The thymus is an organ that supports the differentiation and selection of T cells (1). The thymic development of T cells consists of several processes that require the relocation of developing lymphocytes into, within, and out of the different environments of the thymus (2–4). First, the thymic seeding progenitors (TSPs) enter the subcapsular cortical areas, where they encounter networks of cortical epithelial cells. Second, CD4+CD8+ double-positive (DP) thymocytes generated in the outer cortex are mobile, interacting with stromal cells that are localized in the cortex for positive and negative selection. Third, positively selected thymocytes interact with medullary thymic epithelial cells to complete thymocyte development. Last, mature T cells export from medulla to peripheral lymphoid tissues. The thymus provides the functional microenvironment to selectively induce T lineage differentiation from the TSPs (5).

Notch signaling regulates lineage decisions at multiple stages of lymphocyte development, and Notch activation requires the endocytosis of Notch ligands in the signal-sending cells. Four E3 ubiquitin ligases, Mind bomb (Mib) 1, Mib2, Neuralized (Neur) 1, and Neur2, regulate the Notch ligands to activate Notch signaling, but their roles in lymphocyte development have not been defined. We show that Mib1 regulates T and marginal zone B (MZB) cell development in the lymphopoietic niches. Inactivation of the Mib1 gene, but not the other E3 ligases, Mib2, Neur1, and Neur2, abrogated T and MZB cell development. Reciprocal bone marrow (BM) transplantation experiments revealed that Mib1 in the thymic and splenic niches is essential for T and MZB cell development. Interestingly, when BM cells from transgenic Notch reporter mice were transplanted into Mib1-null mice, the Notch signaling was abolished in the double-negative thymocytes. In addition, the endocytosis of Dll1 was impaired in the Mib1-null microenvironment. Moreover, the block in T cell development and the failure of Dll1 endocytosis were also observed in coculture system by Mib1 knockdown. Our study reveals that Mib1 is the essential E3 ligase in T and MZB cell development, through the regulation of Notch ligands in the thymic and splenic microenvironments.
In addition, Notch signaling regulates the final differentiation step of BM-derived B cells in the spleen, where it facilitates the generation of marginal zone B (MZB) cells while it suppresses the generation of follicular B (FOB) cells (14–16).

Notch signaling is a widely used cell–cell signaling pathway that plays a critical role in cell fate determination of various lineages in vertebrates as well as invertebrates (17). In mammals, four Notch receptors (Notch1–4) and five Notch ligands (Dll [Deltalike] 1, Dll3, Dll4, Jag [Jagged] 1, and Jag2) have been identified. Upon binding to their ligands, the Notch receptors undergo sequential proteolytic cleavages that result in the release of the Notch intracellular domain (NICD) and the Notch extracellular domain (NECD) (18). The NICD acts in the nucleus as a transcriptional regulator with RBP-Jk (19), and the NECD seems to undergo transendocytosis along with Delta in the signal-sending cell (20). A surprising, but poorly understood, finding is that the internalization of Delta in the signal-sending cell is required to activate the Notch signaling in the receiving cells (21). To date, two structurally distinct E3 ubiquitin ligases, Neuralized (Neur)-1/2 (Neur in Drosophila melanogaster) and Mind bomb (Mib)—1/2, have been shown to regulate the endocytosis of Notch ligands in vertebrates and invertebrates (21–28). The requirement of Notch signaling for lymphocyte development has been well studied; however, the role of the E3 ubiquitin ligases that regulate T and MZB cell development is unknown.

Studies using Cre-loxP-mediated gene targeting have shown that specific deletion of Notch1 in hematopoietic cells leads to a complete block of T cell development with a substantial increase in B cells in the thymus (11). In contrast, enforced expression of the active form of Notch1 inhibits B cell development in the BM (29). These results demonstrate that Notch1 is indispensable for T cell commitment at the branch point of T cells versus B cells. Dll1 and Dll4, which are expressed in the thymic epithelial cells, have been suggested to be responsible for activating the Notch1 receptor in T cell progenitors (30). Consistent with these findings, the BM stromal cell line OP9 ectopically expressing the Notch ligand Dll1 (OP9-DL1) or Dll4 loses its ability to support B cell lymphopoiesis but acquires the capacity to induce the differentiation of hematopoietic stem cells (HSCs) into T cells (16, 31). However, conditional deletion of Dll1 in mice shows that Dll1 is dispensable for T cell development (16), and analysis of mice with floxed Dll4 has not yet been reported. In addition, although the requirement of Dll1 as well as Notch2 for the generation of MZB cells has been well studied in vivo (16), the microenvironment that supports MZB cell development remains to be elucidated. Therefore, requirement of Notch signaling in the microenvironments to hematopoietic cells for lymphopoiesis in vivo needs to be clarified.

Therefore, to investigate which E3 ubiquitin ligase is essential for T and MZB cell development and which types of cells in the thymus and spleen transduce Notch signals to their progenitors, we analyzed Mib1 conditional KO (Mib1v/), Mib2−/−, Neur1−/−, and Neur2−/− mice. Surprisingly, only the conditional inactivation of Mib1 caused severe defects in T and MZB cell development. Moreover, an analysis of Mib1-deficient hosts reconstituted with WT BM cells revealed that Mib1 regulates T and MZB cell development in the signal-sending cells of the thymic and splenic microenvironments. In addition, Dll1 was not endocytosed but accumulated in the Mib1-null thymic niches. Consistent with the results from the Mib1-null mice, knockdown of Mib1 in OP9-DL1 abolished T cell development and inhibited endocytosis of Dll1. These findings demonstrate that Mib1 is an essential E3 ubiquitin ligase for Notch signaling in T and MZB cell development in the thymic and splenic microenvironments.

