 min. For annexin staining, the DN-enriched thymocytes were washed twice with annexin V binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM CaCl2, 0.1% BSA) prior to labeling with annexin V-FTTC according to the manufacturer’s protocol.

DN-enriched thymocytes from a pool of ≈3 mice were labeled with thymocyte lineage markers, CD4, CD8, CD25, CD44, CD117, and in some experiments TCRβ prior to sorting on an FACSaria (BD Biosciences) by the UBC FACS Facility. Cell populations were typically ≥99% pure by flow cytometry.

Coculture of progenitor and OP9 stromal cells

Coculture assays were essentially performed as described (22). Sorted cells (1–5 × 105) were seeded into 12-well tissue culture plates containing a near-confluent monolayer of OP9 or OP9-DL1 stromal cells in the presence of 1 mg/ml IL-7 and 5 mg/ml Flt3L, or in the presence and absence of 1 mg/ml IL-7. Cells were transferred to new wells containing a fresh monolayer of stromal cells on days 4 and 8. Cocultures were harvested at the indicated time points and stained with CD4, CD25, CD117, and 7AAD for analysis by flow cytometry. Stromal cells were excluded by analysis of GFP+ cells and live cells (7AAD−) were counted on day 6 or as indicated.

In vivo proliferation assay

BrdU (1 mg in 100 μl PBS) per day for 2 d was administered to mice by i.p. injection and in their drinking water (1 mg/ml BrdU). 48 h later, mice were sacrificed and their thymi were harvested. DN-enriched thymocytes were stained with surface markers prior to fixation and permeabilizing with 1% w/v PFA/0.1% v/v Tween-20/PBS at 1 h at room temperature. Cells were then washed twice with FACS buffer before being incubated at 37°C for 30 min with 350 μl 50 Kunitz/ml DNase (Roche, Laval, Quebec, Canada).
Canada). Cells were then stained with 25 μL anti-Brdu– or isotype control-FITC (BD Pharimingen) for 30 min, washed twice with FACS buffer before being analyzed by flow cytometry.

Migration assays

Migration assays were performed using a Transwell system (Costar, Corning, NY) with a 6.4 mm diameter insert and 5 μm pore size polycarbonate membrane. DN-enriched thymocytes (1 × 10^6) pooled from ≥3 mice were added to the top chamber with 100 μl chemotaxis medium (RPMI 1640 containing 0.1% w/v BSA). Chemotaxis medium (600 μl) containing 0 or 50 ng/ml CXCL12 was added to the bottom chamber. Each condition was done in triplicate. Cells were allowed to migrate for 3 h at 37˚C, and then labeled with lineage markers, CD4, CD8, CD44, CD25, CD117, and 7AAD for flow cytometric analysis. Percentage migration was calculated by dividing the number of cells migrated by the total number of cells added.

In vivo development of noncanonical thymic progenitor populations

Purified donor cells were prepared from CD45+/+ EGFP+/− mice by cell sorting as described previously. Between 0.3–1 × 10^6 of sorted thymic subsets (Lin−CD44+CD25+CD117+TCRε+ or Lin−CD44+CD25−CD117−TCRε+) along with 1 × 10^6 IL-7R+/− splenocytes as carrier cells were resuspended in 100 μl HBSS and i.v. injected into nonirradiated IL-7R−/− recipients. Three weeks after adoptive transfer, thymus, spleen, and lymph nodes from recipient mice were harvested and analyzed for the expression of lineage markers in EGFP+ donor cells by flow cytometry.

Competitive bone marrow reconstitution

Total RBC-lysed bone marrow cells (1 × 10^7) from CD45+/+ EGFP+/− and CD45−/− EGFP+/− mice were resuspended in 200 μl PBS and i.v. injected into nonirradiated IL-7R−/− recipients. Three weeks after bone marrow reconstitution, the thymus of recipient mice was harvested and analyzed by flow cytometry.

Statistical analysis

Unless indicated otherwise, results are expressed as the mean ± SEM of at least three independent experiments and analyzed for statistical significance using an unpaired two-tailed Student t test. The p values ≤0.05 were considered statistically significant and indicated as follows: *p ≤ 0.05; **p ≤ 0.01; and ***p ≤ 0.001.

