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Mind bomb 1 in the lymphopoietic niches is essential for T and marginal zone B cell development

Ran Song,[1] Young-Woong Kim,[1] Bon-Kyoung Koo,[1] Hyun-Woo Jeong,[1] Mi-Jeong Yoon,[1] Ki-Jun Yoon,[1] Dong-Jae Jun,[1] Sun-Kyoung Im,[1] Juhee Shin,[1] Myoung-Phil Kong,[1] Kyong-Tai Kim,[1] Keejung Yoon,[2] and Young-Yun Kong[1,3]

[1]Department of Life Science, Pohang University of Science and Technology, Pohang, Kyungbuk 790-784, South Korea
[2]Center for Biomedical Sciences, National Institutes of Health, Seoul 122-701, South Korea
[3]Department of Biological Sciences, Seoul National University, Seoul 151-747, South Korea

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.

CORRESPONDENCE
Young-Yun Kong:
ykong@snu.ac.kr

Abbreviations used: DN, double negative; DP, double positive; ETP, early T lineage progenitor; FOB, follicular B; HSC, hematopoietic stem cell; Mib, mind bomb; MMTV, mouse mammary tumor virus; MZB, marginal zone B; NECD, Notch extracellular domain; Neur, neuralized; NICD, Notch intracellular domain; pIpC, polyinosinic-polycytidylic acid; siRNA, small interfering RNA; SP, single positive; TNR, transgenic Notch reporter; TSP, thymic seeding progenitor.

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 motile, 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 in the thymus influences lineage decisions at multiple stages of T cell development, the generation of early T lineage progenitors (ETPs), αβ/γδ specification, and pre–T cell receptor signaling (6–9). At the T cell–B cell branchpoint in the thymus, Notch signaling is delivered to progenitors with T and B potential. These progenitors undergo the T lineage development after receiving Notch signals and the B lineage development in the absence of Notch signals (10, 11). Moreover, Notch signaling regulates the generation of ETPs after thymic entry of TSPs in the thymus (12, 13).

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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-Jκ (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 ($Mib1^{f/f}$), $Mib2^{-/-}$, $Neur1^{-/-}$, 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;*$Mib1^{f/f}$ mice

To elucidate the requirement of E3 ligases in T cell development, we used $Mib1^{f/f}$, $Mib2^{-/-}$, $Neur2^{-/-}$ (34), and $Neur1^{-/-}$ mice (35). To investigate the effect of Mib1 in T cell development, the $Mib1^{f/f}$ 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;*$Mib1^{f/f}$ 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;*$Mib1^{f/f}$, $Mib2^{-/-}$, $Neur1^{-/-}$, and $Neur2^{-/-}$ mice. Unexpectedly, the thymocyte development,

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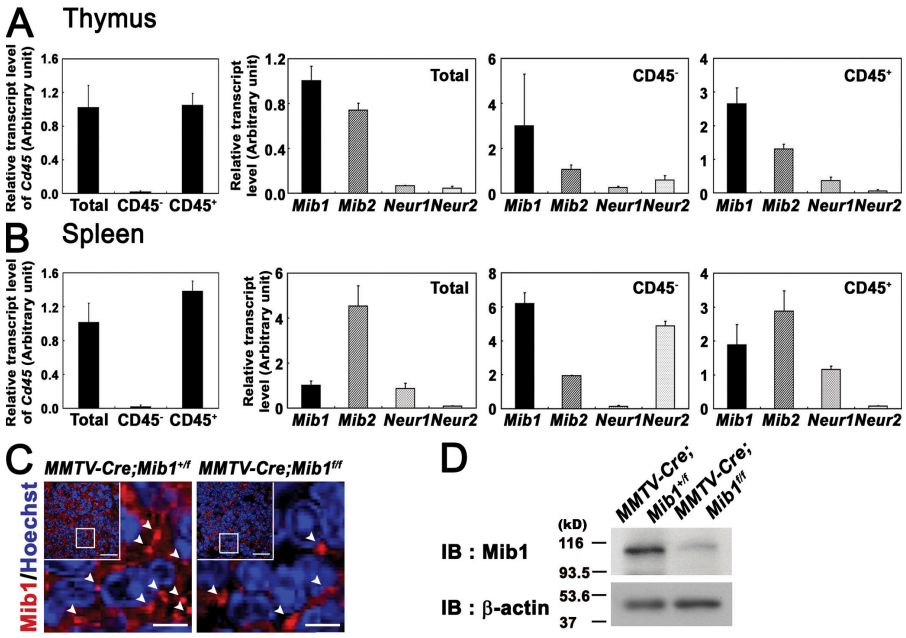