RESULTS
The expression of E3 ubiquitin ligases of Notch signaling in lymphoid tissues
The expression of the E3 ubiquitin ligases of Notch signaling in lymphoid tissues suggests the involvement of these molecules in lymphopoiesis. To test the relevance of the E3 ligases in lymphocyte development, we examined the expression of E3 ligase transcripts in lymphoid tissues, thymus, and spleen. Because we expected that the E3 ligases might work in the nonhematopoietic stromal cells, we measured the expression levels of E3 ligases in isolated cell populations by quantitative real-time RT-PCR. In the thymus, transcripts of all four E3 ligases were detected in both CD45− nonhematopoietic and CD45+ hematopoietic cells (Fig. 1 A). In particular, both the Mib1 and Mib2 transcripts were highly expressed, whereas the Neur1 and Neur2 transcripts were slightly expressed in the CD45− and CD45+ compartments of thymocytes as well as the entire thymus.

In addition, transcripts of the four E3 ubiquitin ligases were also detected in the spleen (Fig. 1 B). Although the Mib2 transcripts were the most abundant in the total spleen and CD45+ hematopoietic cells, the Mib1 and Neur2 transcripts were highly expressed in the CD45− nonhematopoietic cells. These results suggest that Mib1, Mib2, Neur1, and Neur2 might be involved in lymphocyte development and raise the possibility that the four E3 ubiquitin ligases might control Notch signaling in the thymic epithelial cells that express the Notch ligands (30, 32, 33).

Block in T cell development in the mouse mammary tumor virus (MMTV)−Cre;Mib1v/ mice
To elucidate the requirement of E3 ligases in T cell development, we used Mib1v/, Mib2−/−, Neur2−/− (34), and Neur1−/− mice (35). To investigate the effect of Mib1 in T cell development, the Mib1v/ mice were bred with MMTV-Cre transgenic mice, expressing Cre recombinase under the control of the MMTV long terminal repeat promoter, which is active in various cell lineages (36). Mib1 expression was reduced in the thymi and spleens from MMTV-Cre;Mib1v/ mice (Fig. 1, C and D).

To determine which E3 ligase is required for T cell development, flow cytometric analyses were performed on the thymocytes from the MMTV-Cre;Mib1v/, Mib2−/−, Neur1−/−, and Neur2−/− mice. Unexpectedly, the thymocyte development,
The expression of CD4 single-positive (SP), CD8 SP, CD4+CD8+ DP, and TCRγδ T and NK cells in Mib2−/−, Neur1−/−, Neur2−/−, and even Neur1−/−; Neur2−/− mice was comparable to that of WT controls (Fig. 2 A and not depicted), demonstrating that Neur1, Neur2, and Mib2 are dispensable for T cell development. In contrast, T cell development was severely affected in the MMTV-Cre;Mib1−/− mice. The percentage of DP thymocytes was dramatically reduced, whereas the percentage of CD4−CD8− double-negative (DN) cells was relatively increased (Fig. 2 A). In addition, the percentage of B220+ cells was increased ∼7.8-fold in the CD4−CD8− DN thymocytes from the MMTV-Cre;Mib1−/− mice, as compared with the MMTV-Cre;Mib1+/− mice (Fig. 2 B). The absolute numbers of total thymocytes were reduced 3.6-fold in the MMTV-Cre;Mib1−/− mice as compared with those of the control mice. The CD4 SP, CD8 SP, and CD4+CD8 DP thymocytes were also reduced 2.7-fold, 2.1-fold, and 4.3-fold, respectively, whereas the DN cells remained relatively unaffected (Fig. 2 C). Because most of the DN cells of the MMTV-Cre;Mib1−/− mice are B cells, DN numbers appear to be unaffected because of the increase in the number of B cells rather than a DN to DP transition defect.

When DN thymocytes were analyzed for the expression of CD44 and CD25, MMTV-Cre;Mib1−/− mice showed a block at CD44+CD25− DN1 thymocytes, as compared with the MMTV-Cre;Mib1+/− mice (Fig. 2 C). Because the expression of CD44 is not restricted to immature T cell progenitors but is also expressed on B cells (37), DN1 thymocytes in the MMTV-Cre;Mib1−/− mice should contain increased B cells. Collectively, these results suggest that Mib1 is important for T cell development, even though the individual differences of defect in T cell development and generation of B cells exist in the MMTV-Cre;Mib1−/− mice, probably because of variable gene deletion efficiency by MMTV promoter.

To further examine the essential role of Mib1 in T cell development, we used another Mx1-Cre transgenic line. The interferon-inducible promoter Mx1 facilitates the expression of Cre recombinase in the various hematopoietic systems in response to interferon or interferon-inducing agents, such as polyinosinic-polycytidylic acid (pIpC) (38). 6–8-wk-old Mx1-Cre;Mib1−/− mice received four i.p. injections of the IFNα inducer pIpC at 2-d intervals to inactivate the Mib1 gene. Consistent with the results from the MMTV-Cre;Mib1−/− mice, the block in T cell development and the increase of B cells in the CD4−CD8− DN subsets were also found in the thymi of the Mx1-Cre;Mib1−/− mice at 12 wk after the last pIpC injections (Fig. 2, E and F). The absolute numbers of total thymocytes, CD4 SP, CD8 SP, and CD4+CD8+ DP thymocytes were decreased in the Mx1-Cre;Mib1−/− mice as compared with those of the control mice (Fig. 2 A, available at http://www.jem.org/cgi/content/full/jem.20081344/DC1). Collectively,
these results demonstrate that Mib1, among the four E3 ligases, is essential for T cell development.

