RBPJ-dependent Notch signaling initiates the T cell program in a subset of thymus-seeding progenitors

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Abstract

T cell specification and commitment requires Notch signaling. Although the requirement for Notch signaling during intrathymic T cell development is known, it is still unclear whether the onset of T cell priming can occur in a pre-thymic niche and whether RBPJ-dependent Notch signaling has a role during this event. Here we established an Rbpj-inducible system that allowed the temporal and tissue-specific control of the responsiveness to Notch in all hematopoietic cells. Using this system, we found that Notch signaling was required prior to the early T cell progenitor stage in the thymus. Lymphoid-primed multipotent progenitors in the bone marrow underwent Notch signaling with Rbpj induction, which inhibited development towards the myeloid lineage in thymus-seeding progenitors. Thus, our results indicated that the onset of T cell differentiation occurred in a pre-thymic setting, and that Notch played an important role during this event.
Lin−Sca-1+c-Kit+Flt-3hi lymphoid-primed multipotent progenitors (LMPPs)\textsuperscript{13} and Lin−Sca-1lo-c-KitloFlt-3loIL-7Rα+ common lymphoid progenitors (CLPs)\textsuperscript{14}. Upon entry into the thymus, TSPs are referred to as early T cell progenitors (ETPs) and are found within CD4−CD8− double negative (DN)1a/b cells\textsuperscript{15}, which are defined as Lin−CD44+CD25−c-KithiCD24−lo. ETPs efficiently develop into T cells and have limited B cell potential\textsuperscript{15}, suggesting that TSPs receive Notch instructive signals in a pre-thymic setting or immediately after thymic entry.

To further elucidate the role of Notch in this regard, here we generated an Rbpj-inducible mouse model, which renders all hematopoietic cells unresponsive to Notch signaling and also allows the establishment of their responsiveness in an inducible and temporally-regulated manner. The system reported known Notch-dependent lineage decisions in the hematopoietic system and allowed us to address the temporal and tissue-specific requirements for Notch during T cell development. We found that Notch provided a key pre-thymic signal for the development of ETPs that could generate T cells in the thymus. In addition, we found that BM LMPPs, which represent the likely candidate for adult TSPs\textsuperscript{16}, underwent Notch signaling in the BM, preventing myeloid lineage skewing within a subset of LMPPs. These findings establish a pre-thymic role for Notch in directing the generation of T lineage competent TSPs.

**Results**

**RBPJ\textsuperscript{ind} mice allow for controlled Notch responsiveness.**

Genetic ablations of Dll1, Dll4, Jag1, Notch1, Notch2 and Rbpj result in embryonic or neonatal lethality in mice\textsuperscript{17, 18, 19, 20, 21, 22}. To overcome these limitations and to allow the induction and temporal control of Notch responsiveness, and based on the fact that RBPJ interacts with all four Notch receptors\textsuperscript{23}, we generated a mouse model that incorporated conditional deletion of Rbpj and inducible expression of a transgene encoding RBPJ. To conditionally delete Rbpj in hematopoietic cells, RBPJ\textsuperscript{f/f} mice\textsuperscript{11} were bred to Vav-iCre transgenic (Tg) mice\textsuperscript{24}, generating RBPJ\textsuperscript{f/f}Vav-iCre mice (Supplementary Fig. 1a). To induce Notch responsiveness in Rbpj-deficient hematopoietic cells, we generated RBPJ-HA Tg mice, in which expression of an HA-tagged RBPJ transgene is under the control of a tetracycline responsive element. Fibroblasts from these mice showed reverse tetracycline-controlled transactivator (rtTA)- and doxycycline (Dox)-dependent expression of the RBPJ-HA transgene (Supplementary Fig. 1b). ROSA26-rtTA mice, in which expression of rtTA is coupled to that of GFP upon Cre-dependent removal of a loxP-stop-loxP cassette within the ROSA26 locus\textsuperscript{25}, were bred to RBPJ-HA Tg mice, generating Tet\textsuperscript{on}RBPJ-HA mice (Supplementary Fig. 1a). RBPJ\textsuperscript{f/f}Vav-iCre mice were bred to Tet\textsuperscript{on}RBPJ-HA mice to generate RBPJ\textsuperscript{f/f}Vav-iCreTet\textsuperscript{on}RBPJ-HA mice (hereafter RBPJ\textsuperscript{ind}), in which expression of RBPJ-HA in hematopoietic cells can be regulated through presence or absence of Dox \textit{in vivo} (Supplementary Fig. 1a).

Conditional deletion of RBPJ in RBPJ\textsuperscript{f/f}Mx-Cre mice leads to arrest of T lymphopoiesis at the DN1 stage, loss of CD4+ and CD8+ T cells and B cell accumulation in the thymus\textsuperscript{11}. Compared to RBPJ-sufficient mice (RBPJ\textsuperscript{f/+}Vav-iCreTet\textsuperscript{on}RBPJ-HA; hereafter RBPJ\textsuperscript{Ctr}), the thymus of RBPJ\textsuperscript{ind} mice not treated with Dox (hereafter RBPJ\textsuperscript{ind}-noDox) displayed a block
at the CD44+CD25−DN1 stage and a reduction or near absence of c-KithiCD24−lo DN1a/b cells (Fig. 1a), indicating Notch-RBPJ is required for the generation or maintenance of ETPs. Development of CD4 and CD8 double positive (DP) and single positive (SP) cells, as well as γδ T cells, was abrogated, along with the detection of B220+CD19+ B cells and a significant decrease in thymocyte cellularity in the thymus of RBPJind-noDox mice compared to RBPJCtr mice treated with Dox (hereafter RBPJind-Dox mice) (Fig. 1a,b). In RBPJind mice treated with Dox for 6 weeks (hereafter RBPJind-Dox6wk) we detected progression of DN1 cells to CD44+CD25−DN2, CD44−CD25+DN3 and CD44−CD25−DN4 stages, an increase in the percentage of DN1a/b cells (~4-fold), the presence of DPs, SPs and γδ T cells, a decrease in the percentage of B cells (~35-fold), as well as a significant restoration in thymocyte cellularity compared to RBPJind-noDox mice (Fig. 1a,b). RBPJind mice treated with Dox for 3 weeks and analyzed 3 weeks after stopping the Dox treatment (hereafter RBPJind-Dox3wk-noDox3wk) once again displayed a block at the DN1 stage, lacked DN1a/b cells almost entirely and lacked DPs, while CD4+ and CD8+ SPs and γδ T cells were still present (Fig. 1a). The percentage of thymic B cells was similar to that in RBPJind-noDox mice, and thymocyte cellularity was decreased compared to RBPJCtr-Dox and RBPJind-Dox6wk mice, but higher compared to RBPJind-noDox mice (Fig. 1a,b).

