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There was an error published in *Development* **139**, 373-384.

In the legend to Fig. 10, the authors incorrectly stated that donor cells were injected intrathymically rather than intravenously. The correct figure legend appears below.

Fig. 10. Intrathymic DC development from ETP, DN1d and DN1e subsets following intravenous tail vein injections. PBS, ETP, DN1d or DN1e CD45.2 donor cells were injected intravenously into nonirradiated CD45.1 recipient mice. Thymus sections were analyzed for the presence of donor-derived DCs (CD11c$^+$ CD45.2$^+$) in proximity to medullary thymic epithelial cells (keratin 5$^+$) 7 days following injection. Cells were stained with DAPI (blue) and for CD11c (green), CD45.2 (red), keratin-5 (purple). Images are representative of triplicate experiments. Arrowheads indicate donor-derived DCs.

The authors apologise to readers for this mistake.

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SUMMARY

Specialized dendritic cells (DCs) within the thymus are crucial for the deletion of autoreactive T cells. The question of whether these cells arise from intrathymic precursors with T-cell potential has been hotly debated, and the regulatory pathways and signals that direct their development remain unclear. Here, we compared the gene expression profiles of thymic DC subsets with those of four early thymic precursor subsets: early T-cell precursors (ETPs), double-negative 1c (DN1c), double-negative 1d (DN1d) and double-negative 1e (DN1e) subsets. We found that the DN1d subset expressed Spi-B, HEBCan, Ccr7 and Ccr4, similar to thymic plasmacytoid DCs, whereas the DN1e subset expressed Id2, Ccr7 and Ccr4, similar to thymic conventional DCs. The expression of Ccr7 and Ccr4 in DN1d and DN1e cells suggested that they might be able to migrate towards the medulla (low in Dll proteins) and away from the cortex (high in Dll proteins) where early T-cell development occurs. We therefore assessed the sensitivity of developing DC precursors to Dll-Notch signaling, and found that high levels of Dll1 or Dll4 were inhibitory to DC development, whereas medium levels of Dll4 allowed DC development but not myeloid development. To evaluate directly the lineage potential of the ETP, DN1d and DN1e subsets, we injected them into nonirradiated congenic hosts intrathymically or intravenously, and found that they were all able to form medullary DCs in vivo. Therefore, DN1d and DN1e cells are transcriptionally primed to home to the thymus, migrate into DC-permissive microenvironments and develop into medullary DCs.

KEY WORDS: Dendritic cell development, Notch, Chemokine receptors, Transcription factors, T-cell precursors, Mouse

INTRODUCTION

The thymus is a specialized organ that uniquely supports T-cell development, in part by providing Dll (Delta-like) ligands for Notch signaling to uncommitted precursors. The thymus is also thought to support the development of several types of dendritic cells (DCs), but the regulatory pathways and precursors of these cells have been controversial (Schlenner and Rodewald, 2010). DCs are professional antigen-presenting cells that bridge the gap between the innate and adaptive immune systems by detecting pathogens and by activating T cells. They are also responsible for maintaining T-cell tolerance in the thymus and periphery (Coquerelle and Moser, 2010). Conventional DCs (cDCs), which consist of CD8$^+$ and CD8$^-$ subsets, and plasmacytoid DCs (pDCs) are the major DC subsets that reside in the thymus and the spleen under steady state conditions. Although thymic DC subsets resemble splenic DCs in phenotype, microarray data has revealed that these subsets have distinct gene expression patterns (Edwards et al., 2003; Elpek et al., 2011). However, the developmental and functional relationships of these cells are unclear.

Multiple upstream precursors have been identified for splenic DCs (Diao et al., 2004; Liu et al., 2009; Naik et al., 2007; Onai et al., 2007), whereas thymic DC precursors remain elusive. The earliest T-cell precursors in the thymus are double-negative (DN; CD4$^-$CD8$^-$) cells, which can be further subdivided into seven distinct

populations by the expression of the cell surface receptors CD44, CD25 (Il2ra – Mouse Genome Informatics), CD117 (Kit – Mouse Genome Informatics) and CD24. There are four main types of DN1 (CD44$^+$CD25$^-$) thymocytes: DN1a/b cells, collectively known as ETPs (early T-cell precursors; CD44$^+$CD25$^-$CD117$^+$CD24$^{-/int}$), DN1c cells (CD44$^+$CD25$^-$CD117^{int}CD24^{high}), DN1d cells (CD44$^+$CD25$^-$CD117$^-$CD24$^+$) and DN1e cells (CD44$^+$CD25$^-$CD117$^-$CD24$^-$) (Allman et al., 2003; Porritt et al., 2004). In the presence of strong Dll-Notch signals, ETPs give rise to T-lineage-specified cells, which continue to require Dll-Notch signals to become committed T-cell precursors (Schmitt et al., 2004). DN1c cells are thought to derive from blood-borne myeloid CDPs (common dendritic cell progenitors) and can give rise to CD8$^+$ cDCs in the thymus (Luche et al., 2011). Although DN1d and DN1e cells have very little T-cell potential, the developmental relationships between DN1d cells, DN1e cells and thymic DCs are unclear.

DC lineage potential is clearly present in ETPs and DN2a cells (Ardavin et al., 1993; Donskoy and Goldschneider, 2003; Li et al., 2009; Masuda et al., 2007; Yui et al., 2010). The latent DC potential in the thymus has, furthermore, been revealed by conditional ablation of Notch1, which increases DN1c cells and DCs in the thymus (Feyerabend et al., 2009; Radtke et al., 2000). However, other experiments have suggested that a more complex relationship exists between Notch signaling and DC development (Cheng et al., 2010). In mammals, there are four Notch receptors, Notch1, -2, -3 and -4, and five Notch ligands, Delta-like-1 (Dll1), Dll3, Dll4, Jagged 1 and Jagged 2 (Yuan et al., 2010). Previous in vitro studies suggested that high Jagged 1 levels supported the development of DC precursors from hematopoietic progenitors, whereas Dll1-expressing stroma favored the maturation of committed DC precursors (Cheng et al., 2007; Cheng et al., 2003).

Therefore, levels of Notch signaling and the types of ligands encountered in the thymus are likely to be crucial for modulating lineage choice in the thymus.

Thymic seeding progenitors enter the thymus at the cortico-medullary junction (CMJ) (Petrie and Zuniga-Pflucker, 2007). Although the majority of these precursors transit to the cortex for T-cell development (Prockop and Petrie, 2000), some subsets might preferentially migrate to the medulla and, thus, escape the T-cell fate. Dll4 is expressed throughout the thymus primarily on cortical thymic epithelial cells (cTECs), but also at lower levels on medullary thymic epithelial cells (mTECs) (Koch et al., 2008), whereas Dll1 is expressed mostly within the cortex with the highest levels expressed in the CMJ (Harman et al., 2003; Schmitt et al., 2004). The mTECs also secrete the chemokines Ccl17, Ccl19 and Ccl21, which ensure proper trafficking of single-positive T cells to the medulla (Campbell et al., 1999; Kurobe et al., 2006). Xcr1 and Cxcr4 have also been implicated in the localization of thymic DCs to the medulla (Lei et al., 2011). Although the majority of mature thymic DCs are present within the medulla (Kurobe et al., 2006), the influences of Dll1 and Dll4 on DC development are largely unknown.

One of the roles of Dll-Notch signaling in T-cell development is to constrain the activity of the transcription factor PU.1 (Sfpi1 – Mouse Genome Informatics) in the early stages of T-cell development (Franco et al., 2006). PU.1 is expressed in ETPs and at lower levels in DN2 cells (Yui et al., 2010), and $PU.1^{-/-}$ embryos exhibit a partial block in T-cell development (Spain et al., 1999). Spi-B (Spib – Mouse Genome Informatics), an Ets family member that is highly related to PU.1, is also expressed in DN2 and DN3 cells, but $Spib^{-/-}$ thymocyte populations appear normal (Su et al., 1997). Roles for PU.1 and Spi-B in the T/DC lineage choice in the thymus are suggested by studies in which the overexpression of either PU.1 or Spi-B during the early stages of T-cell development diverted T cells to the DC lineage (Lefebvre et al., 2005; Rothenberg and Dionne, 2002). Interestingly, however, PU.1 and Spi-B appear to have distinct roles in DC development. $PU.1^{-/-}$ embryos lack CD11b$^+$ cDCs, but retain functional fetal thymic CD8$^+$ cDCs (Guerriero et al., 2000), whereas Spi-B is a key regulator of splenic pDC development (Schotte et al., 2004). Thymic DCs are present in $Spib^{-/-}$ embryos, but the impact of Spi-B deficiency on specific thymic DC subsets is unknown.