Defective ETP generation in the MMTV-Cre;Mib1f/f mice

T cells are generated in the thymus after colonization from the blood by BM-derived progenitors (39, 40). Recent studies have suggested that Notch signaling is required for the generation of ETPs after the thymic entry of TSPs (12, 13). To investigate which E3 ubiquitin ligase among the four E3 ubiquitin ligases is required for the generation of ETPs, CD44hiCD25−/H11002 DN1 thymocytes from the MMTV-Cre;Mib1f/f, Mib2−/−, Neur1−/−, and Neur2−/− mice were analyzed for the expression of c-kit and CD24 because the c-kit+CD24− and c-kit+CD24+ subpopulations, among the DN1 subsets, appear to most closely resemble canonical T cell progenitors in terms of proliferative capacity, early T lineage gene expression, and TCR rearrangements (41).

Although the ETP generation was not disturbed in the Mib2−/−, Neur1−/−, Neur2−/−, and even Neur1−/−;Neur2−/− mice, the MMTV-Cre;Mib1f/f mice exhibited a dramatic decrease of the ETP populations, as compared with the controls (Fig. 3 A). Although the ETP populations in the thymus were markedly decreased, the LSK progenitors in the BM and blood were preserved in the MMTV-Cre;Mib1f/f mice, indicating that the defective ETP generation is not caused by a decrease in LSK progenitors in the BM and blood (Fig. 3 B). Moreover, the ETP generation was also dramatically reduced

Figure 3. Reduction of early T cell progenitors in the thymus of MMTV-Cre;Mib1f/f mice. (A) The thymocytes from the littermate control (left) and mutant (right) mice indicated were stained for CD4, CD8, CD44, and CD24. ETPs were identified by CD24 and c-kit expression gated on CD4hiCD8loCD25−CD44+ cells. Percentages indicated are mean ± SD from five independent experiments. (B) Leukocytes from the BM (top) and blood (bottom) of the MMTV-Cre;Mib1f/f mice were stained for Sca-1 and c-kit gated on lineage-negative cells to identify LSK cells. Numbers indicate representatives of three independent experiments.
in the Mx1-Cre;Mib1+/f mice (unpublished data). These results demonstrate that Mib1, among the four E3 ligases, is required for the ETP generation.

MZB cell defect in the MMTV-Cre;Mib1+/f mice

Several recent reports demonstrated that Notch2 is critical for the generation of MZB cells, which is mediated through a specific interaction with Dll1 (14–16). To determine which E3 ubiquitin ligase is required for MZB cell development by regulating Dll1, we examined B cell differentiation in the MMTV-Cre;Mib1+/f, Mib2−/−, Neuro1−/−, and Neuro2−/− mice. When the splenocytes from the mice were analyzed, the fraction of B220+CD21+CD23lo−MZB cells was markedly reduced only in the MMTV-Cre;Mib1+/f mice, with a concomitant increase in the fraction of B220+CD21+CD23+ FOB cells (Fig. 4 A). The impaired MZB cell development in the MMTV-Cre;Mib1+/f mice was further confirmed with other markers, CD1d and CD9, which are expressed at high levels in MZB cells (Fig. 4 B) (42, 43). Notch signaling reportedly induces the expression of CD21 (44, 45), and the inactivation of Notch2 in the spleen down-regulates the expression of CD21 (14). Consistent with these findings, the FOB cells in the spleens from the MMTV-Cre;Mib1+/f mice also showed reduced expression of CD21 (Fig. 4 C). Consistent with the results from the MMTV-Cre;Mib1+/f mice, the defect in MZB cell development was also found in the spleen of the Mx1-Cre;Mib1+/f mice at 12 wk after the last plpC injections (Fig. S2, A–C, available at http://www.jem.org/cgi/content/full/jem.20081344/DC1). Surprisingly, MZB cell development was not disturbed in the other mutant mice, the Mib2−/−, Neuro1−/−, Neuro2−/−, and even Neuro1−/−;Neuro2−/− mice (Fig. 4 A). Therefore, our results demonstrate that Mib1 is essential for MZB cell specification, whereas the other three E3 ligases are dispensable.

B lymphopoiesis occurs in the BM and yields newly formed or transitional B cells that emigrate to the spleen (46). Two types of transitional mature B cell precursors exist in the spleen (47). T1 (Type 1) transitional B cells, which are recent immigrants from the BM, develop into T2 transitional B cells in the spleen. These transitional B cells are the splenic precursors of FOB and MZB cells, whereas cycling T2 B cells, a subset of transitional cells, might not be a critical intermediate precursor of FOB cells (48). To further analyze the late stages of B cell development, we examined the distribution of T1 and T2 B cells in the MMTV-Cre;Mib1+/f mice. Although no significant abnormality was observed in the fraction of CD23+CD21−IgM+ T1 B cells, the fraction of CD23+CD21+IgM+ T2 B cells was dramatically reduced in the MMTV-Cre;Mib1+/f mice, as compared with the control mice (Fig. 4 D). Despite the decrease of T2 B cells in the MMTV-Cre;Mib1+/f mice, however, FOB cell compartment was normal, suggesting that cycling T2 B cells might be decreased, whereas AA4.1+ T2 transitional B cells maintained. Therefore, we analyzed AA4.1+ transitional B cell subset in the spleen of the MMTV-Cre;Mib1+/f mice. Expression of the AA4.1 marker has been shown to identify a pool of splenic B cells with typical characteristics of transitional B cells (49). As expected, the AA4.1+IgMhiCD23+ T2 transitional B cell population was not decreased, which is in contrast to the significant reduction of cycling T2 cells in the MMTV-Cre;Mib1+/f mice (Fig. 4 E). These results demonstrate that Mib1 is indispensable for MZB lineage development and the generation of cycling T2 B cells but not AA4.1+ transitional B cell subsets.