The organization of cytokeratin 8 (K8)+β5t+ cortical TECs (cTECs) and K5+UEA-1+ medullary TECs (mTECs) was disrupted in the thymus of RBPJind-noDox mice compared to RBPJCtr-Dox mice, while RBPJind-Dox6wk mice displayed a restoration of thymic architecture (Fig. 2), indicating that lymphopoiesis induced mature mTEC and cTEC differentiation. K5+UEA-1+ mTECs were detected along with K5+K8− immature cTECs in RBPJind-Dox3wk-noDox3wk mice, while mature β5t+ cTECs were absent (Fig. 2), suggesting that maintenance of mature cTECs was dependent on constant supply of T cell progenitors. The thymus of RBPJind-noDox mice contained B220+ B cells that were not confined to the cortico-medullary junction (CJM) or the perivascular space (PVS; indicated by Tomato Lectin+ endothelial cells), as in RBPJCtr-Dox mice, but instead were dispersed throughout the thymus parenchyma (Fig. 2). Similar to RBPJCtr-Dox mice, B cells were restrained to the CJM and PVS in RBPJind-Dox6wk mice, while in RBPJind-Dox3wk-noDox3wk mice, B cells were localized in the expanded cortical niche, similar to RBPJind-noDox mice (Fig. 2).

Sorted BM Lin−Sca-1+c-Kit+ cells (LSKs) from RBPJind-noDox mice cultured on OP9 cells expressing Delta-like-1 (OP9-DL1) for 12 days without Dox did not differentiate into CD44+/−CD25+ DN2/DN3 cells, respectively, and became CD19+ B cells, in contrast to LSKs from RBPJCtr mice (Supplementary Fig. 1c), indicating that controlled Notch responsiveness in RBPJind mouse thymus was recapitulated in vitro. RBPJind-noDox LSKs cultured with Dox for 12 days developed into DN2/DN3 cells, while B cells were not detected (Supplementary Fig. 1c), similar to RBPJCtr LSKs. Culture of RBPJind-noDox LSKs with Dox for 8 days followed by removal of Dox for 4 days led to loss of DN2/DN3 development, without the emergence of B cells (Supplementary Fig. 1c), suggesting that initial Notch responsiveness eliminated the B lineage potential. RBPJind-noDox LSKs cultured on OP9 cells with Dox for 8 days did not give rise to DN2/DN3 cells (Supplementary Fig. 1d), indicating that RBPJ-HA transgene expression did not induce T cell development in the absence of Notch ligands.
CD4+ T cells, CD8+ T cells and γδ T cells were detected in the spleens of RBPJind-Dox6wk mice, but not in RBPJind-noDox mice (Supplementary Fig. 2a). These three T cell populations were detected in the spleen of RBPJind-Dox3wk-noDox3wk mice (Supplementary Fig. 2a), indicating that Notch was dispensable for the survival of mature T cells. B220+IgM+CD21hiCD23− marginal zone B (MZB) cells were only detected in RBPJind-Dox6wk mice (Supplementary Fig. 2a), confirming that Notch directs the survival of MZB cells.28, 29 No significant differences in splenocyte cellularity were observed between the mouse groups analyzed (Supplementary Fig. 2b). These results indicated that the RBPJind system allowed for temporal regulation of Notch responsiveness in vivo and in vitro.

Functional ETPs are absent in the RBPJind-noDox mouse thymus.

Because the number of DN1a/b cells was markedly reduced in RBPJind-noDox mice, we investigated whether maintenance of ETPs required intrathymic Notch signals for their survival or pre-thymic Notch signals for their emergence. To address this, we analyzed the T cell developmental kinetics in RBPJind-Dox mice. CD44+CD25+ DN2 cells were first detected at day 5 post-Dox (Fig. 3a). CD44−CD25+ DN3 cells were detected by day 7, while CD44−CD25− DN4 cells and CD4+CD8+ DPs appeared robustly by day 11 (Fig. 3a–c), reflecting expected kinetics. Two weeks post-Dox, the distribution of DNs, DPs and SPs in the RBPJind-Dox mouse thymus began to resemble steady-state wild-type thymus (Fig. 3a–c). Because it took 5 days for DN2 cells to appear in the RBPJind-Dox mouse thymus, we investigated whether RBPJind-noDox mice lacked ETPs. Sorted ETPs and LMPPs, which give rise to TSPs and thus ETPs,16 from RBPJind mouse differentiated into CD44+CD25+ DN2 cells within 1 day and 3 days, respectively, when cultured on OP9-DL4 cells with Dox (Supplementary Fig. 3a), indicating that if ETPs were present in RBPJind-noDox mice, then DN2 cells would have appeared within day 1-3 post-Dox, and that the delay in generation of DN2 cells in RBPJind-Dox mice suggested an absence of ETPs prior to Dox treatment. ~40% of RBPJind-Dox thymocytes were RBPJ-HA+ within 4 hours post-Dox, ~80% by 8 hours, and ~100% by 24 hours (Supplementary Fig. 3b), indicating that the delayed emergence of DN2 cells in the thymi of RBPJind-Dox mice was not due to a lag in RBPJ-HA transgene expression.

To investigate whether the delayed appearance of DN2 cells in RBPJind-Dox mice was due to disrupted thymic architecture, we performed mixed BM chimeras by injecting equal numbers of CD45.2−(GFP−) wild-type and CD45.2+(GFP+) Supplemental Fig. 1a) RBPJind-noDox BM cells into lethally irradiated CD45.1+ wild-type mice. Four weeks after transfer, during which recipient thymic structure was maintained by wild-type donor T cells, recipient mice were Dox-treated and appearance of RBPJind-Dox DN2 cells assessed at day 2, 4 and 6 post-Dox initiation. CD45.2+(GFP+) wild-type and CD45.2+(GFP+) RBPJind-noDox BM cells, including LSK-Flt-3hi LMPPs, were detected at day 0, prior to Dox treatment (Fig. 4a). Recipient thymi displayed proper segregation of K8+ cTECs and K5+ mTECs at day 0, with CD45.2+(GFP+) wild-type cells showing normal development of DN2, DN3 and DN4 cells, while CD45.2+(GFP+) RBPJind-noDox cells were blocked at the DN1 stage (Fig. 4b). Notably, CD45.2+(GFP+) RBPJind-Dox DN2 cells were first detected at day 6 post-Dox,
compared to CD45.2\(^+\) (GFP\(^-\)) wild-type DN2 cells which were present at all time-points (Fig. 4b). These results indicated that even within a normal thymic structure, RBPJ\(^{ind}\)-noDox cells did not appear to give rise to ETPs.