One of the target genes of DL-Notch signaling during T-cell development is HEBAlt (alternative form of Tcf12 – Mouse Genome Informatics) (Franco et al., 2006; Wang et al., 2006). HEBAlt belongs to the E protein transcription factor family, which also includes E2A (Tcf3 – Mouse Genome Informatics), E2-2Alt (alternative form of Tcf4 – Mouse Genome Informatics), E2-2Can (canonical form of Tcf4 – Mouse Genome Informatics) and HEBCan (canonical form of Tcf12 – Mouse Genome Informatics). E2-2 has been shown to play a role in splenic pDC development (Cisse et al., 2008), but the influences of the other E proteins on DC development are unknown. HEBAlt promotes T-cell development downstream of Dll-Notch signaling (Bain et al., 1997; Wang et al., 2010; Wang et al., 2006), and is thus a good candidate for influencing the T/DC fate choice towards the T-cell lineage. Conversely, the E protein antagonist Id2, which is thought to be induced by PU.1 (Anderson et al., 2002), promotes DC development (Hacker et al., 2003). Id2-deficient animals exhibit reduced numbers of splenic CD8$^+$ cDCs and increased CD8$^-$ cDC and pDC populations (Hacker et al., 2003; Kusunoki et al., 2003). Therefore, the presence of Notch receptors, E proteins, and the Ets family members PU.1 and Spi-B in distinct precursors are likely to

be crucial for determining the downstream consequences of microenvironmental signals during DC development within the thymus.

MATERIALS AND METHODS
Animals
C57Bl/6 wild-type (WT) mice and Lck-HEBAlt^{Tg} mice (Tg) (Braunstein and Anderson, 2010) were maintained at the Sunnybrook Research Institute (SRI), Toronto, Ontario, Canada. In Tg mice, HEBAlt is under the control of the *lck*-proximal promoter. All animal protocols were approved by the animal care committee at SRI. Mice aged 4-7 weeks were used for splenic and thymic DC and DN1 subset analyses and mice older than 7 weeks were used for LSK isolation from bone marrow precursors.

Dendritic cell isolation and flow cytometric analysis
For ex vivo sorting experiments, thymocytes and splenocytes were depleted of red blood cells (RBCs) using ACK solution (155 mM NH$_4$Cl, 0.1 mM disodium EDTA, 10 mM KHCO) and stained in Hank's balanced salt solution with 2.5% bovine serum albumin (BSA) at 4°C. DCs were positively selected from thymocyte samples using CD11c-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and an autoMACS Pro Separator using the 'posseld' program (Miltenyi Biotec, Bergisch Gladbach, Germany). All samples were incubated with anti-FcγR antibodies to eliminate non-specific staining prior to antibody incubation. Thymic CD11c-selected cells and RBC-depleted splenocytes were stained with the following antibodies: MHC II (H2 – Mouse Genome Informatics)-FITC, CD8α-APC, CD19-Bio, Nk1.1-Bio, Thy1-Bio (SRI Antibody Core Facility, Toronto, ON, Canada); CD11c-PE, CD11b-Alexa700, F4/80-Bio, Gr1-Bio, streptavidin-eFluor450 (eBiosciences, San Diego, CA, USA); CD45-PerCPCy5, B220-APCCy7 (BD Biosciences, Mountain View, CA, USA). DAPI staining was used to differentiate live and dead cells. DC subsets were sorted on a FACS Aria using the following parameters: CD8$^+$ cDCs (CD45$^+$, Lin$^-$, CD11c$^+$, B220$^-$, MHC II$^+$, CD8$^+$, CD11b$^-$); CD8$^-$ cDCs (CD45$^+$, Lin$^-$, CD11c$^+$, B220$^-$, MHC II$^+$, CD8$^-$, CD11b$^+$); pDCs (CD45$^+$, Lin$^-$, CD11c^{int}, B220$^+$, MHC II$^{+/int}$), where Lin = Gr1 (granulocytes), F4/80 (macrophages), Thy1 (T cells), Nk1.1 (NK cells), CD19 (B cells) and Ter119 (erythrocytes). For in vitro-derived DC analysis, cells were first gated on CD45$^+$ Lin$^-$ cells. Non-DC myeloid cell analysis was performed after gating on total CD45$^+$ CD11c$^-$ cells, without the Lin$^-$ gate.

DN1 thymocyte subset isolation
Thymocytes were harvested and depleted of CD4$^+$ and CD8$^+$ cells using biotin-conjugated antibodies (SRI Antibody Core Facility, Toronto, Ontario), streptavidin-microbeads and an autoMACS Pro Separator using the 'depletes' program (Miltenyi Biotec, Bergisch Gladbach, Germany). Depleted cells were incubated with anti-FcγR antibodies, washed, then incubated with the following antibodies: CD44-FITC, CD24-PE, NK1.1-Bio, CD8α-Bio, CD4-Bio, TCRβ-Bio, CD11b-Bio, Ter119-Bio, CD25-APC, CD117-Alexa750 and streptavidin-PerCP (antibodies obtained from eBioscience, San Diego, CA; BD Biosciences, Mountain View, CA and SRI antibody core facility, Toronto, Ontario). DN1 subsets were sorted using a FACS Aria using the following parameters: ETP (Lin$^-$ CD44$^+$ CD25$^-$ cKit$^+$ CD24$^{-/int}$); DN1c (Lin$^-$ CD44$^+$ CD25$^-$ cKit^{int} CD24$^+$); DN1d (Lin$^-$ CD44$^+$ CD25$^-$ cKit$^-$ CD24$^+$); DN1e (Lin$^-$ CD44$^+$ CD25$^-$ cKit$^-$ CD24$^-$), where Lin$^-$ = NK1.1$^-$, CD8α$^-$, CD4$^-$, TCRβ$^-$, CD11b$^-$, Ter119$^-$. CD11c$^+$ cells were not specifically gated out in all experiments, but FACS analysis showed that the percentage of CD11c-expressing cells in each population were as follows: ETP: 6.40±0.74%; DN1c: 66.93±6.79%; DN1d: 8.98±1.12%; DN1e: 8.16±0.80%, and we found that CD11c$^-$ DN1 subsets did not differ significantly in gene expression from the unfractionated DN1 subsets.

DC in vitro culture system
Bone marrow cells were depleted of Lin$^+$ cells using biotin-conjugated antibodies against CD19, F4/80, Gr1, Ter119 (Ly76 – Mouse Genome Informatics), streptavidin microbeads and LS MACS columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Lin$^-$ cells were cultured overnight

in OP9 media (RPMI, 10% FBS, 100 U penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 0.1 mM non-essential amino acids, 0.055 mM 2-mercaptoethanol) supplemented with 10 ng/ml SCF, IL-7 and Flt3L. Lin⁻ Sca⁺ cKit⁺ (LSK) precursors were sorted using a FACS Aria. LSK cells (2000 per well) were seeded onto OP9-derived monolayers or placed in stroma-free culture in 6-well plates supplemented with 5 ng/ml SCF, IL-7 and Flt3L. Cells were seeded onto fresh monolayers approximately every 4 days. Flow cytometry for DC phenotype was performed as described above on an LSR II (BD Biosciences, Mountain View, CA, USA).

Gene expression analysis

RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA, USA) and first-strand cDNA was generated from total RNA using Superscript Reverse Transcriptase III (Invitrogen) and 250 ng random primers (Invitrogen). Quantitative real-time RT-PCR (qRT-PCR) was performed using gene-specific primers (supplementary material Table S1; 0.1 µM of each primer per 25 µl reaction), iTaq SYBR Green Supermix with ROX (BioRad, Hercules, CA, USA) and an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). Relative gene expression was determined using β-actin to normalize for cDNA input and the ΔCt-method (Wang et al., 2006). cDNA dilutions yielding β-actin Ct values of ~16-17 were used as templates.

Intravenous and intrathymic injections and immunofluorescence microscopy

ETP, DN1d and DN1e subsets were purified from C57Bl/6 mice, as described above, with these additional lineage markers: CD11c, F4/80, CD19, B220 (CD45R; Ptprc – Mouse Genome Informatics), Gr1, CD3ε. Cells (30,000 per mouse for ETP, 180,000 for DN1d, 160,000 for DN1e) were injected by tail vein intravenous injection. Cells (100,000 per thymic lobe of each recipient mouse for ETP, 75,000 for DN1d, 100,000 for DN1e) were injected intrathymically. Five-week-old CD45.1 (Ptprcᵃ – Mouse Genome Informatics) congenic mice were used as recipients. Thymuses were harvested seven days post-intravenous injection and five days post-intrathymic injection and frozen in OCT. Thin sections (10 µm) were fixed in 2% paraformaldehyde, blocked with PBS with 5% fetal bovine serum (FBS) and 0.05% Triton X-100, and stained with CD11c-FITC, CD45.2/anti-mouse Cy3, anti-Keratin-5/anti-rabbit Cy5 (CD11c-FITC and CD45.2 were obtained from BD Biosciences, Mountain View, CA, USA; anti-mouse Cy3, anti-Keratin-5 and anti-rabbit Cy5 were obtained from Jackson ImmunoResearch Laboratories, West Grove, PA and Covance, Emeryville, CA, USA) and DAPI. Images were acquired with a Zeiss Axiovert 200 Fluorescence Microscope with a final magnification of 200×.