Regulation of T cell development by Mib1 in the thymic microenvironments

The MMTV-Cre and Mx1-Cre transgenic lines delete the floxed genomic sequences in both hematopoietic and nonhematopoietic cells (36, 50), and the Mib1 mRNA is expressed...
in both the CD45+ hematopoietic and CD45- nonhematopoietic cells from the thymus (Fig. 1A). Therefore, the impaired T cell development in the MMTV-Cre;Mib1f/f and Mx1-Cre;Mib1f/f mice could be caused by either an autonomous defect in hematopoietic cells or a nonhematopoietic defect in other components residing in the thymus, such as stromal cells. To distinguish between these possibilities, lethally irradiated CD45.1 WT mice were reconstituted with BM cells from either the MMTV-Cre;Mib1+/f or MMTV-Cre;Mib1f/f mice. At 12 wk after transplantation, all of the CD45.1 recipient mice displayed normal thymocyte development, including B cell and ETP generation (Fig. 5A), indicating that the defective thymocyte development in the MMTV-Cre;Mib1f/f and Mx1-Cre;Mib1f/f mice is not caused by the inactivation of Mib1 in hematopoietic cells.

To investigate possible microenvironmental defects, the lethally irradiated 8–9-wk-old MMTV-Cre;Mib1+/f and MMTV-Cre;Mib1f/f mice were reconstituted with BM cells from CD45.1 congenic mice. At 5–6 wk after transplantation, both the MMTV-Cre;Mib1+/f and MMTV-Cre;Mib1f/f recipient mice were reconstituted by >95% donor origin cells, as assessed by flow cytometry (unpublished data). Interestingly, thymocyte development was severely affected in the MMTV-Cre;Mib1f/f recipient mice, whereas the WT recipient mice showed normal thymocyte development, as in the untransplanted WT mice (Fig. 5B). Consistent with this finding, the absolute numbers of total thymocytes were dramatically reduced (50.8-fold) in the MMTV-Cre;Mib1f/f recipient mice, as compared with those of the control mice. Furthermore, the B220+ cells were increased in the DN populations of the mutant recipient mice (Fig. 5B). These results show that Mib1 regulates T cell development in the nonhematopoietic thymic microenvironments.

To clarify whether the defect in T cell development is caused by the Mib1 deletion in the thymic stromal cells, we prepared genomic DNA from the CD45- cell populations of the MMTV-Cre;Mib1+/f and MMTV-Cre;Mib1f/f mice, and performed genomic PCR with primers that amplify the allele deleted by Cre recombinase–mediated excision. As expected, Mib1 was deleted ~52% in isolated CD45- cell populations.

![Figure 5](image-url) Regulation of T cell development by Mib1 in the thymic microenvironment. (A) The lethally irradiated CD45.1 mice were injected intravenously with BM cells from the 12–15-wk-old MMTV-Cre;Mib1+/f and MMTV-Cre;Mib1f/f mice. At 12 wk after transplantation, the thymocytes were analyzed by flow cytometry for the expression of CD4 and CD8 from four independent experiments. (B) The lethally irradiated MMTV-Cre;Mib1+/f and MMTV-Cre;Mib1f/f mice were transplanted with CD45.1 BM cells. At 5–6 wk after reconstitution with CD45.1 BM cells, the thymocytes were stained for CD4 and CD8 (top) and B220 gated on the CD4+ CD8- DN thymocytes (middle), and CD4+ and CD8+ gated on the CD4+ CD8-CD25+ CD44+ cells (bottom). Numbers indicate mean ± SD from four independent experiments. (C) Genomic DNA was prepared from the CD45- cells sorted from thymi. Quantitative real-time PCR was performed to analyze the Mib1 deletion using deleted allele-specific primers. Expression of nondeleted allele served as a control for relative quantification. Data are mean ± SD from triplicate experiments. *, P < 0.01.

![Figure 6](image-url) Regulation of MZB cell development by Mib1 in the splenic microenvironment. (A) The lethally irradiated CD45.1 mice were injected i.v. with BM cells from the 12–15-wk-old MMTV-Cre;Mib1+/f (left) and MMTV-Cre;Mib1f/f (right) mice. At 12 wk after transplantation, the splenocytes were stained for the expression of CD21 and CD23 gated on the CD45.1+ B220+ cells (top), CD1d and CD9 gated on the CD45.1+ B220+ cells (middle), and CD21 and IgM gated on CD45.1+CD23- cells (bottom). Percentages indicated are mean ± SD from five independent experiments. (B) The lethally irradiated MMTV-Cre;Mib1+/f (left) and MMTV-Cre;Mib1f/f (right) mice (7–9 wk old) were transplanted with CD45.1 BM cells. At 5–6 wk after reconstitution with CD45.1 BM cells, the splenocytes were analyzed as in A. Percentages indicated are mean ± SD from five independent experiments. (C) Genomic DNA was prepared from the CD45- cells sorted from spleens and was analyzed by quantitative real-time PCR with deleted allele-specific primers. Data are mean ± SD from triplicate experiments. *, P < 0.0001.
Because Mib1 regulates the endocytosis of Notch ligand (26), we examined whether Delta is endocytosed into the endocytic pathway targeted to Hrs-positive vesicles (52, 53) in the thymus. To clearly find out the defect in the Mib1-null microenvironment, the lethally irradiated MMTV-Cre;Mib1+/f mice as compared with that of the control mice (Fig. 5 C). Collectively, these results suggest that Mib1 functions in the thymic microenvironment.

