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Runx1/Cbfβ2 Complexes Are Required for Lymphoid Tissue Inducer Cell Differentiation at Two Developmental Stages

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Hematopoietic lymphoid tissue inducer (LTI) cells are essential for the development of secondary lymphoid tissues including lymph nodes and Peyer’s patches. Two transcription factors, the helix-loop-helix inhibitor Id2 and the retinoic acid-related orphan receptor γt (Rorγt), have been shown to be crucial for LTI cell development. However, it remains unclear how the specification of multipotent hematopoietic progenitor cells toward the LTI lineage is programmed. In this study, we report impaired lymphoid tissue organogenesis in mice in which the function of Runx1/Cbfβ transcription factor complexes was attenuated by the loss of either the distal promoter-derived Runx1 or Cbfβ2 variant protein. We found that LTI progenitors in fetal liver, defined previously as a lineage-marker-negative αβ7 integrin (αβ7)+ IL-7Rα-chain (IL-7Rα)+ population, can be subdivided into Rorγt-expressing IL-7Rαhigh cells and nonexpressing IL-7Rαmid cells. Whereas Id2 and Rorγt are required to direct αβ7+IL-7Rαmid cells to become αβ7+IL-7Rαhigh cells, Runx1/Cbfβ2 complexes are necessary for the emergence of αβ7+IL-7Rαmid cells. In addition, the loss of Cbfβ2, but not P1-Runx1, resulted in an inefficient upregulation of Rorγt in residual αβ7+IL-7Rα+ LTI cells at anlagen. Our results thus revealed that Runx1/Cbfβ2 complexes regulate the differentiation of LTI cells at two stages: an early specification of hematopoietic progenitors toward the LTI lineage and a subsequent activation of Rorγt expression at anlagen.

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differentiation of several hematopoietic lineage cells, including hematopoietic stem cells, B-lymphocytes, NK T cells, and cytotoxic T cells (19–22). Runx complexes are composed of a DNA-binding Runx protein and its non–DNA-binding partner Cbfβ (Supplementary Fig. 1). All three mammalian Runx genes (Runx1, Runx2, and Runx3) are transcribed from the distal (P1) and proximal (P2) promoters (23). In addition to the differential expression pattern of P1- and P2-derived Runxl variants (P1-Runxl and P2-Runxl, respectively) (24), P1-Runxl and P2-Runxl variants differ in their N-terminal end sequences (Supplemental Fig. 1). Conversely, alternative RNA splicing of the Cbfβ transcript generates two Cbfβ splice variants, Cbfβ1 and Cbfβ2, which differ only at their C termini (Supplemental Fig. 1) (25).

In this study, we report that mice lacking either the P1-Runxl or Cbfβ2 variant exhibit impaired lymphoid tissue organogenesis due to the impaired differentiation of LTi cells. Our results demonstrate that a Lin-ε4B7-IL-7Rxα population in the fetal liver can be divided into Rorγ IL-7Rxα and Rorγε4B7-IL-7Rxα subsets. Interestingly, only of P1-Runxl and Cbfβ2, but not Rorγ nor Id2, results in the severely impaired differentiation of Lin-ε4B7-IL-7Rxα cells, indicating that Runx1/Cbfβ2 complexes are involved in the early specification to the LTi lineage prior to the developmental stage at which Rorγ and Id2 further program LTi cell development. In addition, full induction of Rorγ expression at anlagen is dependent on Cbfβ2. These results reveal two Runx1/Cbfβ2-dependent regulatory mechanisms underlying LTi cell development: an early specification toward the LTi lineage in multipotent progenitors and an involvement in an induction of Rorγ expression later at anlagen.

Materials and Methods

Mouse strains

Rorγ0/0m, Id2−/−, and Runx1P1N/P1N mice have been described previously (15, 18, 26). The generation of Cbfβ2m2m mice by homologous recombination in embryonic stem cells will be described elsewhere. In brief, to generate the Cbfβ2m allele, the splice donor signal, the GTTAG sequences, at the end of the exon 5 in the Cbfβ gene, was mutated to AAATG. Because the targeted mutations inhibited RNA splicing generating Cbfβ2m mRNA, no Cbfβ2 variant protein is produced in the Cbfβ2m2m mice.

Whole-mount staining

Whole-mount immunostaining was performed as previously described (7). In brief, excised fetuses were microwave irradiated at 500 W for 30 s in ice-cold fixation solution (2% paraformaldehyde in PBS) and incubated in the same solution for 30 min at 4°C. After removing the serosa of the adult small intestine, the endogenous peroxidase activity was blocked with 0.3% H2O2 in methanol for 30 min at 4°C. The rehydrated specimens were then stained with anti-CD71, anti-IL-7Rxα, or anti–VCAM-1 mAb, which was visualized with an HRP-conjugated secondary Ab.

Abs and flow cytometry

The Abs used for flow cytometry were from BD Pharmingen or ebioscience: CD3ε (clone 145-2C11), CD11c (HL3), CD45RB/B20 (RA3-6B2), NK-1.1 (PK136), Gr-1 (RB3-8C5), TER-119 (TER-119), αB7 (DAATK2), IL-7Rα (ATRA734), CD16/CD32 (2.4G2), c-Ki (2B8), Sc-Al (E13-161.7), CD4 (L3T4), CD8α (Ly-2), CXCR5 (2G8), and TCPR (H57-597). Surface staining was performed for 15–20 min with the corresponding mixture of fluorescein isothiocyanate-labeled Abs. Data were acquired on an FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Cell preparation from fetal organ

All organs of embryos were dissected under a stereomicroscope and then dissociated with dispase (BD Pharmingen) for 15 min at 37°C, as previously described (10, 13). After gently pipetting, the dissociated cells were washed with PBS containing 2% FCS. When staining for CXCR5 and CD4, the organs were dissociated with collagenase (Wako Chemicals).