Calculations and statistics

Percentages of DC populations were calculated by dividing the number of each population by the number of CD45⁺ cells within the sample. Standard deviation was calculated for all data and represented with error bars. Unpaired *t*-test statistical analysis of replicates was used to determine statistical significance.

RESULTS
Regulatory factors expressed by thymic and splenic DC subsets

Analysis of the transcriptional fingerprints of splenic and thymic DC subsets using microarray techniques indicate that DC function is heavily shaped by the microenvironments in which the cells reside (Edwards et al., 2003; Elpek et al., 2011; Hacker et al., 2003). However, little is known about the regulatory factors that control DC development within the thymus. We therefore set out to characterize the expression of a set of key regulatory factors in DC subsets in order to identify subset-specific patterns. Owing to the small percentage of DCs within the thymus, we first positively selected cells expressing CD11c, a global marker of DCs, from the thymuses of wild-type mice using magnetic sorting. Next, CD8⁺ cDC, CD8⁻ cDC and pDC subsets were sorted from the DC-

enriched fraction using fluorescence-activated cell sorting (FACS) according to the following parameters: CD8⁺ cDCs (CD45⁺ Lin⁻ CD8α⁺ CD11b⁻ B220⁻ CD11c⁺ MHC II⁺), CD8⁻ cDCs (CD45⁺ Lin⁻ CD8α⁻ CD11b⁺ B220⁻ CD11c⁺ MHC II⁺) and pDCs (CD45⁺ Lin⁻ B220⁺ CD11cⁱⁿᵗ MHC IIⁱⁿᵗ) (Fig. 1A). Other lineages (Lin = CD19, NK1.1, Thy1, F4/80, Gr1, Ter119) were electronically excluded during sorting. These three DC populations were also sorted from splenocytes according to the same parameters for a comparative analysis.

Quantitative real-time RT-PCR (qRT-PCR) was used to assess gene expression in each subset. PU.1, Spi-B and Id2 were robustly expressed within one or more of the DC subsets (Fig. 1B). As these three factors have been shown to be required for DC development in some or all subsets, they were chosen as a diagnostic tool to indicate DC potential in precursor subsets. PU.1 was expressed in all subsets except the thymic pDCs. Interestingly, PU.1 levels were higher in CD8⁻ splenic and CD8⁻ thymic cDCs than in the other subsets, consistent with a myeloid gene program for these cells. Spi-B, a hallmark for pDCs, was expressed in both thymic and splenic pDC subsets. Id2 levels, by contrast, were higher in the CD8⁺ thymic and splenic DC subsets compared with the other DC populations. We also examined the expression of HEBCan and HEBAlt in these six subsets. Unexpectedly, HEBCan was expressed at very high levels within thymic pDCs, but not in splenic pDCs, in contrast to Id2 and HEBAlt, which were absent from the thymic pDCs. Interestingly, HEBAlt was present within the splenic CD8⁻ cDCs and splenic pDCs, but low in thymic CD8⁻ cDCs.

To determine the potential for Notch signaling in mature thymic and splenic DC subsets, we evaluated the presence of all four Notch receptors. Notch receptors 1, 2, 3 and 4 were all expressed predominantly in thymic pDCs, but not in splenic pDCs. Our results therefore demonstrate that although both thymic and splenic pDCs express high levels of Spi-B, mature thymic pDCs are unique by virtue of their repertoire of Notch receptors. To determine whether Notch signaling was functional in thymic pDCs, we analyzed the expression of the Notch target gene Hes1, and found that it was expressed at high levels in thymic pDCs and at low levels in thymic cDCs (Fig. 2A). These results indicate that pDCs are receiving Notch signals within the thymus, and suggest that Notch signaling plays a role in thymic pDC function and/or homeostasis.

Thymic DCs and specific DN1 subsets share transcriptional regulators

Next, we set out to identify candidate precursors of thymic DCs. Previous studies have suggested that thymic precursors can give rise to DCs as well as T cells (Ardavin et al., 1993; Donskoy and Goldschneider, 2003; Li et al., 2009; Wu et al., 1996). We reasoned that uncommitted T-cell precursors that exhibit similar transcriptional profiles to mature thymic DCs would be expected to have greater DC-lineage potential and decreased T-cell potential. We therefore examined gene expression in the ETP, DN1c, DN1d and DN1e subsets, and compared these patterns to those in mature thymic DC subsets. To obtain DN1 subsets, we first enriched DN (CD4⁻ CD8⁻) cells by depleting thymocytes expressing CD8 and/or CD4 by magnetic sorting. ETP (DN1a/b), DN1c, DN1d and DN1e subsets were then isolated by FACS as previously described (Fig. 3A) (Porritt et al., 2004).

PU.1 was present in all DN1 subsets (Fig. 3B), with the highest levels in the DN1c subset. By contrast, Spi-B was expressed at much higher levels within the DN1d cells than in the other DN1
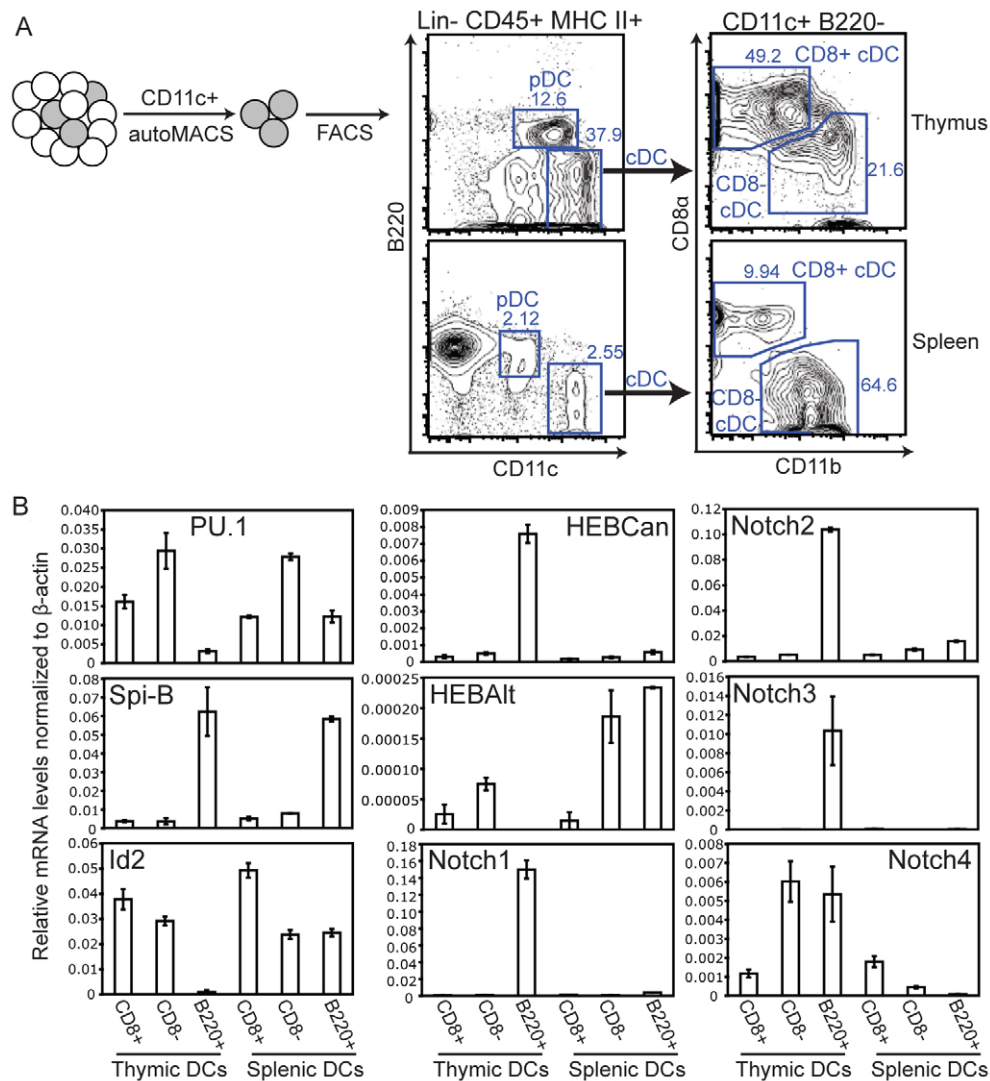
Fig. 1. Regulatory factor mRNA expression in mature thymic and splenic DC subsets. (**A**) Sorting parameters for thymic and splenic DCs. CD11c$^+$ cells were magnetically selected from pooled thymuses. Specific DC subsets were sorted from RBC-depleted splenocytes and CD11c-selected thymocytes. DC subsets were gated first by FSC/SSC profiles and DAPI$^-$. Lin$^-$ (Lin = CD19, F4/80, Gr1, NK1.1, Thy1) CD45$^+$ MHC II$^+$ cells were separated into pDC and cDC populations. cDC cells were further differentiated into CD8$^+$ cDCs and CD8$^-$ cDCs. Numbers refer to percentages. (**B**) Gene expression levels, as determined by qRT-PCR, were normalized to β-actin. Values shown are mean ± s.d. (*n*=3).

subsets. Id2 exhibited a different expression profile, with the highest levels in the DN1e subset. HEBCan was present in all populations, but was expressed at lower levels in the DN1c subset. Like Id2, HEBAlt was expressed almost exclusively in DN1e cells. Notch1 was expressed primarily in the ETPs, with low level expression in the DN1d and DN1e subsets as well. Notch2 was more widely expressed, also peaking in the ETP subset. Like Spi-B, Notch3 was the highest in the DN1d subset, whereas Notch4 was highest in the DN1c and DN1e subsets. However, Hes1 expression, which suggests active Notch signaling, was observed only in the ETPs (Fig. 2B). Therefore, DN1c, DN1d and DN1e cells appear to be transcriptionally primed towards the DC lineage(s), and awaiting the proper signals to fully differentiate.