Figure 1. The expression of E3 ubiquitin ligases and the deletion of Mib1 in the lymphoid tissues. (A and B) The expression of E3 ligases in the thymus (A) and spleens (B). Total RNAs were prepared from the thymus and spleen (total) and nonhematopoietic ($CD45^-$) and hematopoietic ($CD45^+$) fractions sorted from collagenase-treated thymi and spleens. They were subjected to quantitative real-time RT-PCR using specific primers for *Cd45*, *Mib1*, *Mib2*, *Neur1*, and *Neur2*. Data are mean ± SD from triplicate experiments. The mean expression level of *Mib1* mRNA in the total thymus and spleen was defined as 1 arbitrary unit, as compared with the expression levels of the E3 ubiquitin ligases. (C) Immunohistochemistry was performed on the cryosections from the *MMTV-Cre;Mib1*$^{+/f}$ and *MMTV-Cre;Mib1*$^{f/f}$ thymi with the anti-Mib1 antibody. Note that Mib1 expression (arrowheads) was reduced in the *MMTV-Cre;Mib1*$^{f/f}$ mice thymus. Bars, 5 µm. (D) Total protein extracts of the spleens from the *MMTV-Cre;Mib1*$^{+/f}$ and *MMTV-Cre;Mib1*$^{f/f}$ mice were immunoblotted with the anti-Mib1 antibody.

as well as the percentages and numbers 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*$^{f/f}$ 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*$^{f/f}$ mice, as compared with the *MMTV-Cre;Mib1*$^{+/f}$ mice (Fig. 2 B). The absolute numbers of total thymocytes were reduced 3.6-fold in the *MMTV-Cre;Mib1*$^{f/f}$ mice as compared with those of the control mice. The CD4 SP, CD8 SP, and CD4CD8 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*$^{f/f}$ 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*$^{f/f}$ mice showed a block at $CD44^+CD25^-$ DN1 thymocytes, as compared with the *MMTV-Cre;Mib1*$^{+/f}$ 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*$^{f/f}$ mice should contain increased B cells. Collectively, these results suggests 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*$^{f/f}$ 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*$^{f/f}$ 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*$^{f/f}$ 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*$^{f/f}$ 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*$^{f/f}$ mice as compared with those of the control mice (Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20081344/DC1). Collectively,

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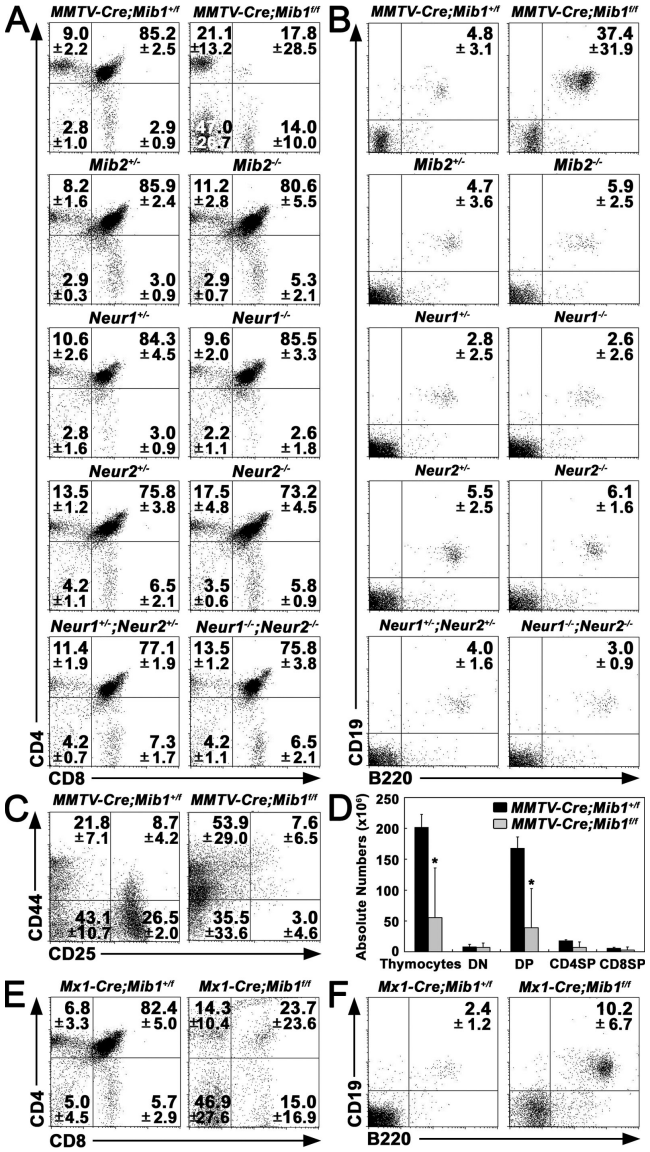