**Emergence of functional ETPs depends on Notch signaling.**

To further investigate whether Notch signaling initiates T cell differentiation pre-thymically, we examined the T lineage potential of the few Lin\(^-\)CD44\(^-\)c-Kit\(^{hi}\)CD24\(^{-}\)lo DN1a/b cells in the thymi of RBPJ\(^{ind}\)-noDox mice. We used stringent criteria for the isolation of c-Kit\(^{hi}\) DN1a/b cells to exclude c-Kit\(^{lo}\) DN1c cells (Supplementary Fig. 4). Sorted DN1a/b cells and BM LSKs from RBPJ\(^{Ctr}\) and RBPJ\(^{ind}\)-noDox mice were cultured on OP9-DL4 cells in the presence or absence of Dox. RBPJ\(^{Ctr}\) LSKs and DN1a/b cells cultured on OP9-DL4 cells gave rise to CD44\(^-\)CD25\(^+\) DN3 cells at day 8 and CD4\(^+\)CD8\(^+\) DPs at day 14, irrespective of Dox (Fig. 5a,b). RBPJ\(^{ind}\)-noDox LSKs on OP9-DL4 cells developed into DN3 cells by day 8 and DPs by day 14 only in the presence of Dox, while RBPJ\(^{ind}\)-noDox DN1a/b cells on OP9-DL4 cells did not develop into T lineage cells despite provision of Dox (Fig. 5a,b). These observations indicated that the few thymic DN1a/b cells in RBPJ\(^{ind}\)-noDox mice were not T cell progenitors, and that the delayed appearance of DN2 cells following Dox treatment could reflect the requirement to recruit TSPs that had experienced Notch signals prior to thymic entry. To test this, we sorted DN1a/b cells from RBPJ\(^{ind}\) mice treated with Dox for 6 days (hereafter RBPJ\(^{ind}\)-Dox\(^{6d}\)) and cultured them on OP9-DL4 cells in the presence or absence of Dox. RBPJ\(^{ind}\)-Dox\(^{6d}\) DN1a/b cells differentiated into DN3 cells at day 8 and DPs at day 14 on OP9-DL4 cells supplemented with Dox, in contrast to RBPJ\(^{ind}\)-noDox DN1a/b cells which failed to do so (Fig. 5a,b). RBPJ\(^{ind}\)-Dox\(^{6d}\) LSKs differentiated into DN3 cells and DPs on OP9-DL4 cells supplemented with Dox, similar to RBPJ\(^{ind}\)-noDox LSKs (Fig. 5a,b). These results indicated that thymic appearance of functional ETPs requires Notch signaling pre-thymically.

We also sorted thymic Lin\(^-\)CD44\(^+\)CD25\(^-\)c-Kit\(^{lo}\)CD24\(^+\) DN1c cells and cultured them on OP9-DL4 cells with or without Dox. RBPJ\(^{Ctr}\) and RBPJ\(^{ind}\)-Dox\(^{6d}\) DN1c cells did not develop into DN3 cells (Fig. 5c), consistent with observations that these cells have inefficient T cell potential\(^{15}\). RBPJ\(^{ind}\)-noDox DN1c cells also did not develop into DN3 cells, albeit CD44\(^{lo}\)CD25\(^-\) B cells were detected in the absence of Dox (Fig. 5c). These experiments excluded the possibility that c-Kit\(^{lo}\) TSPs with T cell potential entered the thymus of RBPJ\(^{ind}\)-noDox mice in the absence of Notch responsiveness. RBPJ\(^{Ctr}\), RBPJ\(^{ind}\)-noDox and RBPJ\(^{ind}\)-Dox\(^{6d}\) BM LSKs on OP9 cells developed into CD19\(^+\) B cells at day 14 (Fig. 5d), while RBPJ\(^{Ctr}\) and RBPJ\(^{ind}\)-Dox\(^{6d}\) DN1a/b cells on OP9 did not develop into B cells (Fig. 5d), as expected\(^{15}\). RBPJ\(^{ind}\)-noDox DN1a/b cells on OP9 also lacked B cell potential (Fig. 5d). These results indicated that the loss of T cell differentiation from RBPJ\(^{ind}\)-noDox DN1a/b cells was not due to their divergence to the B cell lineage.

**RBPJ\(^{ind}\)-noDox DN1a/b cells have myeloid bias.**

To determine whether thymic DN1a/b cells from RBPJ\(^{ind}\)-noDox mice had dendritic cell (DC) potential\(^{30}\), sorted BM LSKs and DN1a/b cells from RBPJ\(^{Ctr}\), RBPJ\(^{ind}\)-noDox and RBPJ\(^{ind}\)-Dox\(^{6d}\) mice were cultured on OP9-DL1\(^{lo}\) cells with Dox, which support DC differentiation. RBPJ\(^{Ctr}\) DN1a/b cells differentiated into CD11c\(^+\)MHC-II\(^+\) DCs at day 8.
to CD11b^{+}MHC-II^{−} cells by day 8, suggesting a strong myeloid potential, while RBPJ^{Ctr} DN1a/b cells had a limited myeloid potential (Supplementary Fig. 5a). BM LSKs from all mice developed into myeloid cells with similar efficiency (Supplementary Fig. 5a). RBPJ^{ind}-noDox and RBPJ^{ind}-Dox^{6d} DN1a/b cells generated less MHC-II^{lo}B220^{+} plasmacytoid DCs and more MHC-II^{hi}B220^{−}CD11b^{+} myeloid DCs than RBPJ^{Ctr} DN1a/b cells, while BM LSK from all mice gave rise to these subsets with similar efficiency (Supplementary Fig. 5b).

To investigate the transcriptional signature of thymic DN1a/b cells, we performed RNA sequencing analysis on sorted RBPJ^{Ctr}, RBPJ^{ind}-noDox and RBPJ^{ind}-Dox^{6d} DN1a/b cells. Gene expression analysis between samples (≥2-fold different, P<0.05) identified 66 genes differentially expressed between RBPJ^{Ctr} and RBPJ^{ind}-noDox. RBPJ^{Ctr} DN1a/b cells had high expression of Notch-target genes (Notch1, Hes1) and T lineage genes (Tcf7, Lck^{31, 32}), while RBPJ^{ind}-noDox DN1a/b cells had high expression of myeloid-specific genes (Mpo, Ctsg, Elane, Prtn3^{33, 34}), four genes were enriched in RBPJ^{ind}-Dox^{6d} compared to RBPJ^{Ctr} (Mfsd2b, Gata2, Apoe, Asph), and nine genes were enriched in RBPJ^{ind}-Dox^{6d} compared to RBPJ^{ind}-noDox (Notch1, Tcf7) (Fig. 6a,b and Supplementary Tables 1–3). Genes that were highly expressed in RBPJ^{Ctr} DN1a/b cells (Hes1, Tcf7) or RBPJ^{ind}-noDox DN1a/b cells (Mpo, Elane) were intermediately expressed in RBPJ^{ind}-Dox^{6d} DN1a/b cells (Fig. 6a), likely due to a mix of Notch-signaled, T cell-competent ETPs and myeloid-specific progenitors in the thymus of these mice. GO analysis on transcripts enriched in RBPJ^{Ctr} or RBPJ^{ind}-noDox DN1a/b cells compared to each other determined that pathways involving genes enriched in RBPJ^{Ctr} included “T cell differentiation” and “αβ T cell differentiation”, while pathways involving genes enriched in RBPJ^{ind}-noDox included “myeloid cell differentiation” and “myeloid cell homeostasis” (Fig. 6c and Supplementary Table 4). These results suggested that the few thymic DN1a/b cells from RBPJ^{ind}-noDox mice lacked T cell potential, but were instead strongly biased toward the myeloid lineage.