Results

Altered DN1 populations in the CD45-deficient thymus

To evaluate the role of CD45 in early thymic development, the numbers and populations of thymocytes were compared between C57BL/6 and the exon 9 CD45-deficient mice (17) backcrossed nine times onto the C57BL/6 background (hereafter referred to as CD45+/+ and CD45−/− mice, respectively). As previously reported (17), there was a significant 2-fold decrease in the total number of thymocytes, a significant reduction in the percentage and number of SP CD4 or CD8 thymocytes and a 2-fold increase in the percentage of CD4−/CD8− DN cells from CD45−/− mice compared with CD45+/+ mice (Table I). However, the DN population is known to be heterogeneous, containing non-T lineage cells, mature γδ T cells, and NKT cells in addition to the developing γδ T cell progenitors. These non-γδ T lineage cells were depleted using a mixture of Abs against the lineage markers NK1.1, γδ TCR, CD19, Gr-1, and CD11b. Comparison of Lin− DN cells revealed no statistically significant difference between the numbers of CD45+/+ and CD45−/− DN thymocytes (4.6 ± 0.6 × 10^6 and 3.1 ± 0.5 × 10^6 cells, respectively, n = 7). However, this was equivalent to a 1.5-fold increase in the percentage of Lin− DN cells in the CD45−/− thymus compared with the CD45+/+ thymus (3.5 ± 0.3% and 2.4 ± 0.2%, respectively), as there is half the number of total thymocytes in the CD45−/− mice. Within the Lin− DN population, we observed reduced numbers and percentages of DN1, DN2, and DN4 populations in the CD45−/− mice. An increase in the percentage of the DN3 population and a corresponding decrease in the DN4 population was reported previously (17).

Independent analysis of the lineage positive (Lin+) DN cells using Gr-1, CD19, NK1.1, CD11b, γδ TCR, and CD3εAbs, revealed a significant increase in the amount of NK cells and a significant decrease in NKT cells in addition to the developing CD4+ T cell progenitors. There was no detectable difference in the number of γδ T cells in the CD45−/− thymus compared with the CD45+/+ thymus, there was a small but significant increase in the percentage of γδ T cells within the DN population (Fig. 1B). Using downregulation of CD24 expression as an indicator of γδ T cell maturation (23), we found a significant increase in the percentage of immature, CD24− γδ T cells and a concomitant decrease in the number and percentage of mature CD24+ γδ T cells in the CD45−/− thymus, suggesting a possible defect in the maturation of γδ T cells. There was no detectable difference in the minute amounts of B cells, granulocytes, and macrophages in the CD45+/+ and CD45−/− thymus (data not shown).

To further examine the effect of CD45 on the numbers of early thymocyte progenitors in the DN1 population, we examined the CD117+ and CD117− DN1 populations, which show reciprocal expression of CD127 (IL-7Rα-chain) (24). As with the CD45+/+ thymus, the majority of CD45−/− DN1 cells were CD117+ and

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Data represents average ± SEM of multiple mice from at least three independent experiments.

*Lineage markers include Gr-1, CD11b, NK1.1, γδ TCR, and CD19.

*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

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CD117. However, there was a significant 2-fold decrease in the number of CD117+ DN1 cells in the CD45+/+ thymus and a significant 5-fold decrease in CD117+ DN1 cells (ETP) in the CD45−/− thymus compared with the CD45+/+ thymus (Fig. 2).

The absence of CD45 did not alter the number of LSK cells in the bone marrow, but did reduce the frequency of CLP and CCR9+ progenitors

To investigate whether the reduced numbers of DN1 cells in the CD45−/− mice were due to reduced progenitors from the bone marrow and/or the blood, the percentage and numbers of LSK cells were examined. There was no significant difference in the frequency or number of LSK cells in either the bone marrow or the blood between the CD45+/+ and CD45−/− mice (Fig. 3 and data not shown). However, there is heterogeneity within the LSK population, which contains cells with varying degrees of self-renewing potential and multipotency (reviewed in Ref. 25). We therefore looked at the CCR9-expressing LSK subset as CCL25, the ligand for CCR9, plays an important role in controlling thymic-gating for T cell progenitors entering the thymus (26). Interestingly, there was a decreased percentage of CCR9-expressing LSK cells in the bone marrow of CD45−/− mice. There was also a decreased percentage of CLP in the bone marrow of CD45−/− mice (Fig. 3C,3D).