Figure 2. Impaired T cell development in the thymi of *Mib1* conditional KO mice. (A and B) Thymocytes from ~8–10-wk-old littermate controls (left) and the mutant (right) mice indicated were stained for CD4 and CD8 (A) and CD19 and B220 gated on $CD4^-CD8^-$ DN thymocytes (B) and were analyzed by flow cytometry. Percentages of each population are indicated in the quadrants. Numbers are mean ± SD from 10 independent experiments. (C) Thymocytes from the indicated mice were analyzed by flow cytometry for the expression of CD44 and CD25 gated on $CD4^-CD8^-$ DN thymocytes. Percentages indicated are mean ± SD from five independent experiments. (D) Absolute cell numbers for total thymocytes and thymocyte subsets of the *MMTV-Cre;Mib1*$^{+/f}$ and *MMTV-Cre;Mib1*$^{f/f}$ mice were calculated and are shown as histograms. The error bars represent mean ± SD from five independent experiments. *, $P < 0.01$. (E and F) The *Mx1-Cre;Mib1*$^{+/f}$ and *Mx1-Cre;Mib1*$^{f/f}$ mice were injected i.p. four times at 2-d intervals with 300 µg pIpC. At ~8–14 wk after the final injection, the thymocytes were analyzed by flow cytometry for the expression of CD4 and CD8 (D) and CD19 and B220 (E) gated on $CD4^-CD8^-$ DN thymocytes. Percentages indicated are mean ± SD from five independent experiments.

these results demonstrate that Mib1, among the four E3 ligases, is essential for T cell development.

Defective ETP generation in the *MMTV-Cre;Mib1*$^{f/f}$ 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, $CD44^{hi}CD25^-$ DN1 thymocytes from the *MMTV-Cre;Mib1*$^{f/f}$, *Mib2*$^{-/-}$, *Neur1*$^{-/-}$, and *Neur2*$^{-/-}$ mice were analyzed for the expression of c-kit and CD24 because the $c\text{-}kit^+CD24^-$ and $c\text{-}kit^+CD24^{lo}$ 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;Mib1*$^{f/f}$ 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;Mib1*$^{f/f}$ 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

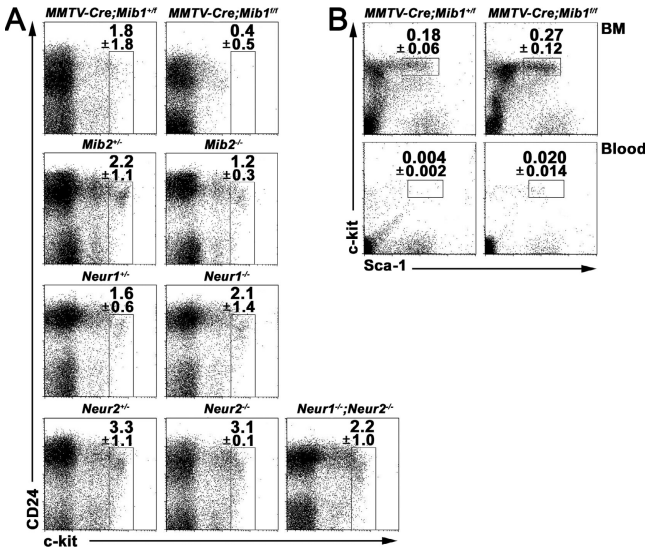

Figure 3. Reduction of early T cell progenitors in the thymi of *MMTV-Cre;Mib1*$^{f/f}$ mice. (A) The thymocytes from the littermate control (left) and mutant (right) mice indicated were stained for CD4, CD8, CD44, CD25, CD24, and c-kit. ETPs were identified by CD24 and c-kit expression gated on $CD4^-CD8^-CD25^-CD44^+$ cells. Percentages indicated are mean ± SD from five independent experiments. (B) Leukocytes from the BM (top) and blood (bottom) of the *MMTV-Cre;Mib1*$^{+/f}$ and *MMTV-Cre;Mib1*$^{f/f}$ 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.