**BM LMPPs undergo Notch signaling in RBPJ^{ind}-Dox mice.**

Because RBPJ^{ind}-noDox mice lacked functional ETPs, we used flow cytometry to examine whether Notch signaling affected BM progenitors with TSP potential, including HSCs, MPPs, LMPPs and CLPs (Supplementary Fig. 6a) in RBPJ^{Ctr} and RBPJ^{ind}-noDox mice. CD62L^{+} LMPPs and Ly6D^{-} CLPs were also analyzed, as these were described to further refine a TSP population^{16, 36}. We observed slight but significant decreases in the numbers of MPPs and LMPPs in RBPJ^{ind}-noDox compared to RBPJ^{Ctr}, while the numbers of HSCs and CLPs were not significantly different (Fig. 7a). Additionally, we observed significant decreases in the percentage and number of CD62L^{+} LMPPs in RBPJ^{ind}-noDox compared to RBPJ^{Ctr}, while the percentage and number of Ly6D^{-} CLPs were not significantly different (Fig. 7b).
We next examined which progenitors up-regulated the expression of Notch-target genes upon gaining Notch responsiveness by qPCR analysis. *Hes1* was expressed in RBPJ<sup>Ctr</sup> HSCs, MPPs, LMPPs and CLPs, while its expression was low in all progenitor subsets from RBPJ<sup>ind</sup>-noDox BM, with LMPPs and CLPs showing significant decreases in RBPJ<sup>ind</sup>-noDox compared to RBPJ<sup>Ctr</sup> (Fig. 7c). *Hes1* expression was not significantly changed in RBPJ<sup>ind</sup>-Dox<sup>6d</sup> HSCs, MPPs and CLPs compared to those from RBPJ<sup>ind</sup>-noDox BM, but its expression was significantly increased in RBPJ<sup>ind</sup>-Dox<sup>6d</sup> LMPPs compared to RBPJ<sup>ind</sup>-noDox LMPPs (Fig. 7c). *Notch1* expression was similar in all progenitor subsets between RBPJ<sup>Ctr</sup>, RBPJ<sup>ind</sup>-noDox and RBPJ<sup>ind</sup>-Dox<sup>6d</sup> mice (Fig. 7c). Sorted RBPJ<sup>ind</sup>-noDox LMPPs differentiated into CD44<sup>−</sup>CD25<sup>+</sup> DN3 cells on OP9-DL4 cells with Dox, and did not generate more CD19<sup>+</sup> B cells on OP9 cells compared to RBPJ<sup>Ctr</sup> and RBPJ<sup>ind</sup>-Dox<sup>6d</sup> LMPPs (Supplementary Fig. 6b). RBPJ<sup>Ctr</sup> and RBPJ<sup>ind</sup>-Dox<sup>6d</sup> LMPPs and CLPs on OP9-DL4 with Dox differentiated into CD44<sup>+</sup>CD25<sup>+</sup> DN2 cells with similar kinetics as RBPJ<sup>ind</sup>-noDox LMPPs and CLPs (day 3) (Supplementary Fig. 6c), suggesting that BM Notch signals do not position LMPPs further ahead the T cell development path. Additionally, limiting dilution analysis of RBPJ<sup>ind</sup>-noDox CD62L<sup>+</sup> LMPPs and CLPs on OP9-DL4 cells with Dox showed the same T cell progenitor frequencies (1/1.22-1/1.44) as the RBPJ<sup>Ctr</sup> counterparts (Supplementary Fig. 7a,b). These results suggested that Notch-unresponsive RBPJ<sup>ind</sup>-noDox LMPPs undergo Notch signaling in the BM and can effectively initiate the T cell program.

**Notch signaling inhibits myeloid potential in BM TSPs.**

To investigate the effect of BM Notch signals in TSPs, we performed single-cell RNA sequencing analysis on sorted LSK-Flt-3<sup>hi</sup> LMPPs from RBPJ<sup>Ctr</sup> (2614 cells), RBPJ<sup>ind</sup>-noDox (2729 cells), RBPJ<sup>ind</sup>-Dox<sup>3d</sup> (1268 cells) and RBPJ<sup>ind</sup>-Dox<sup>6d</sup> (2074 cells) mice. t-SNE analysis of combined cells from all 4 mice identified 8 clusters (cluster 1-8) (Fig. 8a), and gene expression levels were analyzed to identify transcripts enriched in each cluster (≥1.5-fold different, P<0.05) (Supplementary Tables 5–10). Cluster 1 and 2 were not enriched in any particular lineage genes and thus were likely undifferentiated, “stem-like” LMPPs (Fig. 8a). Cluster 3 showed enriched expression of common myeloid progenitor (CMP) genes, e.g., *Cnnpa*, *Cenb2*, *Cd20* and *Tpx2*<sup>34</sup> (Supplementary Table 5). Cluster 4 showed enriched expression of conventional DC genes, e.g., *H2-Eb1*, *H2-Aa*, *Cd74* and *H2-Ab1*<sup>34</sup> (Supplementary Table 6). Cluster 5 showed enriched expression of genes highly expressed by RBPJ<sup>Ctr</sup> thymic DN1a/b cells, e.g., *Mn1*, *Emp1*, *Cd33* and *Smad7* (Supplementary Table 7), suggesting these cells possessed T cell progenitor function. Cluster 6 showed enriched expression of plasmacytoid DC genes, e.g., *Isg15*, *Irf7*, *Ifi1* and *Igfp1<sup>37</sup> (Supplementary Table 8). Cluster 7 showed enriched expression of CLP genes, e.g., *Il7r* and *Rag1*, but also B cell genes, e.g., *Ly6d* and *Ebf1<sup>38</sup> (Supplementary Table 9). Cluster 8 showed enriched expression of granulocyte-monocyte progenitor (GMP) genes, e.g., *S100a8*, *S100a9*, *Ly6g* and *Lyz2*<sup>34</sup> (Supplementary Table 10).