This suggested that reduced DN1 progenitor cells may be due to reduced bone marrow progenitors. To address this, we performed competitive bone marrow repopulation experiments into nonirradiated IL-7Rα−/− recipient mice. We found reduced percentage of DN1 cells contributed by the CD45−/− bone marrow cells, consistent with the possibility of reduced homing of bone marrow progenitors and with the reduced number of DN1 cells present in the CD45−/− mice (Fig. 3E). However, insufficient cell numbers precluded any further breakdown of the DN1 population. In contrast to DN1, the DN2 population was populated by a higher percentage of CD45−/− thymocytes. As expected, CD45−/− thymocytes accumulated at DN3 due to a partial block in the transition to DN4 (17). Similar results were obtained when lethally irradiated congenic BoyJ mice were used as recipients for the competitive bone marrow repopulation experiments (data not shown).
CD45 is a positive regulator of proliferation in vivo

To further explore the effect of CD45 on early DN thymocyte development, we examined their proliferative potential. ETP in particular, have a high proliferative capacity and can expand 1000-fold prior to progression to DN2 (27). Mice were administered BrdU and the percentage of BrdU+ cells in the Lin− DN population was measured 48 h later (Fig. 4A). Significant reduced proliferation was observed in the CD45−/− CD117+ DN1, CD117+ DN1, and DN2 populations compared with CD45+/+ populations. This was consistent with the reduced numbers of CD117+ DN1, CD117+ DN1, and DN2 cells in the CD45−/− thymus. The most dramatic reduction was observed in the CD45−/− CD117− DN1 population. Both CD45+/+ and CD45−/− CD117+ DN1 cells proliferated more than their CD117− DN1 counterparts, consistent with CD117+ DN1 populations having a higher proliferative capacity. Significant reduced proliferation was also observed in the CD45−/− DN3 and DN4 populations (data not shown). This indicates that CD45 has a positive regulatory effect on the proliferation of early DN thymocyte populations.

**CD45 is required for optimal CXCL12 migration in DN1 thymocytes**

Chemokine signaling is necessary for the development of early thymocytes. As CD45 has been shown to positively regulate CXCL12-mediated migration in Jurkat T cells (19), the response of Lin− DN cells to CXCL12 was examined. Transwell migration was assessed using DN-enriched thymocytes (Fig. 4B). Lineage positive cells were gated out during analysis. In the absence of CXCL12, some migratory activity (chemokinesis) was observed in the CD117− DN1 population, with CD45−/− cells showing reduced chemokinesis. CXCL12 induced migration in the DN populations, particularly in the DN1 populations, and this was consistently reduced in the CD45−/− cells. A significant reduction was observed in the CD45−/− CD117− DN1 and DN4 populations. This reduced migration was not due to altered CXCR4 expression levels in the CD45−/− CD117− and CD117− DN1 populations (Fig. 4C). This indicates that CD45 can affect the migration of the DN1 populations, particularly the CD117− DN1 population, presumably by affecting the CXCL12 signaling pathway.

**Identification of an intermediate stage between DN1 and DN2**

As deletion of CXCR4 leads to defects in early thymocyte development, closer analysis of the DN1 population was performed. Analysis of the CD45−/− thymus revealed a distinct CD44−CD25+ population that was not apparent in the CD45+/+ thymus (Fig. 5A). This population had been previously noted in the CD45-deficient mice (28). We had previously determined that the total DN1 population was decreased in the CD45−/− mice. In this study, we show that the DN1 population from CD45−/− mice was divided into two distinct subpopulations based on CD25 expression, which we define in this study as DN1.0 (CD44+CD25−) and DN1.5 (CD44+CD25+). About 80% of the DN1 cells from the CD45+/+ mice resided in the DN1.0 population and 20% in the DN1.5 population, whereas the opposite was true in the CD45−/− mice. This translated to a 7-fold drop in cell numbers in the DN1.0 population in the CD45−/− mice, but no significant increase in cell numbers in the DN1.5 population (Fig. 5B). The different percentage distribution of cells between DN1.0 and DN1.5 in the CD45+/+ and CD45−/− mice suggested that DN1.5 may be an intermediate between DN1.0 and DN2 and that the absence of CD45 not only reduces the number of DN1 progenitors, but may favor their progression and relative accumulation at the DN1.5 stage.