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in the *Mx1-Cre;Mib1$^{f/f}$* 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$^{f/f}$* 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$^{f/f}$*, *Mib2$^{-/-}$*, *Neur1$^{-/-}$*, and *Neur2$^{-/-}$* mice. When the splenocytes from the mice were analyzed, the fraction of B220^{+}CD21^{+}CD23$^{lo/-}$ MZB cells was markedly reduced only in the *MMTV-Cre;Mib1$^{f/f}$* 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$^{f/f}$* 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 splenocytes down-regulates the expression of CD21 (14). Consistent with these findings, the FOB cells in the spleens from the *MMTV-Cre;Mib1$^{f/f}$* mice also showed reduced expression of CD21 (Fig. 4 C). Consistent with the results from the *MMTV-Cre;Mib1$^{f/f}$* mice, the defect in MZB cell development was also found in the spleen of the *Mx1-Cre;Mib1$^{f/f}$* mice at 12 wk after the last pIpC 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$^{-/-}$*, *Neur1$^{-/-}$*, *Neur2$^{-/-}$*, and even *Neur1$^{-/-}$;Neur2$^{-/-}$* 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$^{f/f}$* 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$^{f/f}$* mice, as compared with the control mice (Fig. 4 D). Despite the decrease of T2 B cells in the *MMTV-Cre;Mib1$^{f/f}$* 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$^{f/f}$* 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^{+}IgM^{high}CD23^{+} 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$^{f/f}$* 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

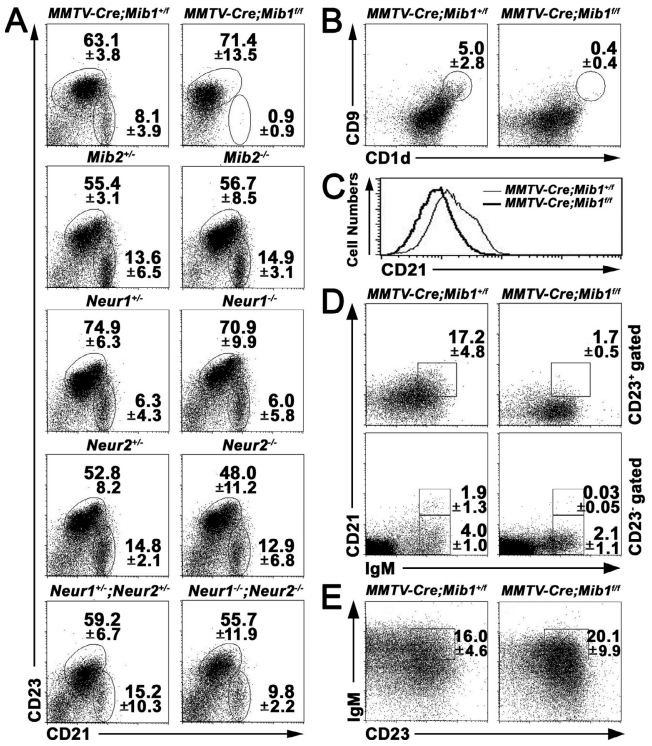

Figure 4. MZB cell defect in the *MMTV-Cre;Mib1$^{f/f}$* mice. (A) The splenocytes from the indicated mice were analyzed by flow cytometry for the expression of CD21 and CD23 gated on B220^{+} cells. MZB and FOB cells were defined as CD21^{hi}CD23$^{lo/-}$ and CD21^{int}CD23^{hi}, respectively. Percentages indicated are mean ± SD from 10 independent experiments. (B) The splenocytes from the *MMTV-Cre;Mib1$^{+/f}$* (left) and *MMTV-Cre;Mib1$^{f/f}$* (right) mice were stained for CD1d and CD9 gated on B220^{+} cells. Note the defective CD1d^{hi}CD9^{hi} MZB cells in the mutant mice. Percentages indicated are mean ± SD from 10 independent experiments. (C) FOB cells from the *MMTV-Cre;Mib1$^{+/f}$* and *MMTV-Cre;Mib1$^{f/f}$* mice were analyzed by flow cytometry for the expression of CD21. A representative of four independent experiments is shown. (D) The splenocytes were stained with antibodies against CD21, CD23, and IgM and were analyzed by flow cytometry for the expression of CD21 and IgM, gated on the CD23^{-} splenocytes for T1 B cells, and gated on the CD23^{+} splenocytes for T2 B cells. Numbers indicate mean ± SD from five independent experiments. (E) The splenocytes from the indicated mice were analyzed by flow cytometry for the expression of CD23 and IgM gated on B220^{+}AA4.1^{+} cells. The transitional T2 B cells were defined as CD23^{+}IgM^{hi}. Percentages indicated are mean ± SD from three independent experiments.