Ccr7 is expressed by thymic DCs and DN1d and DN1e subsets

Migration within the thymus results in differential exposure to Dll4 and Dll1, depending on the route taken. ETPs migrate from the CMJ to the outer cortex, which contains high levels of Dll4. However, if DN1 subsets traveled directly to the medulla from the CMJ, they would encounter a different microenvironment with lower levels of Dll1 and Dll4. To identify a potential mechanism of migration and localization of DCs in the thymic medulla, we

analyzed the mature thymic DC subsets for the expression Ccr4 and Ccr7, which respond to the ligands Ccl17 and Ccl19/21, respectively. Interestingly, we found that Ccr7 was expressed on all thymic DCs (Fig. 4A,B), whereas Ccr4 transcripts were not detected in any thymic DC subset (Fig. 4A). We also examined the presence of *Ccr4* and *Ccr7* mRNA in DN1 subsets. Intriguingly, DN1d and DN1e cells expressed higher levels of both *Ccr4* and *Ccr7* than did ETPs and DN1c subsets (Fig. 4A), suggesting they might be able to home directly to the medulla. These results are consistent with the previously reported absence of Ccr7 from DN1c cells (Luche et al., 2011).

An in vitro system for assessing the impact of graded Dll1- and Dll4-mediated Notch signaling on DC development

Previous studies have assessed the effects of Dll-Notch1 signaling as a binary event using conditional Notch1 deletion strategies. However, migration of thymic precursors to the medulla would expose them to lower levels of Dll1 and Dll4, which could permit different developmental outcomes than would exposure to either high levels or a complete absence of Dll-Notch1 signaling. To determine how graded levels of Dll1 and Dll4 signaling impact DC development, we used an in vitro culture system initially developed
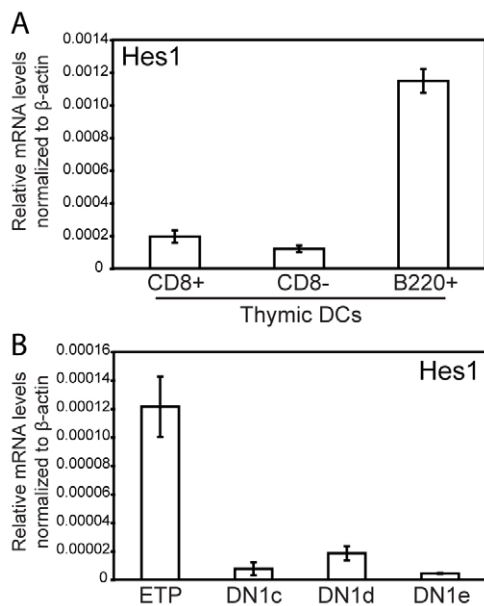
Fig. 2. *Hes1* mRNA expression in thymic DC and DN1 subsets. qRT-PCR of *Hes1* transcripts of (**A**) thymic DC subsets and (**B**) DN1 subsets. Gene expression levels, as determined by qRT-PCR, were normalized to β-actin. Values shown are mean ± s.d. (*n*=3).

using fetal liver precursors (Mohtashami et al., 2010). Lineage-negative Sca1$^+$ c-kit$^+$ (LSK) hematopoietic precursors were sorted from adult bone marrow (BM) and co-cultured with OP9 stromal cells expressing low, medium or high levels of Dll1 or Dll4. OP9 stroma, which was derived from the bone marrow of the *op/op* M-CSF-deficient mouse (Kodama et al., 1994), expresses low levels of Jagged 1 and secretes an array of cytokines and other regulatory factors (Cho et al., 1999). Control OP9 monolayers provided a baseline of DC development on stromal cells in the absence of Dll, whereas stroma-free cultures allowed us to monitor DC development in the absence of stromal cell-derived factors. All cultures were supplemented with the cytokines SCF, IL-7 and Flt3L.

Medium levels of Dll4 support DC development but not myeloid development

We first assessed the appearance of DCs in these cultures by monitoring the expression of CD11c using flow cytometry. After eight days, stroma-free cultures generated the highest percentage of CD45$^+$Lin$^-$ DCs (Fig. 5A,B), whereas OP9 cultures had the highest numbers of DCs (Fig. 5E). Exposure of LSK cells to high levels of either Dll4 or Dll1 strongly inhibited the appearance of DCs (Fig. 5A), which correlated with enhanced T-cell development, as expected (data not shown) (Mohtashami et al., 2010). In all Dll1 cultures, the percentage of DCs at day 8 of culture decreased as the Dll ligand expression levels increased (Fig. 5A,B). The absolute numbers of cells in each Dll-expressing culture did not differ significantly (Fig. 5D). However, cultures expressing medium levels of Dll4 supported the development of DCs to a greater degree than any of the other Dll-bearing stroma, including stroma expressing low levels of Dll proteins, in terms of absolute numbers (Fig. 5A,E). We also analyzed the presence of non-DC myeloid cells (CD11c$^-$ CD11b$^+$) in these cultures. Interestingly, the percentage of CD11c$^-$ CD11b$^+$ cells, which

represent macrophages, monocytes and granulocytes, were more negatively impacted by medium levels of Dll1 and Dll4 than DCs were (Fig. 5A,C). Therefore, thymic niches presenting intermediate levels of Dll4 would be expected to support DC development but exclude myeloid development.

High levels of Dll inhibit the generation of DC-primed precursors

Multiple developmental stages occur during the differentiation of the LSK precursor cells into DCs or T cells during normal development. However, when high levels of Dll1 or Dll4 are present, LSK precursors undergo accelerated T-cell development (Mohtashami et al., 2010) and are thought to bypass earlier developmental stages owing to their immediate exposure to Dll-Notch signaling. Therefore, we examined DC development in switch cultures to determine whether high levels of Dll inhibited the generation of DC precursors or the maturation of DCs. We co-cultured LSK cells with OP9 stroma for 2 days [OP9(2d)Dll1] or 4 days [OP9(4d)Dll1] before transferring the cells to high Dll1-expressing stroma in the presence of SCF, IL-7 and Flt3L (Fig. 6A). Following 8 days of culture, the percentage of DCs generated on OP9(2d)Dll1 was less than that on OP9, but greater than the percentage of DCs that had developed on high Dll1-expressing stroma (Fig. 6B,C). Therefore, high levels of Dll were inhibitory for the in vitro formation of DC precursors, but once these precursors had formed, they were refractory to high levels of Dll.

Medium levels of Dll4 preferentially support immature DCs

Immature and mature DCs can be differentiated by their levels of MHC Class II (Sallusto et al., 1995). We therefore compared the percentages of CD11c$^+$ MHC II$^-$ cells (immature DCs) and the percentage of CD11c$^+$ MHC II$^+$ cells (mature DCs) in cultures expressing high, medium and low levels of Dll1 or Dll4 (Fig. 7). By day 8, populations of mature DCs were apparent in all cultures (Fig. 7A,C). The percentage of mature DCs fell as the levels of either Dll1 or Dll4 rose. However, cultures expressing medium levels of Dll4 at day 8 also contained an immature DC population (CD11c$^+$ MHC II$^-$) (Fig. 7B). Therefore, exposure of precursors to intermediate levels of Dll4 preferentially supports the generation and/or expansion of immature DCs.