To determine whether DN1.5 was an intermediate between DN1.0 and DN2, equal numbers of sorted DN1.0 and DN1.5 cells were plated on OP9 stromal cells expressing the Notch ligand Delta-like−1 (DL1) in the presence of IL-7 and Flt3L, which supports thymocyte development in vitro (22). Fig. 6A (left panel) shows that CD45+/+ and CD45−/− DN1.0 cells had the ability to make DN1.5 and DN2 cells, whereas DN1.5 cells made DN2 and DN3, but not DN1.0 cells. This suggested that DN1.5 was an intermediate between DN1.0 and DN2. When sorted, DN1.0 and DN1.5 cells were plated on OP9 cells not expressing DL1 (Fig. 6A, right panel), DN1.0 cells from both CD45−/− and CD45+/+ mice progressed to the DN1.5 stage, but neither DN1.0 nor DN1.5 cells progressed beyond the DN1.5 stage in the absence of DL1. This demonstrates that Notch sig-

[FIGURE 3. Flow cytometric analysis of LSK populations from CD45+/+ and CD45−/− mice. A, Flow cytometric analysis of bone marrow cells indicating frequency of CD117+Sca-1+ cells within the Lin− compartment ex vivo. Data from one mouse are shown and are representative of 15 mice over five independent experiments. B, Graph of the average percentage and number ± SEM of bone marrow LSK cells from one tibia. C, Graph of the average percentage ± SEM of CCR9-expressing cells within the bone marrow LSK population. D, Graph of the average percentage ± SEM of common lymphoid progenitor cells within the Lin− bone marrow population. Data are an average of six or more individual mice over two to three independent experiments. ***p < 0.001. E, Graph of the average percentage ± SEM of CD45+/+ versus CD45−/− donor cells within the DN1-4 populations, 3 wk after competitive 1:1 bone marrow reconstitution experiments into nonirradiated IL-7R−/− mice. Thymocytes were harvested and analyzed by flow cytometry. Expression of EGFP and CD45 were used to identify donor CD45+/+ and CD45−/− from recipient mice. Data are an average of eight mice from two independent experiments.]
naling is required for the progression of DN1.5 cells to the DN2 stage, but not for the progression of DN1.0 cells to DN1.5.

To determine the importance of IL-7 in the survival and progression of DN1.0 and DN1.5 populations, sorted DN1.0 and DN1.5 cells were cultured on OP9 and OP9-DL1 in the presence or absence of IL-7. IL-7 increased the survival of both DN1.0 and DN1.5 populations and IL-7 together with DL1 further enhanced cell survival or proliferation (Fig. 6B). In the absence of a Notch signal, IL-7 is a survival factor that allows the progression of DN1.0 to DN1.5. However, both IL-7 and Notch signaling were required for progression beyond the DN1.5 stage.

Although both CD45+/+ and CD45−/− DN1.0 cells progressed to DN2 on OP9-DL1 at approximately the same rate in the presence of IL-7 and Flt3L, the progression of CD45−/− DN1.5 cells to DN2 showed a significant lag phase compared with the CD45+/+ DN1.5 cells (Fig. 6C).

The absence of CD45 alters the distribution of CD117+ and CD117− cells within the DN1 populations

To further determine why the DN1.5 population from the CD45−/− mice exhibited a lag phase in generating DN2 cells (Fig. 6C), we examined the distribution of ETP within the DN1.0 and DN1.5 populations. The numbers of CD45−/− CD117+ cells were decreased ~4-fold in both the DN1.0 and DN1.5 populations compared with CD45+/+ cells (Fig. 7A, left panel). The percentage of CD117− cells in both the DN1.0 and DN1.5 populations were significantly different in the CD45−/− mice, compared with the CD45+/+ mice (Fig. 7A, right panel), but this was largely attributed to a decrease in CD117− cells in the DN1.0 population and an increase in the DN1.5 population in the CD45−/− thymus, as these cells represent the majority of cells in the DN1 population (Fig. 7B). The CD45−/− thymus had a significant 6-fold decrease in CD117+ cells in the DN1.0 population and a 3-fold increase in the DN1.5 population in the CD45−/− thymus, suggesting that CD45 may negatively affect CD25 expression and progression of DN1.0 to DN1.5.