We next focused on Sell<sup>+</sup> LMPPs and analyzed expression of three important thymus-homing genes: *Selplg* (PSGL-1), *Ccr9* (CCR9) and *Ccr7* (CCR7)<sup>39, 40</sup>. Total LMPPs and LMPPs expressing Sell, Selplg, Ccr9 or Ccr7 from RBPJ<sup>Ctr</sup> and RBPJ<sup>ind</sup>-Dox<sup>6d</sup> mice had significantly higher *Hes1* expression compared to cells from RBPJ<sup>ind</sup>-noDox mice.
Supplementary Fig. 8a and Supplementary Table 11). Selplg expression was not restricted to a particular cluster, but its expression in cluster 5 overlapped with Ccr9 expression, which was largely restricted to cluster 5 (Fig. 8a). Ccr7 expression was not restricted to a particular cluster, but its expression in cluster 5 overlapped with Selplg and Ccr9 expression, and only Sell+ LMPPs from RBPJCtr, RBPJind-Dox3d and RBPJind-Dox6d BM expressed all three genes (Fig. 8a). Of these cells, 100% RBPJCtr, 50% RBPJind-Dox3d and 75% RBPJind-Dox6d cells were located in cluster 5 (Fig. 8b and Supplementary Fig. 8b). Because the proteins encoding these genes contribute to thymus-homing and are expressed by TSPs and ETPs, these observations suggested that BM Notch signals in LMPPs induce thymus-seeding capacity. However, Sell+Selplg+Ccr9+ LMPPs were still detected in RBPJind-noDox mice (Fig. 8b). Of the 35 Sell+Selplg+Ccr9+ LMPPs in cluster 5, more cells were from from RBPJCtr (16) and RBPJind-Dox6d (12) mice and each formed a clear cluster, compared to fewer cells from RBPJind-noDox mice (5), which did not form a defined cluster (Fig. 8b). Of the overall distribution of Sell+Selplg+Ccr9+ LMPPs, majority of RBPJCtr cells located to cluster 5 (16/24), while RBPJind-noDox cells showed decreased distribution to cluster 5 (5/14) and increased distribution to cluster 3 (3/24 versus 5/14, respectively), indicating CMP-GMP potential (Fig. 8b). RBPJind-Dox6d Sell+Selplg+Ccr9+ LMPPs displayed reduced distribution to cluster 3 compared to RBPJind-noDox (1/24) and, more similar to RBPJCtr, half of RBPJind-Dox6d cells located to cluster 5 (12/24) (Fig. 8b). Distribution of RBPJind-noDox, but not RBPJind-Dox6d, Sell+Selplg+Ccr9+ LMPPs to cluster 5 significantly deviated (P<0.05) from RBPJCtr (Supplementary Table 12).

Among the genes up-regulated (≥1.5-fold, P<0.05) in RBPJind-noDox Sell+Selplg+Ccr9+ LMPPs compared to RBPJCtr LMPPs (Supplementary Table 13), we detected increased expression of Egr1, which was reported to bias MPPs and LMPPs toward the myeloid fate, Klf4 and Klf2, which control differentiation of myeloid progenitors to Ly6C+ and Ly6C− monocytes, respectively, and the AP-1 complex factors, Fos, Fosb, Jun and Junb, which control development of specific myeloid lineages (Fig. 8c). Expression of these genes, including Egr1, Klf4 and Klf2, was reduced in RBPJind-Dox6d Sell+Selplg+Ccr9+ LMPPs compared to RBPJind-noDox LMPPs, and as such, the transcriptional profile of RBPJind-Dox6d was more similar to RBPJCtr (Fig. 8c and Supplementary Tables 14–15). GO analysis on transcripts enriched in RBPJind-noDox Sell+Selplg+Ccr9+ LMPPs compared to RBPJCtr LMPPs determined that pathways involving these genes included “myeloid leukocyte differentiation” (Supplementary Table 16). Thus, Sell+Selplg+Ccr9+ LMPPs were biased toward the myeloid fate in RBPJind-noDox mice, suggesting that BM Notch signaling in TSPs acts to suppress myeloid potential.

**Discussion**

Here we generated RBPJind mice in which hematopoietic cells could be toggled to become responsive to Notch signaling. Using this system, we found that Notch signaling in BM TSPs repressed the myeloid potential of these cells and allowed the up-regulation of a transcriptional program that could coordinate their migration to the thymus. These observations indicated that Notch plays a role in T cell differentiation prior to arrival of TSPs in the thymus.
Mice withDll4conditional deletion have fewer ETPs, but evidence for pre-thymic Notch signals in directing T lineage differentiation in TSPs was not documented. Recently, it was shown that BM osteoblasts express DLL4 and appeared to provide Notch signals for the generation of Ly6D− CLPs, which are candidate TSPs. In our study, we could not detect functional ETPs in the thymus of RBPJind-noDox mice. Thus, Notch signaling appeared to be required to generate TSPs that give rise to functional ETPs. Notch signaling is thought to be the crucial determinant of T versus B cell decisions, as disruption in Notch signaling led to B cell accumulation in the thymus. However, more recent evidence suggests that inhibition of Notch signaling in ETPs converts them to DCs rather than B cells. In our study, the few thymic DN1a/b cells in RBPJind-noDox mice had no T nor B cell potential, but displayed strong myeloid potential and expressed myeloid-specific genes. This finding is consistent with evidence that balance between Notch and PU.1 is important for T cell versus myeloid fate decisions, respectively. Thus, Notch signaling in DN1a/b cells appears to direct T-myeloid, rather than T-B lineage decisions.

Single-cell RNA sequencing of BM LMPPs showed that Notch signals affected the expression of genes that contribute to thymus-homing of TSPs and thus appearance of ETPs in the thymus. This, together with results from the mixed BM chimera mice, suggests that Notch signaling in BM can contribute to the generation of TSPs. Further analysis indicated that RBPJind-noDox, Sell+Selplg+Ccr9+ LMPPs had higher expression of myeloid differentiation genes compared to RBPJCtr and RBPJind-Dox LMPPs, including Egr1 and Klf4. These genes may be targets of PU.1 and also represent a GMP-specific gene signature. Egr1 deficiency resulted in more ETPs in the thymus, and down-regulation of Klf4 was required for full T lymphopoiesis to occur in the thymus, suggesting that suppression of these factors may be required for the generation of ETPs capable of T cell commitment. Additionally, Sell+Selplg+Ccr9+ LMPPs from RBPJind-noDox mice were biased toward the CMP cluster (cluster 3). BM endothelial cells were reported to express DLL4, which was required to prevent myeloid skewing in as early as HSC and MPP stages.

However, because RBPJind-noDox mice lacked LMPPs with efficient thymus-seeding capacity, we cannot exclude the possibility that thymic entry of LMPP TSPs was completely impaired in the absence of Notch responsiveness. In such a scenario, the myeloid bias of RBPJind-noDox DN1a/b cells may be due to presence of CMPs, GMPs or MEPs within the DN1a/b pool, and not necessarily conversion of LMPPs to CMPs. This interpretation is still consistent with the idea that lack of BM Notch signals leads to bias of myeloid-committed progenitors within the subset of cells that seed the thymus. Thus, our results indicate that BM Notch signaling inhibits early events of myeloid differentiation in TSPs, and that without these signals, TSPs become fully committed to the myeloid fate upon thymic entry. Altogether, this work revealed an important pre-thymic role for Notch in the generation of T lineage competent TSPs, such that upon thymic entry, ETP functionality is maintained and T cell development can ensue in full.
Methods

Mice.

All mice were bred and maintained in the Comparative Research Facility of the Sunnybrook Research Institute under specific pathogen-free conditions. All animal procedures were approved by the Sunnybrook Research Institute Animal Care Committee and performed in accordance with the committee’s ethical standards.

Generation of TetOS-RBPJ-HA transgenic mice.