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in both the CD45$^+$ hematopoietic and CD45$^-$ nonhematopoietic cells from the thymus (Fig. 1 A). Therefore, the impaired T cell development in the *MMTV-Cre;Mib1$^{f/f}$* and *Mx1-Cre;Mib1$^{f/f}$* 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;Mib1$^{f/f}$* mice. At 12 wk after transplantation, all of the CD45.1 recipient mice displayed normal thymocyte development, including B cell and ETP generation (Fig. 5 A), indicating that the defective thymocyte development in the *MMTV-Cre;Mib1$^{f/f}$* and *Mx1-Cre;Mib1$^{f/f}$* 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;Mib1$^{f/f}$* 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;Mib1$^{f/f}$* 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;Mib1$^{f/f}$* recipient mice, whereas the WT recipient mice showed normal thymocyte development, as in the untransplanted WT mice (Fig. 5 B). Consistent with this finding, the absolute numbers of total thymocytes were dramatically reduced (50.8-fold) in the *MMTV-Cre;Mib1$^{f/f}$* 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. 5 B). 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;Mib1$^{f/f}$* 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

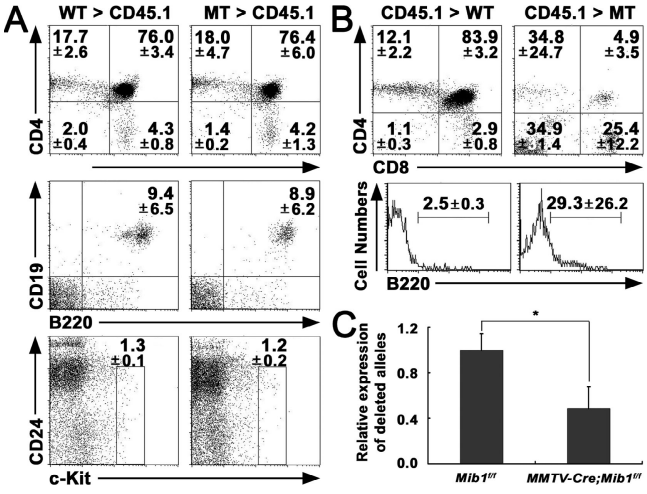

Figure 5. Regulation of T cell development by Mib1 in the thymic microenvironment. (A) The lethally irradiated CD45.1 mice were injected intravenously with BM cells from the 12–15-wk-old *MMTV-Cre;Mib1$^{+/f}$* and *MMTV-Cre;Mib1$^{f/f}$* mice. At 12 wk after transplantation, the thymocytes were analyzed by flow cytometry for the expression of CD4 and CD8 gated on the CD45.2$^+$ populations (top), CD19 and B220 gated on the CD4$^-$CD8$^-$ DN thymocytes (middle), and CD24 and c-kit gated on CD4$^-$CD8$^-$CD25$^-$CD44$^+$ cells (bottom). Numbers indicate mean ± SD from four independent experiments. (B) The lethally irradiated *MMTV-Cre;Mib1$^{+/f}$* and *MMTV-Cre;Mib1$^{f/f}$* 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 (bottom). Numbers indicate mean ± SD from three 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.