Because the distribution of CD117+ and CD117− cells within the DN1.0 and DN1.5 populations was different between CD45+/+ and CD45−/− cells, it was estimated that there was approximately three times more ETP in the CD45−/− DN1.0 population, and seven times less ETP in the CD45−/− DN1.5 population when equal numbers of DN1.0 and DN1.5 cells from CD45+/+ and CD45−/− mice were plated on OP9 and OP9-DL1 cells. This reduction in ETP in the CD45−/− DN1.5 population may help explain the longer lag time observed for this population before it progressed to DN2. To determine whether this was the case, equal numbers of CD117+ DN1.0 populations...
CD45 and DN2 populations was similar for both CD45+/+ and CD45−/− mice. This result suggests that the absence of CD45 did not significantly affect the development of DN1.5 cells. However, when DN1.5 cells were plated on OP9-DL1 cells, the CD45−/− DN1.5 population showed a significant decrease in annexin V labeling compared with the CD45+/+ population (Fig. 9A). This suggests that CD45 is necessary for the survival of DN1.5 cells when plated on OP9-DL1 cells.

To further characterize the role of CD45 in the progression of CD117− DN1 cells, we examined the expression of the pro-apoptotic protein Bcl-2. The CD45+/+ population showed a higher level of Bcl-2 expression compared with the CD45−/− population (Fig. 9B). This result suggests that the absence of CD45 may affect the apoptotic process in DN1.5 cells.

CD45 regulates the progression, survival, and maturation of CD117− DN1 cells

To further investigate the role of CD45 in the progression of CD117− DN1 cells, we performed a flow cytometric analysis of CD44 and CD25 expression in Lin− DN thymocytes ex vivo. CD44+CD25− DN1.0 and CD44+CD25− DN1.5 populations were analyzed. The average numbers and percentage ± SEM of DN1 population and the subpopulations, DN1.0 and DN1.5, from seven mice are shown. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

FIGURE 5. Identification of an intermediate between DN1 and DN2. A. Flow cytometric analysis of CD44 and CD25 expression in Lin− DN thymocytes ex vivo. CD44+CD25− DN1.0 and CD44+CD25− DN1.5 populations are outlined. Data from one mouse are shown and are representative of seven mice over three independent experiments. B. Graph of the average numbers and percentage ± SEM of DN1 population and the subpopulations, DN1.0 and DN1.5, from seven mice. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

and DN1.5 cells were plated on OP9-DL1 cells. Interestingly, all populations progressed rapidly to the DN2 and DN3 stages and by day 6 had proliferated substantially (Fig. 7C). No obvious difference in the rate of progression was observed between the CD45+/+ and CD45−/− thymocytes (data not shown). Similar to total DN1.0 and DN1.5 populations, the progression of CD117− DN1.5 cells to DN2, but not DN1.0 cells to DN1.5, was dependent on Notch signaling, as cells did not progress past the DN1.5 stage when the cells were cocultured on OP9 cells (data not shown). This indicated that CD45 was required to generate optimal numbers of ETP, but was not essential for their progression to the DN2–3 stage in vitro. Because CD117 expression is maintained on ETP as they progress to the DN2–3 stage in vitro. Be-
into CD4+ cells and a slightly higher percentage became CD8+ cells (Fig. 10A, 10B). Interestingly, a substantial percentage of TCRβ+CD117+DN1 cells developed into γδ TCR-expressing cells (Fig. 10C). Overall, the noncanonical CD117+DN1 cells are indeed much less efficient at producing mature T cells with very few single CD4+ or CD8+ T cells being evident. However, they are capable of giving rise to γδ T cells in vivo.