HEBAlt inhibits DC development

During normal T-cell development, HEBAlt levels rise until the β-checkpoint after which it is downregulated (Wang et al., 2006). We have previously shown that HEBAlt-expressing fetal liver-derived LSK cells are inhibited from becoming B cells and myeloid cells in the presence of high levels of Dll1 (Wang et al., 2010). HEBAlt is upregulated by Notch signaling, suggesting that it could influence DC development. We therefore examined the development of DCs in cultures of precursors from HEBAlt-transgenic (Tg) mice. In these mice, HEBAlt expression is under the control of the *Lck* promoter, which drives HEBAlt expression in some DN1 cells and most DN2 and DN3 cells (Braunstein and Anderson, 2010; Shimizu et al., 2001). Following 8 days of culture there were no significant differences between the WT and Tg cultures. On day 18, WT cultures had both immature and mature DCs in the presence of the low and medium levels of Dll1 and Dll4 (Fig. 8A). However, the percentage of DCs in the presence of low Dll1 and medium Dll4 in Tg cultures was lower than in WT cultures (Fig. 8B), suggesting that HEBAlt inhibited DC development even in the presence of low Dll-Notch signaling.
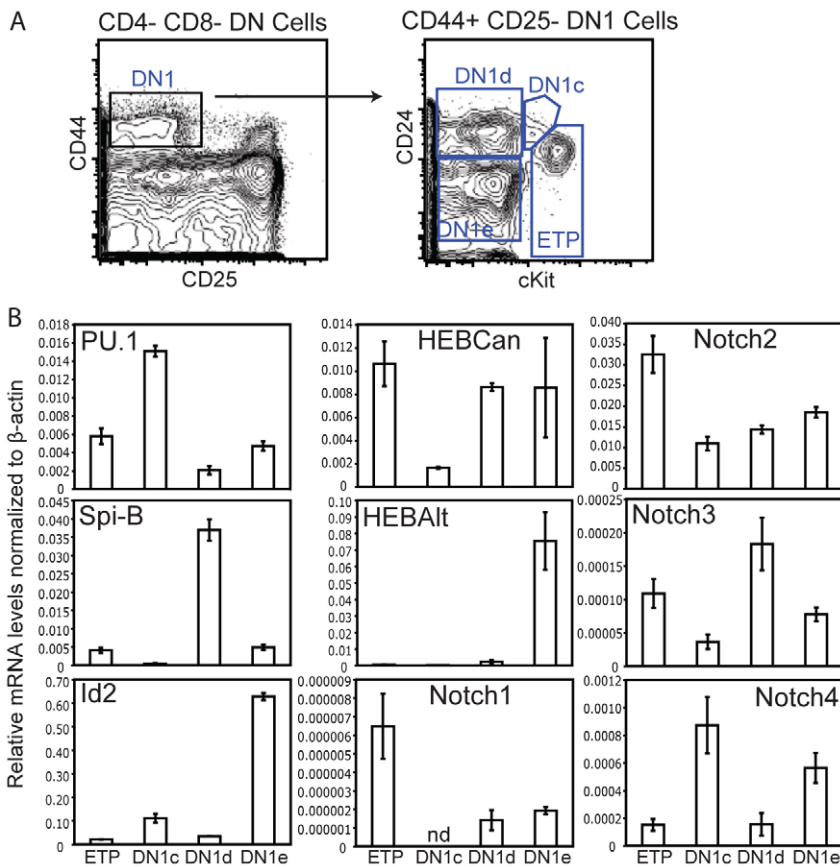
Fig. 3. Regulatory factor mRNA expression in DN1 early thymocyte subsets. (**A**) Sorting parameters for ETP, DN1c, DN1d and DN1e subsets. (**B**) Gene expression levels, as determined by qRT-PCR, were normalized to β-actin. Values shown are mean ± s.d. (*n*=3). nd, not detectable.

We also examined the surface expression of B220 and CD8α to determine whether pDCs and CD8$^+$ DCs, respectively, were generated in these cultures. A lack of B220 expression indicated that canonical pDCs were not present on day 18. However, CD8α expression on CD11c$^+$ cells was observed on WT cells in the presence of medium Dll4 levels (Fig. 8C). Interestingly, HEBAlt inhibited the generation of both CD8$^+$ and CD8$^-$ DCs compared with WT cells. As the lowest levels of Dll4 on OP9 stroma are thought to be most similar to Dll4 expression levels within the medulla (Mohtashami et al., 2010), these results provide evidence

that CD8$^+$ and CD8$^-$ DCs can develop within the thymic microenvironment, and suggest that the upregulation of HEBAlt at the DN2 stage might be, in part, responsible for driving the T/DC fate choice towards the T-cell lineage.

Thymic DCs can develop from ETPs, DN1d and DN1e subsets in vivo

Our data suggests that DN1d and DN1e subsets are transcriptionally primed towards the DC lineage. Although many precursors have been shown to contain DC potential (Ardavin et al., 1993; Donskoy
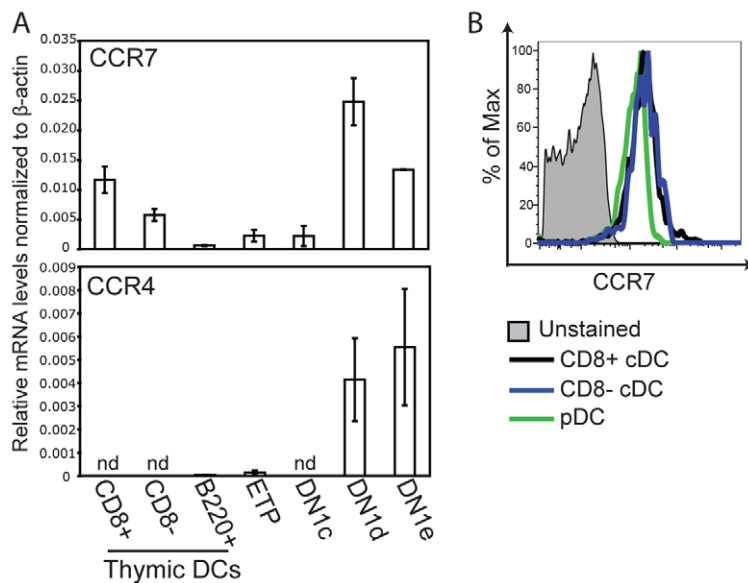

Fig. 4. Chemokine receptor expression in mature thymic DCs and DN1 subsets. (**A**) *Ccr7* and *Ccr4* transcript levels were determined by qRT-PCR, which were normalized to β-actin. Values shown are mean ± s.d. (*n*=3). nd, not detectable. (**B**) Ccr7 surface expression was examined on thymic DC subsets by flow cytometry.
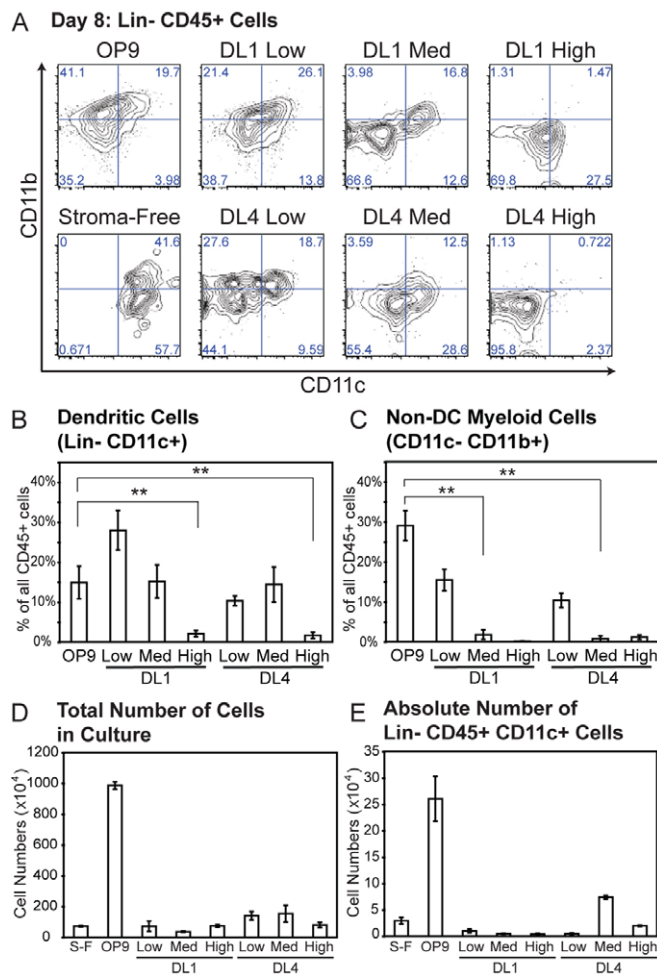
Fig. 5. Non-DC myeloid cells are inhibited by low levels of DL ligand. All data was taken from day 8 of culture. (**A**) LSK cells were cultured with low, medium and high DL1- and DL4-expressing OP9 stroma, control OP9 stroma, or in stroma-free (S-F) conditions. All cultures were supplemented with SCF, IL-7 and Flt3L. Non-DC myeloid cells were assessed by gating on FSC/SSC, DAPI⁻ CD45⁺ cells. (**B,C**) Percentage of (B) DCs and (C) myeloid cells were calculated. Statistically significant differences from the OP9 control, where indicated, were determined. **$P<0.01$. (**D,E**) Total cell numbers in culture (D) and absolute number of DCs (E) are shown. The percentage of CD11c⁺ cells, as determined by flow cytometry, in each condition was multiplied by the total number of cells in each culture. Means were calculated from triplicate samples ± s.d. Data is representative of two independent experiments.