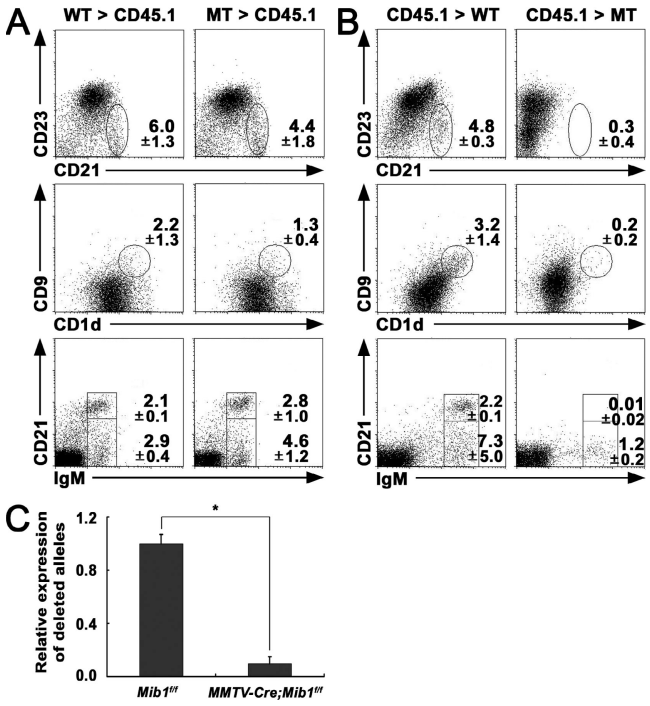

Figure 6. Regulation of MZB cell development by Mib1 in the splenic microenvironment. (A) The lethally irradiated CD45.1 mice were injected i.v. with BM cells from the 12–15-wk-old *MMTV-Cre;Mib1$^{+/f}$* (left) and *MMTV-Cre;Mib1$^{f/f}$* (right) mice. At 12 wk after transplantation, the splenocytes 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;Mib1$^{f/f}$* (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.

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from *MMTV-Cre;Mib1$^{f/f}$* 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$^{f/f}$* 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$^{f/f}$* 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$^{f/f}$* mice were reconstituted with BM cells from CD45.1 congenic mice, B220$^+$CD21$^+$CD23$^{lo/-}$ and B220$^+$CD1d$^+$CD9$^+$ MZB cell development was completely blocked in the *MMTV-Cre;Mib1$^{f/f}$* 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$^{f/f}$* recipient mice (Fig. 6 B and not depicted). These results show that the impaired MZB cell development in the *MMTV-Cre;Mib1$^{f/f}$* mice originated from a nonhematopoietic microenvironment. Consistent with these findings, *Mib1* was deleted ~90% in isolated CD45$^-$ splenic stromal cells from *MMTV-Cre;Mib1$^{f/f}$* 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$^{f/f}$* 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$^{f/f}$* and *Mx1-Cre;Mib1$^{f/f}$* 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$^{+/f}$* and *MMTV-Cre;Mib1$^{f/f}$* 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$^{f/f}$* mice (Fig. 7 A), indicating that the Mib1-null thymic microenvironment cannot activate Notch signaling in T cell progenitors.