Discussion
In this study, we have identified a new role for CD45 in early thymocyte development. We also identified and characterized an intermediate DN1.5 stage in the development of conventional T cell progenitors, ETP, from the DN1 to DN2 compartments, and in the noncanonical progression of CD117+DN1 cells to DN4. In vitro progression of both of these populations beyond the DN1.5 stage was strictly Notch dependent. The absence of CD45 significantly reduced both the ETP and CD117+DN1 populations, indicating an important role for CD45 in maintaining the numbers of these cells in the thymus. Specifically, the loss of CD45 resulted in reduced proliferation of these populations in vivo and decreased migration toward CXCL12 in vitro. CD45 did not affect the in vitro or in vivo progression of ETP, but was required for the
progression of the CD117$^+$ DN1 cells. These CD117$^+$ DN1 cells did not progress beyond the DN1.5 stage in vitro, did not express TCR$^\beta$, and CD117$^+$ DN1.5 cells accumulated in vivo in the CD45-deficient thymus.

Although the thymic seeding progenitor from the blood has not been definitively identified, progenitors phenotypically similar to ETP are present within the LSK population of both the blood and bone marrow, and these can home to and repopulate the thymus (30). Although the heterogeneous LSK population in the bone marrow was not significantly altered, the frequency of the CCR9-expressing LSK population and the CLP progenitors were decreased in the CD45$^{-/-}$ mice. As CCL25 has been shown to play a role in progenitor homing to the thymus (26), this reduced CCR9$^+$ LSK population could lead to decreased numbers of T cell progenitors seeding the thymus. Consistent with this, we did observe less CD45$^{-/-}$ ETPs in this population.

ETPs in the thymus have the ability to develop into T and NK cells (13) and in the CD45$^{-/-}$ mice, we observed reduced numbers of ETP and increased numbers of NK cells in the thymus. Others have reported a similar increase in NK cells and a decrease in NKT cells in the CD45 null spleen (31). We also noted fewer NKT cells in the thymus of CD45 null mice. In contrast to NKT cells, which split off from conventional $\alpha\beta$ T cell development at the DP stage (32), $\gamma\delta$ T cells are thought to branch off at the DN3 stage (33). The frequency of $\gamma\delta$ T cells was increased in the CD45$^{-/-}$ thymus and further analysis revealed an increased percentage of immature, CD24$^+$ $\gamma\delta$ T cells and a decreased percentage of mature, CD24$^{-}$ $\gamma\delta$ T cells, suggesting a role for CD45 in the maturation of $\gamma\delta$ T cells. Similar data were observed in the CD45 null fetal thymus (34). As Notch signaling is thought to block both NK and $\gamma\delta$ T lineage commitment in favor of $\alpha\beta$ T cell commitment (reviewed in Ref. 35), a reduced Notch signal would be predicted to reduce $\alpha\beta$ progenitors in favor of NK and $\gamma\delta$ T cells, as is observed in the CD45-deficient mice. However, IL-7 has also been implicated in

**FIGURE 7.** Altered CD117$^+$ and CD117$^-$ DN1 populations in CD45$^{-/-}$ mice. A and B, Graphical representation of flow cytometric data of CD117$^+$ and CD117$^-$ DN1.0 and DN1.5 populations ex vivo. Graphs show an average $\pm$ SEM of seven individual mice from three independent experiments. *p $\leq$ 0.05; **p $\leq$ 0.01; ***p $\leq$ 0.001. C, Flow cytometric analysis of sorted CD117$^+$ DN1.0 (CD44$^+$CD25$^-$) and CD117$^+$ DN1.5 (CD44$^+$CD25$^+$) thymic populations cocultured for 6 d with OP9-DL1 cells in the presence of 1 ng/ml IL-7 and 5 ng/ml Flt3L. Plots are from one representative experiment of three repeats using cells pooled from a minimum of three mice in each repeat. Axes are in log scale. D, Graphical distribution of CD117$^+$ cells between the three CD44$^+$ DN populations (DN1.0, DN1.5, and DN2) ex vivo determined by flow cytometric analysis and graphed as an average percentage $\pm$ SEM of CD117$^+$ cells within the CD44$^+$ DN population. Data are an average of nine individual mice over three independent experiments.