Fig. 6. Inhibition of DC precursors by high level Dll. (**A**) LSK cells were cultured with OP9 stroma for 2 or 4 days followed by culture with high-expressing DL1 OP9 stroma for a total of 8 culture days. (**B**) DC populations were assayed by MHC II and CD11c expression. Numbers represent percentages. (**C**) Percentages of total DCs (CD11c⁺) were calculated and compared with OP9 and DL1-high stroma co-culture controls. Means were calculated from triplicate samples ± s.d.

and Goldschneider, 2003; Li et al., 2009), in vivo transfer studies demonstrating direct developmental pathways to thymic DCs are lacking. We first sorted ETPs, DN1d and DN1e precursors from wild-type CD45.2 (Ptprc^b – Mouse Genome Informatics) mice and injected them intrathymically into nonirradiated congenic CD45.1 recipients. Thymus sections were taken five days post-injection. Nonirradiated recipient mice were used to provide unperturbed thymic microenvironments (Dakic et al., 2004). We performed immunofluoresence staining using antibodies that detected CD45.2 (donor origin), CD11c (DC phenotype) and keratin 5 (medullary thymic epithelial cells). All three subsets gave rise to CD11c⁺ DCs within the thymus, which indicates that DC potential is present in each subset and that this potential is realized within the setting of the
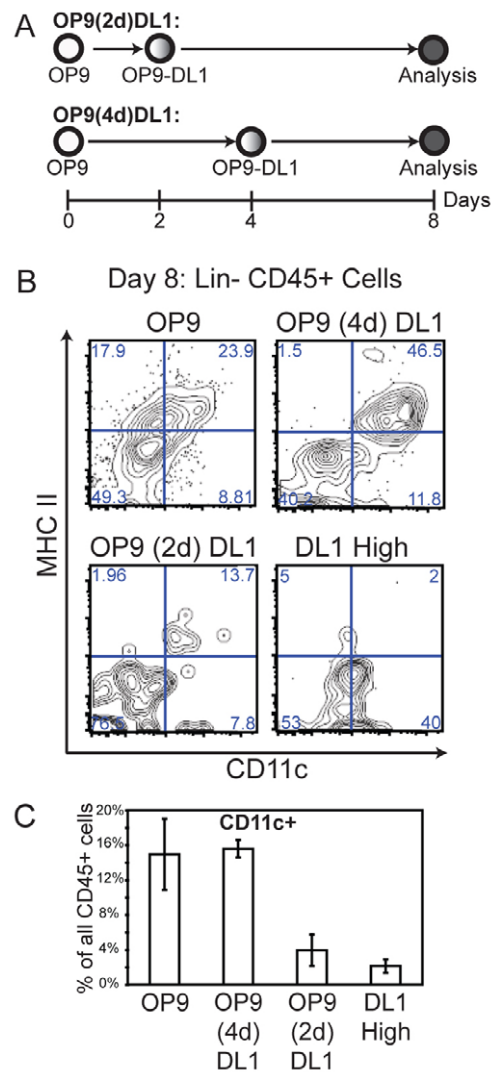
mature structured thymus (Fig. 9). Interestingly, the majority of DCs were present within the medulla or in close proximity to the mTECs, regardless of the donor subset. The presence of mature T cells in the medulla was not examined because the short duration of in vivo development would not have allowed for full T-cell differentiation at that time point. These results show that placement of these precursors directly into the thymus allows them to differentiate into DCs, and suggests that those injected near the medulla might have been preferentially induced to become DCs.

Precursors that enter the thymus through the CMJ would be recruited into either the medullary or cortical environments depending on their repertoire of chemokine receptors. Because DN1d and DN1e cells express Ccr7 whereas ETPs do not, we
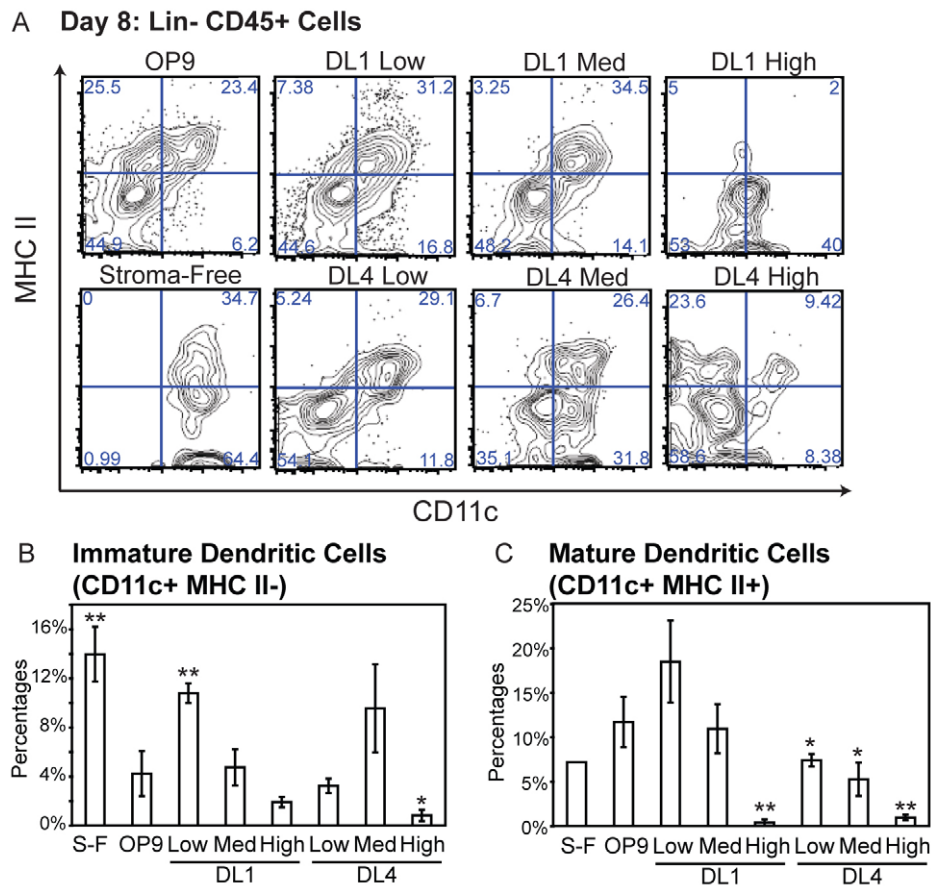
A   Day 8: Lin- CD45+ Cells

B   Immature Dendritic Cells (CD11c+ MHC II-)

C   Mature Dendritic Cells (CD11c+ MHC II+)

forced these cells to enter the thymus through the circulation, thereby more closely mimicking the route by which thymic precursors are initially sorted, as well as testing their ability to home to the thymus. Sorted ETPs, DN1d and DN1e cells were injected intravenously into nonirradiated congenic CD45.1 recipients, and their presence and phenotype were monitored seven days later by immunofluorescence staining. Although all three subsets again had the capacity to generate CD11c⁺ DCs within the thymus, donor-derived DCs were slightly more frequently observed in the thymic medullary regions of animals injected with DN1d or DN1e subsets (Fig. 10). Therefore, our work shows that ETPs, DN1d and DN1e cells can home to the thymus and develop into DCs, and that these precursors or DCs can localize to the medulla.

DISCUSSION

It has been shown conclusively that cDC-restricted precursors are generated in the bone marrow and migrate through the blood to the spleen and lymph nodes (Liu and Nussenzweig, 2010). However, the origins and developmental programs of thymic DC subsets remain obscure. Here, we have established unique transcriptional profiles for splenic and thymic DC subsets and we have used this information to identify candidate precursors in the thymus that are transcriptionally primed towards the DC lineages. We have also shown that DCs can develop in the presence of moderate levels of Dll4-Notch signaling, consistent with development of these cells within the thymic medulla, and that these levels of Dll4 are restrictive for myeloid development. Finally, we have demonstrated that DN1d and DN1e subsets have the capacity to generate thymic DCs in vivo in the context of a fully structured thymus. Our data supports a model in which the expression of Ccr7 and Ccr4 on DC-lineage primed DN1d

and DN1e thymocyte subsets allows them to preferentially migrate to the medulla after entering the thymus, where they are exposed to a microenvironment that favors DC development.