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$^{+/f}$*

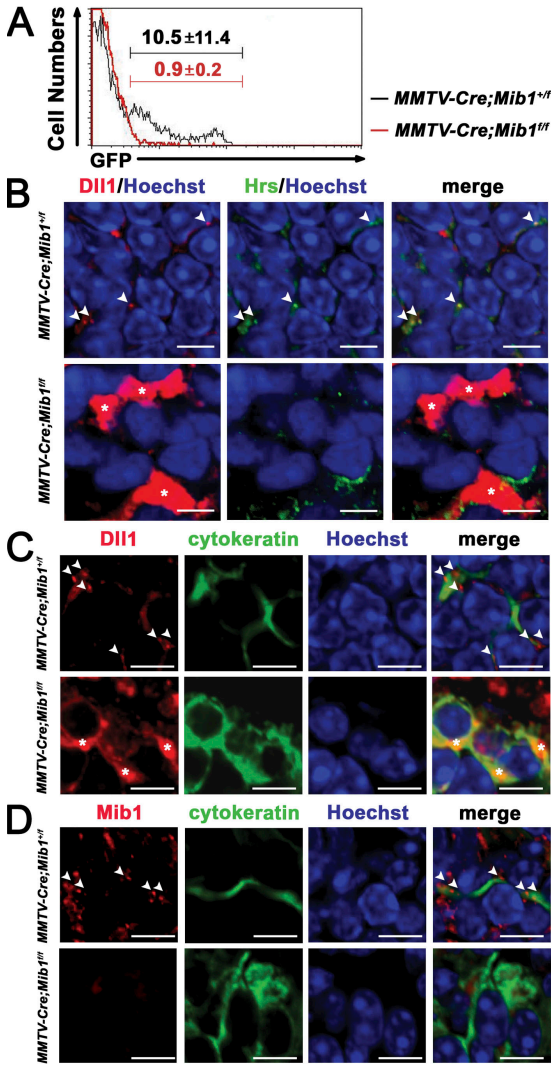

Figure 7. Notch signaling defect in the thymi of *Mib1* conditional KO mice. (A) The lethally irradiated *MMTV-Cre;Mib1$^{+/f}$* (black) and *MMTV-Cre;Mib1$^{f/f}$* (red) mice (7–9 wk old) were injected i.v. with BM cells from the TNR mice (51). At 4 wk after transplantation, the thymocytes were analyzed by flow cytometry for GFP expression gated on the CD4$^-$CD8$^-$ DN thymocytes. Percentages indicated are mean ± SD from three independent experiments. (B–D) The lethally irradiated *MMTV-Cre;Mib1$^{+/f}$* and *MMTV-Cre;Mib1$^{f/f}$* mice were transplanted with CD45.1 BM cells. 6 wk after transplantation, the thymi were fixed and cryosections were immunostained with anti-Dll1 (B and C, red), anti-Hrs (B, green), anti-cytokeratin (C and D, green), and anti-Mib1 (D, red) antibodies with HOECHST (blue). Note that Dll1 was colocalized with Hrs (B, arrowheads) in the *MMTV-Cre;Mib1$^{+/f}$* thymus, whereas it accumulated in the cytokeratin-positive cortical epithelial cells of the *MMTV-Cre;Mib1$^{f/f}$* thymus (B and C, asterisks). The arrowheads in C and D show the expression of Dll1 and Mib1, respectively. A representative of three independent experiments is shown. Bars, 5 µm.

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and *MMTV-Cre;Mib1*$^{f/f}$ 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*$^{+/f}$ recipient mice (Fig. 7 B, arrowheads). In the thymi of the *MMTV-Cre;Mib1*$^{f/f}$ 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;Mib1*$^{f/f}$ mice by immunohistochemistry. In the *MMTV-Cre;Mib1*$^{f/f}$ thymus, Dll1 was accumulated by some of the cytokeratin-positive cortical epithelia 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;Mib1*$^{f/f}$ 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;Mib1*$^{f/f}$ 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-treated OP9-DL1 cells (Mib1 siRNA/OP9-DL1; Fig. 8 A). In addition, when C2C12-Notch1 cells transfected with a CBF-Luc vector carrying RBP-Jκ 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,