Regulation of MZB cell development by Mib1 in the splenic microenvironments

Because Mib1 is expressed in both CD45+ and CD45− cells in the spleen (Fig. 1 B), we investigated whether Mib1 functions in the hematopoietic cells or in the microenvironments for MZB cell development. When the lethally irradiated CD45.1 WT mice were reconstituted with BM cells from either the MMTV-Cre;Mib1+/f or MMTV-Cre;Mib1−/f mice, all of the CD45.1 recipient mice displayed not only T1 B and T2 B cells but also normal MZB cell development (Fig. 6 A and not depicted), indicating that the impaired MZB cell development in the MMTV-Cre;Mib1−/f mice is not caused by an autonomous defect in hematopoietic cells. However, in the reciprocal BMT experiment, in which the lethally irradiated MMTV-Cre;Mib1+/f and MMTV-Cre;Mib1−/f mice were reconstituted with BM cells from CD45.1 congenic mice, B220+CD21+CD23−/− and B220+CD19+CD9+ MZB cell development was completely blocked in the MMTV-Cre;Mib1−/f recipient mice, whereas it was unaffected in the control mice (Fig. 6 B). In addition to MZB cells, cycling T2 B cell development, but not T1 B cell development, was severely affected in the MMTV-Cre;Mib1−/f recipient mice (Fig. 6 B and not depicted). These results show that the impaired MZB cell development in the MMTV-Cre;Mib1−/f mice originated from a nonhematopoietic microenvironment. Consistent with these findings, Mib1 was deleted ~90% in isolated CD45− splenic stromal cells from MMTV-Cre;Mib1−/f mice as compared with that of the control mice (Fig. 6 C), suggesting that Mib1 regulates MZB and cycling T2 B cell development in the splenic microenvironments.

Defective Notch signaling in the MMTV-Cre;Mib1−/f thymic microenvironments

The thymic stroma provides a unique microenvironment for T cell differentiation, and it expresses multiple Notch ligands (30, 32, 33). Several studies suggested that Dll1 and Dll4 in the thymic stroma might be the critical Notch ligands to activate Notch1 in T cell progenitors (16, 32). Because Mib1 regulates multiple Notch ligands, including Dll1 and Dll4, and T lineage commitment is blocked in the MMTV-Cre;Mib1−/f and Mx1-Cre;Mib1−/f mice, we speculated that the inactivation of Mib1 in the thymic microenvironment would prevent the activation of Notch signaling in T cell progenitors. To test this possibility, the lethally irradiated MMTV-Cre;Mib1+/f mice were reconstituted with BM cells from the transgenic Notch reporter (TNR) mice, which express EGFP in cells upon Notch/CBF1 activation (51). We readily observed EGFP expression in the DN thymocytes of the MMTV-Cre;Mib1−/f mice at 4 wk after TNR BM transplantation. In contrast, EGFP expression was almost absent in those of the MMTV-Cre;Mib1+/f mice (Fig. 7 A), indicating that the Mib1-null thymic microenvironment cannot activate Notch signaling in T cell progenitors.
and MMTV-Cre;Mib1f/f mice were reconstituted with BM cells from CD45.1 congenic mice. Expectedly, Dll1 was endocytosed to the cytoplasm as a punctate form and some endocytosed Dll1 was colocalized with Hrs in the thymi of the MMTV-Cre;Mib1+/− recipient mice (Fig. 7 B, arrowheads). In the thymi of the MMTV-Cre;Mib1f/f recipient mice, however, Dll1 was exclusively accumulated and did not colocalize with Hrs (Fig. 7 B, asterisks). The endocytosis of Dll1 to Hrs-positive vesicles was normal in the thymi from the Mib2−/− and Neur1−/−;Neur2−/− mice (Fig. S3 A, available at http://www.jem.org/cgi/content/full/jem.20081344/DC1), suggesting that the endocytosis of Dll1 to Hrs-positive vesicles is dependent on Mib1.

Because Dll1 is expressed on cortical epithelial cells (54), we investigated whether Dll1 accumulates on cortical epithelial cells, and Mib1 expression is abolished in the thymic stroma of the MMTV-Cre;Mib1f/f mice by immunohistochemistry. In the MMTV-Cre;Mib1f/f thymus, Dll1 was accumulated by some of the cytokeratin-positive cortical epithelial cells where the expression of Mib1 was markedly reduced (Fig. 7, C and D) (26). The expressions of ERTR7, a marker of the mesenchyme-derived stromal cells (55), and cytokeratin in the MMTV-Cre;Mib1f/f thymus (Fig. S4, A and B, available at http://www.jem.org/cgi/content/full/jem.20081344/DC1) suggest that Mib1 does not affect the differentiation of the stromal cells. Collectively, these data indicate that Mib1, among the four E3 ligases, plays a critical role in T cell development by regulating the endocytosis of Notch ligands in the thymic microenvironments.