Our finding that a lower threshold of Dll-Notch signaling is required to inhibit myeloid development than to inhibit DC development is consistent with other studies showing that myeloid potential within intrathymic precursors is lost by the DN2 stage (Kawamoto et al., 1998), whereas DC potential is not lost until the DN2b stage (Masuda et al., 2007; Yui et al., 2010). However, although latent DC potential can be revealed in multiple precursors when they are removed from the thymic environment or from the inhibitory impact of high Dll-Notch signaling (Bell and Bhandoola, 2008; Feyerabend et al., 2009; Sambandam et al., 2005), the question of whether any of these constitute the normal precursor pools for thymic medullary DCs remains open. Moreover, there has been a long-standing debate about whether the DCs that exist within the thymus arise from a common precursor with both T-cell and DC potential, or whether they enter the thymus as committed DC progenitors. Studies using parabiotic mice suggested that CD8⁺ thymic DCs arise from intrathymic precursor(s), whereas CD8⁻ DCs and pDCs enter the thymus in a partially differentiated DC state (Li et al., 2009). Conversely, studies using an IL-7R fate-mapping mouse model revealed that nearly all splenic and thymic pDCs exhibited a history of IL-7R expression, indicative of a developmental transition through the common lymphoid progenitor stage, whereas thymic and splenic cDCs did not (Schlenner et al., 2010). However, DN1c, DN1d and DN1e subsets were not examined in these studies, leaving open the possibility that these cells might act as physiological precursors for differentiation into DCs within the thymus.
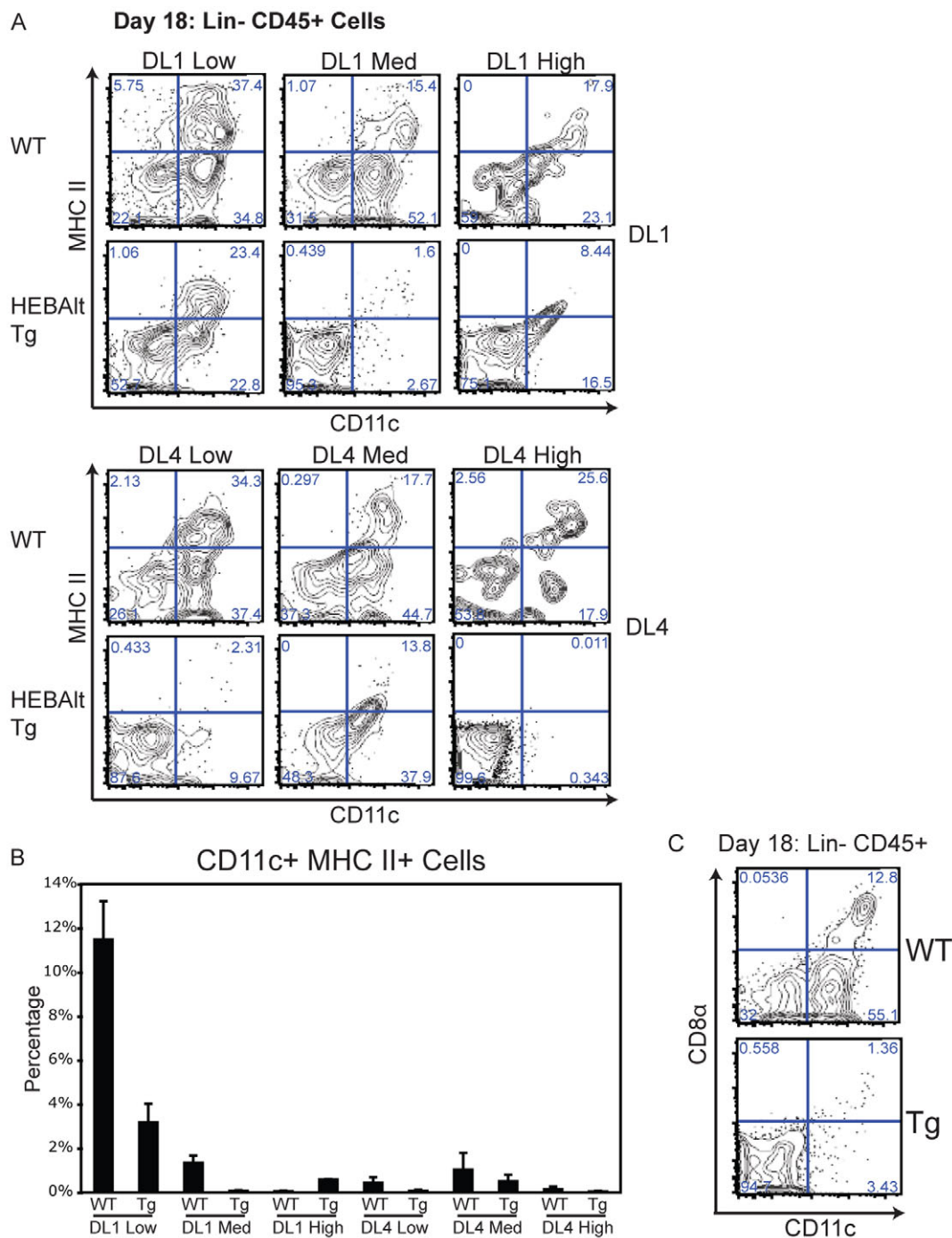
Fig. 8. HEBAlt inhibits DC development. (**A**) LSK cells from wild-type (WT) and HEBAlt-transgenic (Tg) bone marrow were co-cultured with the indicated stroma for 18 days. Cultures were supplemented with SCF, IL-7 and Flt3L. DCs were gated on FSC/SSC, DAPI⁻, CD45⁺ and Lin⁻ (Lin = CD19, F4/80, Gr1, NK1.1, Thy1) cells. Numbers represent percentages. (**B**) Percentage of mature DCs was calculated. Means were calculated from triplicate samples ± s.d. (**C**) CD8⁺ DCs (CD8α⁺ CD11c⁺) DCs were only generated on medium levels of DL4 from WT precursors.

Our work has defined a new set of DC precursors within the thymus. Previous studies showed that a low-CD4 precursor population in the thymus, which contains both DN1c and DN1d cells, can give rise to CD11c⁺ CD8⁺ cells in vivo following intravenous transfer (Ardavin et al., 1993; Wu et al., 1996). Moreover, a recent study elegantly showed that DN1c cells give rise to CD8⁺ CD207⁺ DCs in the thymus (Luche et al., 2011). Other studies have shown that DN1c and DN1d cells also have some B cell potential, suggesting that they are not all committed to the DC lineage (Porritt et al., 2004). Here, we have shown definitively that DN1d and DN1e cells, in addition to ETPs, can independently home to the thymus and differentiate into thymic medullary DCs. Introducing these cells intravenously confirmed that they could enter the thymus through the CMJ and traffic to the medulla, whereas intrathymic injections confirmed that the developmental events by which DN1 subsets became DCs could occur within the thymus. Our
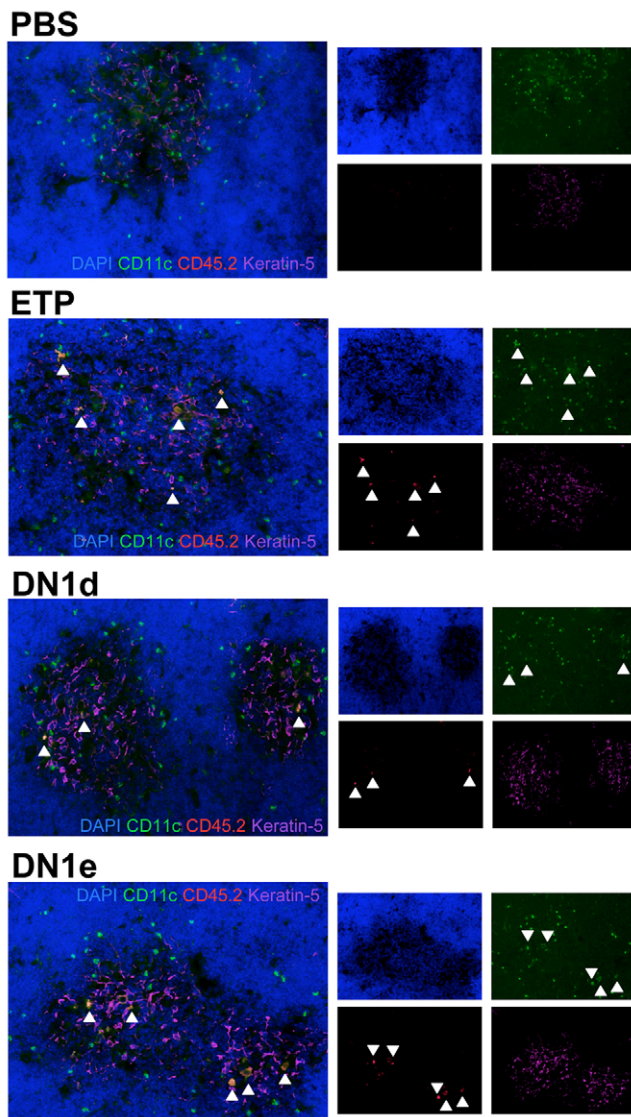
Fig. 9. Intrathymic DC development from DN1 subsets following intrathymic injections. PBS, ETP, DN1d or DN1e CD45.2 donor cells were injected intrathymically into nonirradiated CD45.1 recipient mice. Thymus sections were analyzed for the presence of donor-derived DCs (CD11c+ CD45.2+) in proximity to medullary thymic epithelial cells (keratin 5+) 5 days following injection. Cells were stained with DAPI (blue) and for CD11c (green), CD45.2 (red), keratin 5 (purple). Images are representative of triplicate experiments. Arrowheads indicate donor-derived DCs.
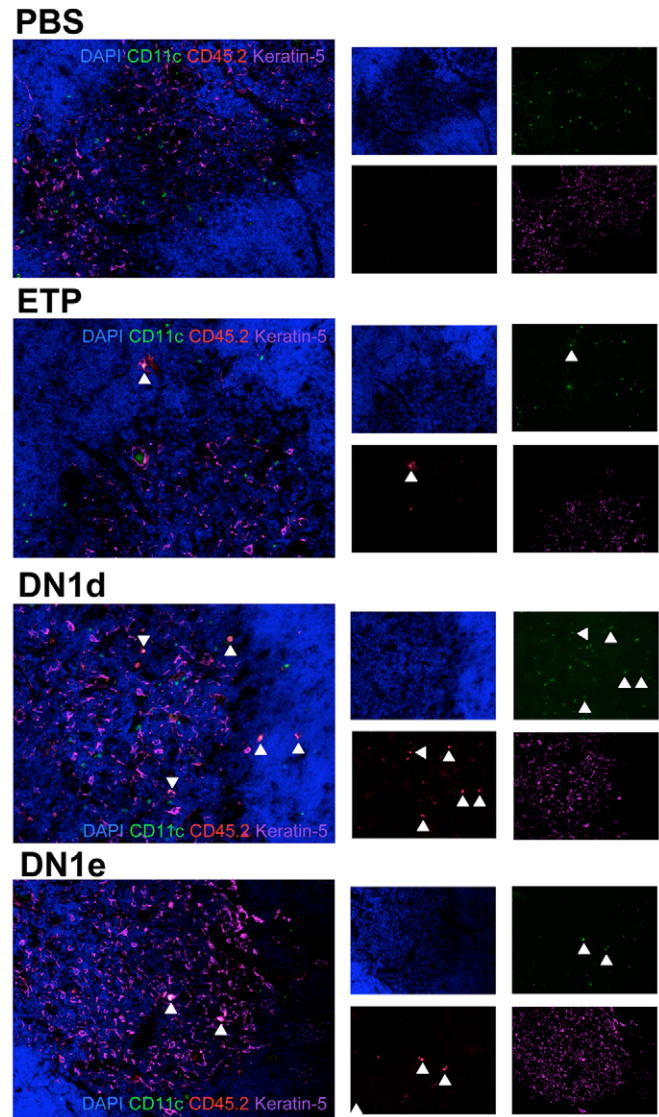