Mouse RBPJ coding sequence (CDS)\textsuperscript{56, 57} lacking a stop codon and carrying a Kozak consensus sequence as well as EcoRI and SnaBI restriction sites at 5’ and 3’ end, respectively, was PCR-amplified with Platinum Pfx Polymerase (Invitrogen) from a whole thymus cDNA preparation using the following set of primers: RBPJ-F 5’-ATAGCGAATTCGCCGCAACCATGGCGCCTGGCTGACATCA3'- and RBPJ-R 5’-TAAATACGTAGGACACCAGGTGGCTCTGTG-3’. The RBPJ CDS was then cloned into the MIY-II vector using the EcoRI and SnaBI restriction sites. The hemagglutinin (HA) tag was generated by annealing complementary oligonucleotides (HA-tag F:5’-TATTATACGTAACCAGCTACCCATACGATGTTCCAGATTACGCTTGAGGATCCTGCA

CATCCTATGGGTTAGCTGTAGTATAATAATGGGTAGCTGGTTACGTATAATAA-3’), which incorporated a threonine-serine linker at its 5’ end as well as SnaBI and BamHI sites, and was subsequently annealed into the MIY-RBPJ construct to allow for transgene (Tg) detection and to differentiate it from the endogenously-encoded RBPJ. RBPJ-HA cassette was further subcloned into the pTetOS vector with the use of EcoRI and BamHI restriction sites. pTetOS-RBPJ-HA was digested with Sall and the cassette, which included the β-globin intron sequence and a polyA sequence, was ligated into an XhoI site of the modified insulator-containing pJC13–1 vector. Insulated TetOS-RBPJ-HA construct was linearized with Sall to remove bacterial DNA elements. Transgenic mice were generated by microinjection of the Tg construct DNA into fertilized eggs obtained from the mating of superovulated C57BL/6J females with C57BL/6J males (The Jackson Laboratory line 000664) at the University of Michigan Transgenic facility, Ann Arbor. Founders and the F1 progeny were screened by PCR for copy number of the Tg by comparing it to the Tg copy standards. Mass of Tg DNA was set as a function of number of base pairs of Tg DNA, the haploid content of a mammalian genome (3 x10\textsuperscript{9} bp) and amount of tail DNA available. Copy number standards were prepared at 0.01, 0.1, 1, 10, 25 and 50 copies. Primer sequences used were: RBPJ-HA F: 5’-ATGACGGGTCATTTACTCC-3’ and RBPJ-HA R: 5’-CAAGCGTAATCTGGAA

CATCCTATGGGTTAGCTGTAGTATAATAATGGGTAGCTGGTTACGTATAATAA-3’.

In vitro induction of RBPJ-HA expression in TetOS-RBPJ-HA founders.

Transgenic founder- and F1-derived fibroblasts were prepared by either digesting mouse tails with collagenase IV overnight or by separating two layers of ear tissue and allowing the fibroblasts to adhere to plastic-coated plates. In both cases, cells were grown in Dulbecco's Modified Eagle Medium (DMEM) substituted with 10% fetal bovine serum (FBS), Penicillin-Streptomycin (Pen-Strep) and Hepsodium Pyruvate-Gentamicin mixture. Ten million fibroblasts were transfected with 4 μg of rtTA-containing pTet-DualON vector...
(Clontech) using Amaxa nucleofector (Lonza, program U23) and cultured in the presence of 1 μg/ml Dox (Clontech) for 72 hours. Cells were collected and stained for HA in Western blots.

**Induction of Notch responsiveness.**

To induce transgenic RBPJ-HA expression *in vivo*, 6 to 8 week old RBPJ*ind* mice were injected with 2 mg/ml Dox (Sigma-Aldrich) intraperitoneally at time 0 and administered 1 mg/ml Dox in drinking water supplemented with 5% Splenda *ad libitum*, with water changed twice a week for the duration of the experiment. Mice not receiving Dox were given drinking water with Splenda alone. RBPJ-HA expression was induced *in vitro* by culturing cells in the presence of 1 μg/ml Dox.

**Cell preparation, flow cytometry and cell sorting.**

Single-cell suspensions were prepared from mouse BM, thymus and spleen. BM were crushed while thymus and spleen were mashed, then passed through cell strainers while in α-Minimum Essential Medium Eagle (αMEM) supplemented with 20% FBS and 1% Pen-Strep. Erythrocytes were lysed using BD Pharm Lyse™. Single-cell suspensions were stained with antibodies while in Hanks’ Balanced Salt Solution (HBSS) supplemented with 1% bovine serum albumin (BSA) and 2mM EDTA. Antibodies were purchased from BD Biosciences, eBiosciences, or BioLegend: CD45.1, CD45.2, MHC-II, CD44, CD25, CD24, CD117, CD4, CD8, CD3, TCRγδ, B220, CD19, IgM, CD21, CD23, NK1.1, CD11b, CD11c, Gr1, Ter119, Sca-1, Flt-3, CD127, CD62L, Ly6D. Anti-HA antibody was purchased from Roche and anti-rat secondary antibody was purchased from Jackson Immunoresearch Laboratories. Intracellular staining for HA was performed using eBiosciences Foxp3/Transcription Factor Staining Buffer Set. Flow cytometry was performed on LSR II (BD Biosciences) and data analyzed with FlowJo software version 9.9.6. Cell sorting was performed using BD FACS Aria IIu.

**Immunofluorescence.**

Whole thymi were embedded in OCT compound (Tissue-Tek) and snapped frozen in liquid nitrogen. 6 μm tissue slices were obtained using Leica CM3050S and then fixed with 2% paraformaldehyde (Electron Microscopy Sciences) prior to staining. Tissue slices were stained with anti-cytokeratin 5 (Covance), anti-cytokeratin 8 (Tromb), Ulex Europaeus Agglutinin I (UEA-1, Vector Laboratories), anti-β5t (MBL International), anti-B220 (BD Biosciences) or Lycopersicon Esculentum (Tomato) Lectin (Vector Laboratories). Secondary antibodies (anti-rabbit and anti-rat) and streptavidin were purchased from Jackson Immunoresearch Laboratories and BD Biosciences, respectively. Tissue slices were then mounted with Dako fluorescent mounting medium prior to imaging using Zeiss Axiovert 200M.

**BM chimera.**

1 million whole BM cells from CD45.2+(GFP+) RBPJ*ind* mice and CD45.2+(GFP−) wild-type mice were mixed and injected into CD45.1+ wild-type hosts that were lethally irradiated at 900 rads. After irradiation and injection, hosts were left for 4 weeks to allow
wild-type donor cells to maintain the host thymus. Afterwards, hosts were treated with Dox to assess appearance of DN2 cells from RBPJ\textsuperscript{ind} donor cells.