**FIGURE 8.** Altered progression of CD117$^-$ DN1 $CD45^{-/-}$ thymocytes. Flow cytometric analysis of sorted CD117$^-$ DN1.0 (CD44$^+$CD25$^-$) and CD117$^-$ DN1.5 (CD44$^+$CD25$^+$) thymic populations cocultured with OP9-DL1 (left) and OP9 (right) cells with 1 ng/ml IL-7 and 5 ng/ml Flt3L at days 6 and 9. Plots are from one representative experiment of three repeats using cells pooled from a minimum of three mice in each repeat. Axes are in log scale.
the maturation of γδ T cells as IL-7 null mice have reduced numbers and frequency of adult thymic γδ T cells (36). IL-7 dependent survival and proliferation of pro-B cells is enhanced in the absence of CD45 (37). If CD45 also negatively regulates IL-7 signaling here, then the absence of CD45 would be predicted to increase the number and maturation of γδ T cells in the CD45-deficient thymus. Whether CD45 affects NK and γδ T cell commitment and development by affecting IL-7 and/or Notch signaling requires further investigation.

FIGURE 9. Altered survival and maturation of CD117− DN1 CD45−/− thymocytes. A, Flow cytometric analysis of annexin V in ex vivo thymocytes (left). Histograms from one representative mouse (right). Graph of the average percentage ± SEM of annexin V+ cells from six individual mice over two independent experiments. *p ≤ 0.05. B, Flow cytometric analysis of Bcl-2 in ex vivo thymocytes. Data from one mouse are shown and are representative of nine mice over three independent experiments. C, Flow cytometric analysis of the DN1 population for CD24 and CD117 expression ex vivo (left). Data from one mouse that are representative of seven mice over three independent experiments (right). Average percentage ± SEM of DN1a–e cells within the DN1 population from seven mice over three experiments. D, Flow cytometric analysis of the CD117− DN1 population for TCRβ expression ex vivo (left). Histogram of CD117− DN1 cells from one mouse, representative of six mice over two independent experiments (right). Graph of the average percentage ± SEM of TCRβ+ cells from six mice. ***p ≤ 0.001.

Interestingly in this report, we showed that the adoptive transfer of the CD117− DN1 population into IL-7Rα−/− mice gave rise to γδ T cells, demonstrating that TCRβ− CD117− DN1 thymocytes have the potential to become γδ T cells. However, because these cells were injected i.v. and were found primarily in the spleen and lymph nodes, it is yet to be established whether this is their normal developmental path in the thymus.

It has been shown that the absence of CD45 leads to a partial developmental block affecting the DN3 to DN4 transition (17, 38).
This has been attributed to its ability to affect Lck and pre-TCR signaling. In the CD45−/− mice, we observed an absence of a TCRβ+ population within the CD117− DN1 thymocytes. Although it is not clear whether this TCRβ-expressing DN1 population are early thymic progenitors or DN thymocytes that have undergone unsuccessful pre-TCR selection, it raises the possibility that CD45 may play a role in regulating the expression of TCRβ.

CD45−/− DN1 cells showed decreased proliferation in vivo but not reduced survival or accelerated progression in vitro when compared with the CD45+/+ DN1 cells. Interestingly, two CD45-deficient cell lines, BW5147 and Jurkat T cells, show decreased proliferation compared with their wild-type counterparts (data not shown). Because the ETP are thought to decrease the pre-TCR and TCR signaling thresholds by dephosphorylating Lck. In this study, we show that CD45 can affect this population. Previous work has shown the effect of CD45 on the DN3 to DN4 transition where pre-TCR signals induce TCRα gene rearrangement and at the DP stage where CD45 regulates αβ TCR signaling (17, 18). In these cases, CD45 is thought to decrease the pre-TCR and TCR signaling thresholds by dephosphorylating Lck. In this study, we show that CD45 can influence thymic development as early as the DN1 stage and raise the possibility that CD45 may regulate TCRβ expression in the CD117−DN1 population. This suggests a new function for CD45 prior to pre-TCR or TCR expression. Overall, we have shown that CD45 regulates the number of CD117+ and CD117− DN1 thymocyte progenitors and is required for the development and progression of the noncanonical CD117− DN1 population.
References