**Regulation of T cell development by Mib1 in the OP9-DL1 stromal cells**

It has been demonstrated that the culture of HSCs on OP9-DL1 cells facilitates T cell development from HSCs (31), whereas progression through the DN stages of T cell development is impaired in the presence of the Notch signaling inhibitor (54). To further examine whether the defect in T cell development in the MMTV-Cre;Mib1f/f mice is caused by inability of Mib1-disrupted stromal cells to activate Notch signaling in hematopoietic cells, we transfected Mib1 small interfering RNA (siRNA) duplexes into the OP9-DL1 cells. Mib1 protein was significantly reduced 36 h after microporation in the Mib1 siRNA/OP9-DL1 cells (Mib1 siRNA/OP9-DL1; Fig. 8 A). In addition, when C2C12-Notch1 cells transfected with a CBF-Luc vector carrying RBP-J/H9260 binding sites were cocultured with the Mib1 siRNA/OP9-DL1 cells, CBF-luciferase reporter activity was markedly reduced compared with that of control siRNA-treated cells (control siRNA/OP9-DL1), suggesting that Mib1 is required for Notch signaling through regulating Dll1 function (Fig. 8 B[reference 56]).

To test whether the Mib1 siRNA/OP9-DL1 cells are able to support the T cell development from HSCs, fetal liver–derived LSK or BM-derived LSK cells were cocultured with either control siRNA/OP9-DL1 or Mib1 siRNA/OP9-DL1 cells. Consistent with the results from the Mib1-null mice, T cell development was blocked at the DN1 stage, when both fetal liver–derived and BM-derived LSK cells were cultured on the Mib1 siRNA/OP9-DL1 cells (Fig. 8 C). In addition,
when either control siRNA/OP9-DL1 or Mib1 siRNA/OP9-DL1 cells were cocultured with C2C12-Notch1 cells, Dll1 was not endocytosed but accumulated in the plasma membrane on the Mib1 siRNA/OP9-DL1 cells, whereas the endocytosis of Dll1 was readily observed as a punctate form on the control siRNA/OP9-DL1 cells (Fig. 8 D). These results recapitulate the defect in T cell development observed in the MMTV-Cre;Mib1f/f mice and provide the evidence that Mib1 in the stromal cells is required for T cell development by regulating Dll1 endocytosis.

**DISCUSSION**

The Notch signaling pathway is involved throughout the hematolymphoid system, from the generation of definitive HSCs to the differentiation of peripheral T and B cells (57, 58). In this study, our data provide in vivo evidence that an E3 ubiquitin ligase of Notch signaling, Mib1, is required for lymphopoiesis, including T lineage commitment and MZB cell development. Conditional inactivation of the Mib1 gene caused a block of T lineage commitment and MZB cell development, whereas their development was not perturbed in the other three mutant mice with inactivation of Mib2, Neur1, and Neur2, and even in the Neur1/2 double KO mice. More importantly, reciprocal BMT experiments revealed that Mib1 in the thymic and splenic microenvironments is essential for Notch signaling to the hematopoietic progenitors. Although the Notch ligands and the E3 ligases are expressed in both hematopoietic progenitors and their microenvironments (Fig. 1, A and B) (59), the defective Notch activation of hematopoietic progenitors and the failure of Notch ligand endocytosis were observed in the Mib1-null microenvironment. These results demonstrate that Notch signaling in the lymphopoietic niches to progenitors, but not the interactions between hematopoietic cells, is required for lymphopoiesis. In addition, the block in T cell development and the defect in the endocytosis of Dll1 were also observed in OP9-DL1 cell by Mib1 knockdown. Collectively, our study provides the first in vivo evidence that Mib1, in the lymphopoietic niches, controls T lineage commitment and MZB cell development by regulating Notch signaling.

The Notch signaling pathway is conserved in all metazoans. So far, four E3 ubiquitin-ligases, Mib1, Mib2, Neur1, and Neur2, which regulate the Notch ligands, have been identified in vertebrates, and three, dMib1, dMib2, and dNeur, have been found in D. melanogaster (21–28). In D. melanogaster, mutations in dNeur result in a defect in developmental defects that closely resemble those of Notch mutants and other Notch pathway mutants (60–62). However, mice with a disrupted Neur1 gene exhibit no abnormal cell fate specifications during neurogenesis and somitogenesis, two processes involving Notch signaling (35, 63). This discrepancy might be caused by the functional redundancy provided by Neur2. We originally expected a subset of Notch phenotypes in the Neur1/2 double KO mice because most mutant mice with disruptions in the key components of the Notch signaling pathway, such as Dll1, Dll4, Jag2, Notch1, Notch2, lunatic fringe, RBP-Jk, MAML1, and MINT, exhibit a subset of defects in lymphopoiesis (58). In this study, however, T and B cell development was not disturbed in the Neur1/2 double KO mice, demonstrating that Neur1/2 is not involved in these cell fate decisions.

In addition to Neur, Mib1 has been identified as another E3 ubiquitin ligase that interacts with Delta to promote its ubiquitination and internalization in the signal-sending cells, using zebrafish mutant models (21). In D. melanogaster, dMib1 is involved in regulating Notch ligand endocytosis, and the ectopic expression of dNeur bypasses the requirement for dMib1, suggesting that they appear to be interchangeable in mediating the ubiquitination and internalization of the Notch ligand (23, 64, 65). However, dMib1 and dNeur are expressed in different patterns (23, 64, 65), suggesting that both dNeur and dMib1 regulate Notch ligand endocytosis in different contexts of Notch-dependent cell fate decisions. In contrast to the results in D. melanogaster, Mib1-null mouse embryos showed completely defective Notch activation in terms of NICD generation and Notch-target gene expression (26), whereas the Neur1/2 double KO mice did not show any gross defects in mammalian development (34). Consistent with these findings, a developmental block in T and MZB cell development, which is a prototype of a Notch signaling defect, was observed only in Mib1-null mice, suggesting an obligatory role of Mib1 in mammalian T and MZB cell development.