**Cell culture.**

BM-derived LSKs, LMPPs and CLPs, and thymus-derived DN1a/b and DN1c cells were purified by flow cytometric cell sorting and cultured on either OP9-DL4, OP9-DL1\textsuperscript{lo} or OP9 cells in αMEM media supplemented with 20% FBS (Gibco) and 1% Pen-Strep, as well as 1 ng/ml of IL-7 and 5 ng/ml of Flt-3L for T-/B- assays, and 100 ng/ml of Flt-3L for myeloid/DC assays (R&D Systems). For T-/B-/myeloid/DC assays from thymic DN1a/b and DN1c cells, ~500 cells were used for each lineage assay for each experiment, with the same numbers used for BM LSK controls. For DN2 differentiation kinetics from ETPs, LMPPs and CLPs, ~2000 cells were used for each day of assessment for each experiment. For single-cell and limiting dilution analysis assays, BM CD62L\textsuperscript{+} LMPPs and CLPs were sorted onto OP9-DL4 cells in 96 well plates at the following doses: 100 cells (12 wells), 30 cells (12 wells), 10 cells (24 wells), 3 cells (24 wells), 1 cell (48 wells), and the T cell progenitor frequency was calculated using ELDA software version 1.58.

**Quantitative RT-PCR.**

mRNA from sorted BM progenitors were extracted using TRIzol Reagent (ThermoFisher Scientific) and purified. cDNA synthesis and qRT-PCR reaction was performed as one-step using Luna Universal One-Step RT-qPCR Kit, and data was collected using Eppendorf Mastercycler Realplex2. Primers for the following genes were used: Notch1 (F: 5’-AGATCGACAACCGGAATGT-3’ and R: 5’-CCCACAGCCCACAAAGAAC-3’), Hes1 (F: 5’-TCCTGACGGCCAATTTGC-3’ and R: 5’-GGAAGGTGACACTGCGTTAGG-3’), β-actin (F: 5’-GGCTCTTTTCCAGCTCTTCCT-3’ and R: 5’-GTCTTTACGGATGTCAACGTCA-3’). Normalized relative expression of Notch1 and Hes1 was determined using β-actin expression as a housekeeping gene.

**RNA sequencing.**

Thymic DN1a/b cells were RNA sequenced using NextSeq, and data was analyzed using R software version 3.5.1 with the package edgeR version 1.59. RNA sequencing products were aligned to the mouse genome (GRCm38) to obtain raw read counts for genes, in which genes not expressed across all samples were removed. Gene expression was normalized between samples to account for variations in library size and sequencing depth. Unsupervised clustering of samples was done, and differential expression analysis of genes was performed after filtering for those that showed at least 2-fold changes between samples, and that were statistically significant.59

**Single-cell RNA sequencing.**

BM LMPPs were RNA sequenced at single-cell resolution using 10X Genomics, and data was analyzed using R software version 3.5.1 with the package Seurat version 2.4.60. Each sample, from a total of 2739 (RBPJ\textsuperscript{Cir}), 2808 (RBPJ\textsuperscript{ind-noDox}), 1319 (RBPJ\textsuperscript{ind-Dox}3d), 2166 (RBPJ\textsuperscript{ind-Dox}6d) cells, was first filtered to remove cells with low gene counts that arise from aborted sequencing, and gene expression was normalized between cells. Afterwards,
variable expression of genes was determined. All samples were then merged and aligned, and dimensions for t-distributed stochastic neighbor embedding (t-SNE) were calculated to identify unique cell clusters. Cell subsetting based on gene expression was done to identify and analyze TSP populations to determine their cluster location and differential gene expression between samples.

**Statistical analysis.**

The data and error bars are presented as mean ± standard deviation (SD). To determine statistical significance, a two-tailed unpaired t-test was performed using Prism software version 6. Statistical significance was determined as: ns (P>0.05), *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Tests of significance for RNA sequencing and single-cell RNA sequencing data was performed using empirical Bayes moderated t-statistics and Wilcoxon rank sum test, respectively, under R software version 3.5.1, where P<0.05 was considered significant.

**Reporting Summary.**

Further information on research design is available in the “Life Sciences Reporting Summary” linked to this article.

**Data Availability**

The data that support the findings of this study are available from the corresponding author upon request. Raw and processed RNA sequencing and single-cell RNA sequencing data are available at the Gene Expression Omnibus database under accession number GSE128964.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. RBPJ\textsuperscript{ind} mice allow for controlled T cell development.
(a) Flow cytometry analysis of the thymic phenotype of RBPJ\textsuperscript{Ctr}-Dox, RBPJ\textsuperscript{ind}-noDox, RBPJ\textsuperscript{ind}-Dox\textsuperscript{6wk} and RBPJ\textsuperscript{ind}-Dox\textsuperscript{3wk}-noDox\textsuperscript{3wk} mice. Left to right: analysis of the DN compartment (DN gated), the DN1 compartment (DN1 gated), DPs/SPs, γδ T cells (DN gated) and B cells (DN gated). DN gated: gated on CD4$^-$CD8$^-$. DN1 gated: gated on Lin$^-$ (CD8, CD3, NK1.1, B220, CD19, CD11b, CD11c, Gr1, Ter119) CD44$^+$CD25$^-$. Data are representative of three independent experiments (n=3 mice per group). (b) Total thymic cellularity of RBPJ\textsuperscript{Ctr}-Dox, RBPJ\textsuperscript{ind}-noDox, RBPJ\textsuperscript{ind}-Dox\textsuperscript{6wk} and RBPJ\textsuperscript{ind}-Dox\textsuperscript{3wk}.
noDox\textsuperscript{3wk} mice showing mean ± standard deviation (n=3 mice per group). *$P<0.05$, **$P<0.01$ (two-tailed unpaired t-test).
Figure 2. Regulation of T lymphopoiesis in RBPJ\textsuperscript{ind} mice induces thymic architectural changes. Immunofluorescence analysis of K5, K8, UEA-1 and β5t (left), UEA-1, β5t and B220 (middle; 10x magnification; scale bars denote 100μm) and β5t, B220 and Tomato Lectin (right, 20x magnification; scale bars denote 50μm) in thymic sections from RBPJ\textsuperscript{Ctrl}-Dox, RBPJ\textsuperscript{ind}-noDox, RBPJ\textsuperscript{ind}-Dox\textsuperscript{6wk} and RBPJ\textsuperscript{ind}-Dox\textsuperscript{3wk}-noDox\textsuperscript{3wk} mice. The white arrows indicate cortex and medulla boundaries. Data are representative of three independent experiments (n=3 mice per group).
Figure 3. Appearance of DN2 cells in RBPJ\textsuperscript{ind} mouse thymus is delayed upon induction of Notch responsiveness.
(a-b) Flow cytometry analysis of the day-by-day progression of (Lin\textsuperscript{-} pre-gated) DN (a) and DP development (b) in the thymus of RBPJ\textsuperscript{ind}-Dox mice after Dox treatment for 1 to 14 days (as indicated). Data are representative of three independent experiments (n=3 mice per group). (c) Percentages of DN subsets and DPs in the thymus of RBPJ\textsuperscript{ind}-Dox mice after Dox treatment for 1 to 14 days (as indicated), showing mean ± standard deviation (n=3 mice per group).
Figure 4. Appearance of RBPJ\textsuperscript{ind} DN2 cells in WT thymus is delayed upon induction of Notch responsiveness.  