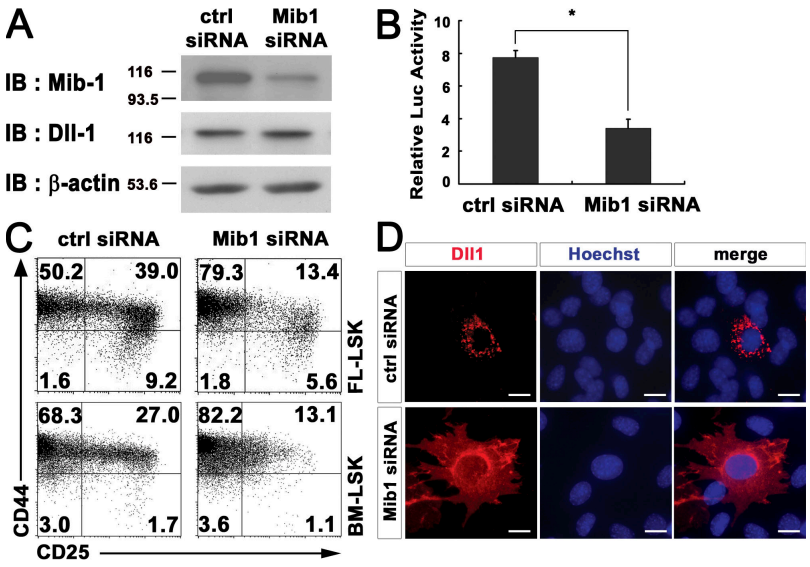

Figure 8. Block in T cell development at DN1 stage in knockdown Mib1 in OP9-DL1 cells. (A) Immunoblot of Mib1 protein in OP9-DL1 cells 36 h after microporation with control (ctrl) or Mib1 siRNA. Dll1 expression was not affected. (B) Control siRNA/OP9-DL1 or Mib1 siRNA/OP9-DL1 cells were cocultured with C2C12-Notch1 cells transfected with 8× WT CBF-Luc vectors. 24 h after coculture, luciferase activities were measured. Data are mean ± SD from triplicate experiments. *, $P < 0.001$. (C) Lin$^-$Sca-1$^+$c-Kit$^+$ fetal liver (FL-LSK) or BM (BM-LSK) cells were prepared and cultured on control siRNA/OP9-DL1 or Mib1 siRNA/OP9-DL1 cells. Growing cells collected on days 5 or 7, respectively, were stained for CD44 and CD25 gated on CD4$^-$CD8$^-$ DN cells and analyzed by flow cytometry. A representative of three independent experiments is shown. (D) C2C12-Notch1 cells were cocultured on control siRNA/OP9-DL1 or Mib1 siRNA/OP9-DL1 cells. After 12 h, the cells were stained with Dll1 antibody. Bars, 10 μm.

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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;Mib1$^{f/f}$* 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 variety of 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-Jκ, 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 *Mib^{ta52b}* 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 nonfunctional. 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 transplantation experiments revealed that the Mib1-null lymphopoietic niches cannot support T lineage commitment and MZB cell specification. This inability of Mib1-null microenvironments

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might be caused by the malfunction of Dll1 and Dll4. Consistent with this finding, the Dll1 was accumulated in the *MMTV-Cre;Mib1$^{f/f}$* 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$^{f/f}$* 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 thymi 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 depends 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$^{f/f}$* 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$^{+/f}$* and *Mx1-Cre;Mib1$^{f/f}$* 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$^{-/-}$* mice (35), and the TNR mice (51) 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 (30-F11)-FITC; CD4 (RM4-5)-FITC, -PE, and -biotin; CD8α (53–6.7)-FITC and -biotin; CD19 (1D3)-biotin; B220 (RA3-6B2)-FITC, -PE, -biotin and -APC; CD25 (7D4)-biotin; CD44 (IM7)-FITC and -biotin; CD24 (M1/69)-PE; CD117 (2B8)-PE and -APC; Sca-1 (E13-161.7)-FITC; CD3 (145-2C11)-biotin; Ter119 (Ly-76)-biotin; CD11b (M1/70)-biotin; Gr-1 (RB6-8C5)-biotin; CD21 (7G6)-FITC; CD23 (B3B4)-biotin; CD9 (KMC8)-biotin; CD1d (1B1)-PE; IgM (II/41)-APC; CD45.1 (A20)-FITC and PE; CD45.2 (104)-FITC; and C1qRp (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 Biosciences).

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 Sensiscript RT; QIAGEN) with oligo-dT priming. GAPDH mRNA served 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 DNeasy Blood & Tissue kit (QIAGEN). For detection and quantification, a MyiQ 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 M 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 × 10^6 BM cells from the *MMTV-Cre;Mib1$^{+/f}$* and *MMTV-Cre;Mib1$^{f/f}$* mice i.v. Inversely, the lethally irradiated *MMTV-Cre;Mib1$^{+/f}$* and *MMTV-Cre;Mib1$^{f/f}$* 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), Dll1 (sc-9932; 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 transfected into C2C12-Notch1 cells with pRL-TK vector using Lipofectamine (Invitrogen). Luciferase activities were measured with a Dual Luciferase kit (Promega). For OP9-DL1 cell coculture, Lin$^-$Sca-1$^+$c-Kit$^+$ 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 cocultures with C2C12-Notch1, 10^5 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;Mib1$^{f/f}$* mice. Fig. S2 shows flow cytometric analysis which exhibits the MZB cell defect in the *Mx1-Cre;Mib1$^{f/f}$* mice. Fig. S3 shows the normal endocytosis of Dll1 in the thymi from the *Mib2$^{-/-}$* and *Neur1$^{-/-}$;Neur2$^{-/-}$* mice by immunostaining. Fig. S4 shows the expression of the stromal cell markers cytokeratin and ERTR7, suggesting that Mib1 did not affects the differentiation of the stromal cells in the thymus of the *MMTV-Cre;Mib1$^{f/f}$* mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20081344/DC1.

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The authors have no conflicting financial interests.

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