In addition to Mib1, we previously identified Mib2 as an E3 ligase that regulates Delta (27). Overexpression of Mib2 rescues both the neuronal and vascular defects in the zebrafish Mib2 mutants (27), suggesting that they are interchangeable in mediating the ubiquitination and internalization of the Notch ligand. Mib2 is highly expressed in adult tissues, whereas Mib1 is expressed in both embryonic and adult tissues (27). Therefore, although Mib1 is an essential core component of the mammalian Notch pathway that controls multiple Notch ligands (26), it is necessary to determine whether Mib1 regulates multiple ligands beyond embryonic development. The existence of Mib2, which has functional similarities to Mib1 and is highly expressed in adult tissues with Mib1, brings us to speculate that both Mib1 and Mib2 might have functional redundancy in the adult tissues (27). However, in vivo studies using KO mice have revealed the essential role of Mib1 in Notch-mediated lymphocyte development beyond embryonic development.

We previously found that Mib1 is an essential regulator for generating functional Notch ligands to activate Notch signaling (26, 34). In this study, the conditional inactivation of Mib1 disturbed both T lineage commitment and MZB cell specification, suggesting that Dll1 and Dll4 should be non-functional. Indeed, the BM transplantation experiments using the TNR mice (51) demonstrate that the Mib1-null thymic microenvironment cannot initiate Notch signaling to thymic progenitors. Moreover, our reciprocal BM transplanta- tion experiments revealed that the Mib1-null lymphopoietic niches cannot support T lineage commitment and MZB cell specification. This inability of Mib1-null microenvironments
might be caused by the malfunction of Dll1 and Dll4. Consistent with this finding, the Dll1 was accumulated in the MMTV-Cre;Mib1−/− thymus, although it is unlikely that most of Dll1 was present at the plasma membrane. This abnormal localization of Dll1 might result from the continual accumulation of Dll1 in the ER, Golgi, or other organelle in the absence of Mib1. Furthermore, the block in T cell development and the failure of Dll1 endocytosis were also found in OP9-DL1 cells by Mib1 knockdown, which recapitulates the defect in T cell development observed in the MMTV-Cre;Mib1−/− mice. Collectively, our data suggest that Mib1 controls Notch signaling in the thymic progenitors from the thymic stromal cells, through the regulation of Dll1 and Dll4.

We previously proposed that Mib1 and Neur2 may play a cooperative role in the endocytic pathway of Delta using COS-7 cell lines (25). In this study, however, the endocytosis of Dll1 was impaired in the thymus from only Mib1-null mice but not Neur1−/−, Neur2−/−, or Neur1−/−;Neur2−/− mice. In addition, Mib2−/− mice also showed normal endocytosis of Dll1, despite Mib2 readily inducing its endocytosis in vitro (27). This discrepancy might be caused by the difference between in vivo and in vitro systems. Our in vivo study using KO mice would represent more relevant physiology of the Notch ligand endocytosis than in vitro observation.

Many studies have reported that maintenance of HSCs and regulation of their self-renewal and differentiation depend on their specific microenvironment (66, 67). Notch ligand–receptor interactions between the BM microenvironment and hematopoietic cells are thought to have a role in the maintenance of HSCs (68). In this paper, we have clearly shown that Notch signaling between the lymphopoietic niches and hematopoietic cells is required for T and MZB cell specification. This study will help elucidate the exact cellular sources in the lymphopoietic niches that trigger Notch signaling to their progenitors.

MATERIALS AND METHODS

Mice. The Mib1−/− mice (34) were bred with MMTV-Cre and Mx1-Cre mice (Jackson ImmunoResearch Laboratories) for removal of the floxed allele. 8-wk-old Mx1-Cre;Mib1−/− and Mx1-Cre;Mib1+/+ mice were injected i.p. four times at 2-d intervals with 300 μg pIpC. The Mib2−/− and Neur2−/− mice were previously generated by our group (34). CD45.1 mice (Jackson ImmunoResearch Laboratories), the Neur1−/−, Neur2−/−, and Neur1−/−;Neur2−/− mice (35), and the TNR mice (35) were used. All of the mouse experiments were performed in the animal facility under POSTECH institutional guidelines.

Flow cytometry and cell sorting. The following conjugated monoclonal antibodies were purchased from BD Biosciences unless otherwise indicated: CD45 (56-70-F11)-FITC; CD4 (RM4-5)-FITC; -PE and -biotin; CD90 (53-6.7)-FITC and -biotin; CD19 (1D3)-biotin; B220 (RA3-6B2)-FITC, -PE, -biotin and -APC; CD25 (7D4)-biotin; CD44 (MT7)-FITC and -biotin; CD24 (M1/69)-PE; CD117 (2B8)-PE and -APC; Sca-1 (E13-161.7)-FITC; CD3 (145-2C11)-biotin; Ter-119 (Ly-76)-biotin; CD11b (M1/70)-biotin; Gr-1 (RB6-8C5)-biotin; CD21 (7G6)-FITC; CD23 (B3B4)-biotin; CD9 (KMCS)-biotin; CD14 (1B1)-PE; IgM (II/41)-APC; CD45.1 (A20)-FITC and PE; CD45.2 (104)-FITC; and C1q/Rp (AA4.1, eBioscience)-FITC. Biotin-conjugated monoclonal antibodies were detected with streptavidin-PerCP (BD Biosciences). Single-cell suspensions were stained with the respective antibodies and were analyzed using a FACSCalibur flow cytometer (Becton Dickinson). Cells were sorted with a FACSVantage flow cytometer (BD Biosciences). The data were analyzed using the CellQuest software (BD Bioscience).