Fig. 10. Intrathymic DC development from ETP, DN1d and DN1e subsets following intravenous tail vein injections. PBS, ETP, DN1d or DN1e CD45.2 donor cells were injected intrathymically into nonirradiated CD45.1 recipient mice. Thymus sections were analyzed for the presence of donor-derived DCs (CD11c+ CD45.2+) in proximity to medullary thymic epithelial cells (keratin 5+) 7 days following injection. Cells were stained with DAPI (blue) and for CD11c (green), CD45.2 (red), keratin-5 (purple). Images are representative of triplicate experiments. Arrowheads indicate donor-derived DCs.

OP9-Dll co-culture results indicate that DCs, including CD8+ DCs, can be generated under conditions that mimic the levels of Dll4 found in the thymic medulla (Mohtashami et al., 2010). Collectively, these results strongly support the identity of DN1d and DN1e cells as intrathymic precursors of DCs. Their capacity to develop into other cell types within the thymus remains to be determined.

To test our model further, we conducted a preliminary analysis of thymic DC subsets in $Ccr7^{-/-}$ mice, but found that they did not exhibit any significant differences from wild-type mice. This might be owing, in part, to the ability of Ccr4 to respond to medullary Ccl22 and, thus, provide at least partial compensation for the loss of Ccr7. Moreover, others have shown that although $Ccr7^{-/-}$ mice

exhibit a more disorganized thymus, medullas are still present (Misslitz et al., 2004). Therefore, a $Ccr7^{-/-}$ $Ccr4^{-/-}$ mouse model will be required to test fully the hypothesis of chemokine-mediated migration of intrathymic precursors. In other preliminary studies, we placed DN1 subsets into fetal thymic organ cultures and found that they were unable to generate DCs (A.J.M. and M.K.A., unpublished observations), which strengthens the case that a mature intact thymic structure is necessary to create the conditions required for intrathymic DC development.

Our gene expression studies further support the ability of DN1d cells and DN1e cells to act as precursors of thymic DCs. The similarity between DN1d cells and thymic pDCs is particularly

striking: both have little or no PU.1, Id2 or IL7R, and high levels of Spi-B, Notch3 and Ccr7. DN1c cells, by contrast, have high levels of PU.1 and undetectable levels of Spi-B. Strong Dll-Notch signaling downregulates PU.1 and extinguishes the myeloid gene program (Franco et al., 2006). Interestingly, DN1c cells do not express Ccr7 or Ccr4 but also lack Notch1, which could render them ignorant to strong Dll. These differences suggest that there might be distinct routes by which intrathymic precursors adopt different DC lineage fates. Although ETPs clearly have DC potential, they do express high levels of Notch1 (Tan et al., 2005). They also lack Ccr7, which is, in part, responsible for directing cells towards the medulla and away from the cortex. PU.1 is required early in hematopoiesis to activate Flt3, which is crucial for DC development (Carotta et al., 2010). However, our studies suggest that in thymic pDCs (and perhaps splenic pDCs), this function might be mediated by Spi-B, thus allowing Flt3 expression without activation of PU.1-driven myeloid genes. The influence of graded Dll-Notch signaling on Spi-B expression and pDC development is unclear (Dontje et al., 2006; Olivier et al., 2006). However, our results indicate that although Dll-Notch signaling is weak in DN1d cells in spite of the presence of Notch3, mature thymic pDCs are receiving strong Dll-Notch signals. Therefore, collaboration between Spi-B and Notch factors might be involved in pDC maturation within the thymus.

We also investigated the expression of the E proteins HEBCan and HEBAlt in thymic DCs. Although the impact of HEB-deficiency on thymic DC development has not been formally tested, our results suggest that studies on DC development using currently available HEB-knockout mouse models, in which both HEBAlt and HEBCan are deleted, could be misleading. We found that thymic pDCs express very high levels of HEBCan, but lack HEBAlt altogether, whereas both thymic cDC subsets have high Id2 expression. Moreover, transgenic expression of HEBAlt interferes with DC development even under conditions of low Dll-Notch signaling. These results suggest that whereas HEBCan might play a role in segregating the pDC and cDC phenotypes, HEBAlt acts downstream of high Dll-Notch signaling to constrain DC potential in developing T cells.

Our results support a model in which thymic precursors are directed to different microenvironments within the cortex or the medulla depending on their expression of Ccr7 and Ccr4. The way in which they respond to those environments are regulated by their repertoire of receptors and the transcription factors that they express, such that ETPs respond to high Dll by activating a T-lineage program, whereas DN1d and DN1e cells respond to moderate levels of Dll4 by generating immature DCs. More work will be needed to confirm the precursor-product relationships of DN1d and DN1e cells with specific subsets of thymic DCs, and to evaluate their latent T-cell potential, but our results clearly show that these cells are functionally equipped with the gene programs necessary for differentiation into the DC lineage within the thymus.

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Table S1. Sequences of gene-specific primers used for qRT-PCR experiments

Gene	Sequence	Product size (bp)
PU.1	S: ACACCATGTCCACAACAACGA A: GTGCATCTGTTCCAGCTCCAT	100
Spi-B	S: CTGCAAGCCCTTCAGTTACC A: AAAGGCAGCAGTAGCAGGAT	104
Id2	S: TCCGGTGAGGTCCGTTAGGAA A: GCTTGGAGTAGCAGTCGTTCATG	122
HEBAlt	S: GTGCTTATCCTGTCCCTGGAATG	131
HEBCan	S: TCATTCTCTGCCACAAGTTCGA	177
HEB*	A: TGGCTTGGGAGATGGGTAAC	–
Notch1	S: AGTGCAACCCCCTGTATGAC A: CTAGGCCATCCCACTCACAT	98
Notch2	S: CGTGTGAGAATGCTGCTGTT A: TTGTGGCAGACACCATTGTT	148
Notch3	S: GCCGTACGGGTAGTCACTGT A: CATAGCCAGCTGGACACTCA	120
Notch4	S: GCCCGATGTGAGAAAGACAT A: GGGGTCACTCAGACATTCGT	156
Ccr4	S: AGGCAAGGACCCTGACCTAT A: GCAGTACGTGTGGTTGTGCT	122
Ccr7	S: GTGGTGGCTCTCCTTGTCAT A: AGTCCACCGTGGTATTCTCG	88
Hes1	S: TCCTGACGGCCAATTTGC A: GGAAGGTGACACTGCGTTAGG	121

PCR product size of each pair of primers is indicated.

S, sense primer; A, antisense primer.

HEBCan and *HEBAlt* have the same antisense primer.