(a) Flow cytometry analysis of BM chimerism of CD45.2\textsuperscript{+}(GFP\textsuperscript{+}) RBPJ\textsuperscript{ind}-noDox donor cells and CD45.2\textsuperscript{+}(GFP\textsuperscript{−}) wild-type (WT) donor cells in lethally irradiated CD45.1\textsuperscript{+} WT mice 4 weeks post-irradiation and BM reconstitution and prior to start of Dox treatment (day 0). (b) Immunofluorescence analysis of host thymic architecture at day 0 (10x magnification; scale bars denote 100\textmu m) and flow cytometry analysis of thymic chimerism of RBPJ\textsuperscript{ind}-noDox cells and WT cells on Day 0 (top), and flow cytometry analysis of
kinetics of appearance of $\text{RBP}^{\text{ind}}$-Dox DN2 cells in host mice following 2, 4, and 6 days of Dox treatment ($\text{Lin}^{-}$ pre-gated) (bottom). Data are representative of three independent experiments ($n=3$ mice per group).
Figure 5. Notch signaling is required for thymic appearance of T lineage competent ETPs.
(a-b) Flow cytometry analysis of DN3 and DP development from RBPJ\textsuperscript{Ctr}, RBPJ\textsuperscript{ind-noDox} and RBPJ\textsuperscript{ind-Dox}\textsubscript{6d} thymic DN1a/b cells and BM LSKs cultured on OP9-DL4 with or without Dox for 8 days (a) and 14 days (b). (c) Flow cytometry analysis of DN3 development from RBPJ\textsuperscript{Ctr}, RBPJ\textsuperscript{ind-noDox} and RBPJ\textsuperscript{ind-Dox}\textsubscript{6d} thymic DN1a/b cells cultured on OP9-DL4 with or without Dox for 8 days. (d) Flow cytometry analysis of B cell development from RBPJ\textsuperscript{Ctr}, RBPJ\textsuperscript{ind-noDox} and RBPJ\textsuperscript{ind-Dox}\textsubscript{6d} thymic DN1a/b cells and BM LSKs cultured on OP9 for 14 days. Data are representative of three independent
experiments (n=2 mice pooled for RBPJ<sup>Ctr</sup> and n=8 mice pooled for RBPJ<sup>ind</sup>-noDox and RBPJ<sup>ind</sup>-Dox<sup>6d</sup> for each experiment).
Figure 6. RBPJ\textsuperscript{ind}-noDox thymic DN1a/b cells are myeloid biased.

(a) Heatmap analysis of genes enriched in DN1a/b cells sorted from RBPJ\textsuperscript{Ctr}, RBPJ\textsuperscript{ind}-noDox or RBPJ\textsuperscript{ind}-Dox\textsuperscript{6d} mouse thymi. (b) Genes differentially expressed between thymic DN1a/b cells of RBPJ\textsuperscript{Ctr}, RBPJ\textsuperscript{ind}-noDox and RBPJ\textsuperscript{ind}-Dox\textsuperscript{6d} mice. For gene expression fold-change values, see Supplementary Tables 1–3. (c) GO analysis of biological pathways involving genes enriched in RBPJ\textsuperscript{Ctr} DN1a/b cells compared to RBPJ\textsuperscript{ind}-noDox DN1a/b cells, or genes enriched in RBPJ\textsuperscript{ind}-noDox DN1a/b cells compared to RBPJ\textsuperscript{Ctr} DN1a/b cells. Full names of the biological pathways are described in Supplementary Table 4. Data are
from one independent experiment, where each group was done in duplicates (n=2 mice pooled for RBPJ\textsuperscript{CTR} and n=8 mice pooled for RBPJ\textsuperscript{ind-noDox} and RBPJ\textsuperscript{ind-Dox6d} for each replicate).
Figure 7. BM LMPPs undergo Notch signaling.
(a) Total numbers of BM HSCs, MPPs, LMPPs and CLPs in RBPJ\textsuperscript{Ctr} and RBPJ\textsuperscript{ind-noDox} mice, showing mean ± standard deviation (n=7 mice per group). (b) Flow cytometry analysis of BM CD62L\textsuperscript+ LMPPs and Ly6D\textsuperscript− CLPs (left) and percentages and total numbers of BM CD62L\textsuperscript+ LMPPs and Ly6D\textsuperscript− CLPs (right) from RBPJ\textsuperscript{Ctr} and RBPJ\textsuperscript{ind-noDox} mice. Data are representative of four independent experiments (n=7 mice per group; left) and showing mean ± standard deviation (n=7 mice per group; right). (c) qPCR analysis of Hes1 and Notch1 gene expression by BM HSCs, MPPs, LMPPs and CLPs from RBPJ\textsuperscript{Ctr}, RBPJ\textsuperscript{ind-noDox} and
RBPJ\textsuperscript{ind}.Dox\textsuperscript{6d} mice, showing mean ± standard deviation. Gene expression levels were normalized relative to β-actin (n=3 mice pooled for each group for each of the three independent experiments). ns, not significant (P>0.05), *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (two-tailed unpaired t-test).
Figure 8. Notch signaling in BM inhibits myeloid skewing in TSPs. (a) t-SNE analysis of 8 clusters from total BM LMPPs (n=8685 cells), analysis of Selplg, Ccr9 and Ccr7 gene expression in Sell+ LMPPs (n=2918 cells), and analysis of Ccr9 and Ccr7 gene expression in Sell+Selplg+ Ccr9+ LMPPs from RBPJ_Ctr mice (n=734 cells), RBPJind_noDox mice (n=516 cells), RBPJind_Dox3d mice (n=273 cells) and RBPJind_Dox6d mice (n=498 cells). For genes enriched in clusters 3-8, see Supplementary Tables 5–10. (b) t-SNE analysis of Sell+Selplg+ Ccr9+ LMPPs from RBPJ_Ctr, RBPJind_noDox, RBPJind_Dox3d and RBPJind_Dox6d mice, and overall cluster distribution of Sell+Selplg+ Ccr9+ LMPPs and Sell
Selplg+ Ccr9+ Ccr7+ LMPPs from RBPJCtr mice (n=24 and 4 cells, respectively), RBPJind-noDox mice (n=14 cells), RBPJind-Dox3d mice (n=6 and 2 cells, respectively) and RBPJind-Dox6d mice (n=24 and 4 cells, respectively) (e) Heatmap analysis of expression of myeloid differentiation genes in Selplg* Ccr9* LMPPs from RBPJCtr mice (n=24 cells), RBPJind-noDox mice (n=14 cells), RBPJind-Dox3d mice (n=6 cells) and RBPJind-Dox6d mice (n=24 cells), and GO analysis of the top 10 biological pathways involving genes enriched in RBPJind-noDox Selplg* Ccr9* LMPPs compared to RBPJCtr LMPPs. Full names of the biological pathways are described in Supplementary Table 16. For all genes differentially expressed between RBPJCtr, RBPJind-noDox and RBPJind-Dox6d Selplg+ Ccr9+ LMPPs, see Supplementary Tables 13–15. Names of the biological pathways in the “leukocyte differentiation” cluster and the genes involved are described in Supplementary Table 16. Data are from one independent experiment (n=3 mice pooled for each group).