Real-time PCR analysis. For the real-time RT-PCR, RNA was extracted from the total thymus, spleen, and the FACS-sorted cells using an RNeasy Micro kit (Qiagen) according to the manufacturer’s instructions. Aliquots of 1 or 2 μg RNA were used for RT (Omniscript RT or Sensript RT; Qiagen) with oligo-dT priming. GAPDH mRNA was used as a control for relative quantification. For the genomic DNA PCR, genomic DNA was prepared from the CD45− cells sorted from collagenase-treated thymus and spleen using a DNaseasy Blood & Tissue kit (Qiagen). For detection and quantification, a MiYq real-time PCR detection system (Bio-Rad Laboratories) was used. PCR reactions were performed using an iQ SYBR Green Supermix kit (Bio-Rad Laboratories). Primer information will be provided upon request.

Western blotting. For Western blotting, the spleens were homogenized in lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, and 5 mM EDTA) containing a protease inhibitor mixture (Roche). Generally, 50 μg of protein from the supernatants were separated by size, blotted with primary and secondary antibodies, and visualized with ECL plus (GE Healthcare). The primary antibodies used were anti-Mib1 (provided by P.J. Gallagher, Indiana University School of Medicine, Indianapolis, IN) and anti-actin (Sigma-Aldrich) antibodies.

BM chimeras. CD45.1 mice (8–12-wk-old) were exposed to 960 rads of γ irradiation ~4–6 h before receiving 5 × 106 BM cells from the MMTV-Cre;Mib1−/− and MMTV-Cre;Mib1+/+ mice i.v. Inversely, the lethally irradiated MMTV-Cre;Mib1+/− and MMTV-Cre;Mib1−/− mice were i.v. injected with BM cells from the CD45.1 and TNR mice (51). Mice were maintained on antibiotics in drinking water for 7 d.

Immunohistochemistry. For histological analysis, tissues were fixed in 4% paraformaldehyde overnight at 4°C and embedded in OCT for sectioning. For immunohistochemistry, cryosections were incubated in blocking solution (3% BSA, 5% horse serum, and 0.5% Tween-20 in PBS) at room temperature for 3 h, followed by an additional incubation with various antibodies against the following proteins: Mib1 (provided by J. Peng, School of Medicine, Emory University, Atlanta, GA [reference 69]), multi-keratin (clone C-11; NeoMarkers), DBA-59932; Santa Cruz Biotechnology, Inc.), pan-reticular fibroblast marker (clone ER-TR7; Cedarlane) and Hrs (provided by M.J. Clague, University of Liverpool, Liverpool, UK). Specific binding was detected with Alexa 594– and 488–conjugated secondary antibodies (Invitrogen). Images were taken using confocal microscopy (FV1000; Olympus).

siRNA inhibition of Mib1 and OP9 cell cocultures. For siRNA-mediated silencing, we used SMART-pool Mouse Mib1 siRNA and siCONTROL non-targeting siRNA pool (Thermo Fisher Scientific). These siRNA duplexes were electroporated into OP9-DL1 cells using Microporator apparatus and buffers recommended by the manufacturer (Digital Bio Technology). 36 h after electroporation, to allow siRNA silencing, Western blot analysis, luciferase assay, and OP9-DL1 cell coculture were performed. For the CBF-Luc assay, the 8× CBF-Luc vectors were transfectected into C2C12-Notch1 cells with pKL-TK vector using Lipofectamine (Invitrogen). Luciferase activities were measured with a Dual Luciferase kit (Promega). For OP9-DL1 cell coculture, Lin−Sca-1−/− cells of fetal liver or BM were seeded on a monolayer of OP9-DL1 cells electroporated with siRNA and cultured as described (31) for 5 or 7 d, respectively. After culture, growing cells were collected and analyzed by flow cytometry. For OP9-DL1 cell cocultures with C2C12-Notch1, 106 C2C12-Notch1 cells were seeded into one well of a 24-well plate containing a monolayer of OP9-DL1 cells and cultured. After 12 h, the cells were immunostained with Dll1 antibody and then visualized with Alexa 594–conjugated secondary antibody (Invitrogen). Images were taken using microscopy (Axioskop2 Plus; Carl Zeiss, Inc.).
Online supplemental material. Fig. S1 shows the reduction of T cell subsets in the thymus of the Mx1-Cre;Mib1f/f mice. Fig. S2 shows the cytometric analysis which exhibits the MZB cell defect in the Mx1-Cre;Mib1f/f mice. Fig. S3 shows the normal endocytosis of Dll1 in the thymus from the Mib2−/− and Neu1−/−;Neu2−/− mice by immunostaining. Fig. S4 shows the expression of the stromal cell markers cytokeratin and ERTR7, suggesting that Mib1 does not affect the differentiation of the stromal cells in the thymus of the MMTV-Cre;Mib1f/f mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20081344/DC1.

We thank Drs. Y.N. Jan, N. Gaiaron, J. Peng, and J.C. Zuriga-Pflucker for kindly providing the Neu1−/− mice, the TNR mice, Mib1 antibody, and the OP9-DL1 cells, respectively.

This work was supported by the Korea Science and Engineering Foundation grant funded by the Korea government (R11-2001-090-03001-0; M10641000003-H11002-08110).

The authors have no conflicting financial interests.

Submitted: 20 June 2008
Accepted: 19 August 2008

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