Results

Impaired lymphoid organogenesis in Runx mutant mice

To investigate the function of P1-Runxl mutant protein, we replaced the exon encoding-specific N-terminal sequences for

![FIGURE 1. Impaired development of secondary lymphoid tissues in Runx1P1N/P1N and Cbfβ2m2m mice. A, Representative gross image of PPs on adult intestine from 12-wk-old control (Wt), Runx1P1N/P1N, and Cbfβ2m2m mice showing reduced size of remaining PPs in Runx1P1N/P1N and Cbfβ2m2m mice. Scale bars, 1 mm. B, Whole-mount staining of adult intestine from Wt, Runx1P1N/P1N, Cbfβ2m2m mice using anti-CD71 Ab. C, Statistical summary of the numbers of PPs in the littermate control, Runx1P1N/P1N, and Cbfβ2m2m mice. D, The number of mice with normal lymph nodes at the indicated positions. Although the mesenteric LNs were present in all Runx1P1N/P1N and Cbfβ2m2m mice, the development of other peripheral LNs was impaired in these mice. ***p < 0.0001.](http://www.jimmunol.org/)

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P1-Runx1 with the neo' gene (the Runx1\textsuperscript{P1N} allele) (26). Although NKT cell development was almost abolished in the Runx1\textsuperscript{P1N/P1N} mice, as reflected in the conditional inactivation of the Runx1 gene in CD3\textsuperscript{+}CD8\textsuperscript{+} double-positive thymocytes (21), other phenotypes, such as thymic hypopcellularity, CD4 derepression in double-negative thymocytes, and the reduction of CD4\textsuperscript{+} T cell numbers, were milder than those caused by inactivation of Runx1 gene (Supplemental Fig. 2) (27, 28). Thus, the effect of P1-Runx1 deficiency varied among T cell subsets and the developmental stages, presumably because of differences in either a redundant function or in the sensitivity to the dosage of Runx1 protein.

During phenotypic analyses of T cell development, we noticed that the formation of PPs was impaired in the Runx1\textsuperscript{P1N/P1N} mice. Immune staining of whole adult intestines with anti-B220 Ab confirmed that both the number and size of PPs were reduced in the Runx1\textsuperscript{P1N/P1N} mice (Fig. 1A–C). Similarly, formation of peripheral LNs, such as the inguinal and axillary LNs, was impaired in the Runx1\textsuperscript{P1N/P1N} mice, whereas the mesenteric LNs were present in all Runx1\textsuperscript{P1N/P1N} mice examined (Fig. 1D).

Because the activity of Runx1 protein is exerted as a heterodimeric complex with a non-DNA partner, the Cbf\beta protein (29), we wished to examine the role of Cbf\beta in lymphoid tissue organogenesis. By targeting mutations to the splicing donor signals that paired by loss of the Cbf\beta variant (Fig. 2B). Similarly, although LN anlagen were detected as an aggregates of IL-7R\textsuperscript{+} LTo cells in the para-aortic regions of the control mice as paired dense spots, a reduced number of diffuse spots or no significant signal was detected in the Runx1\textsuperscript{P1N/P1N} and Cbfb\textsuperscript{2m/2m} mice (Fig. 2C). Ectopic anlagen formation was occasionally observed in these mutant mice, consistent with the ectopic locations of para-aortic LNs in some adult mutant mice (M. Tachibana and H. Yoshida, unpublished observations). Consistent with the presence of MLN in adult Cbfb\textsuperscript{2m/2m} mice, formation of MLN anlagen was not impaired by loss of the Cbf\beta variant (Fig. 2D). These results...

**FIGURE 2.** Impaired organization of secondary lymphoid tissue anlagen at 17.5 dpc fetuses of Runx1\textsuperscript{P1N/P1N} and Cbfb\textsuperscript{2m/2m} mice. Whole-mount staining of intestine with anti–VCAM-1 Ab (A) and anti–IL-7R\textsuperscript{α} Ab (B) and staining of para-aortic region with anti–IL-7R\textsuperscript{α} Ab (C) from control (Wt), Runx1\textsuperscript{P1N/P1N}, and Cbfb\textsuperscript{2m/2m} newborn mice. The brown spot indicated with the arrows in A and C represent clusters of LTo cells at PP anlagen and clusters of LTi cells at the renal region, respectively. The blue spot indicated with arrows in left three panels in B represents aggregates of IL-7R\textsuperscript{α} LTo cells at PP anlagen. Images with large magnification indicate a relatively scattered distribution of reduced number of IL-7R\textsuperscript{α} LTo cells in the Cbfb\textsuperscript{2m/2m} mice (B, right two panels; scale bars, 100 µm). In both Runx1\textsuperscript{P1N/P1N} and Cbfb\textsuperscript{2m/2m} mice, the numbers of PP and LN anlagen were reduced. The positions of the residual para-aortic LN anlagen, shown by the arrows, were sometimes ectopic in the Runx1\textsuperscript{P1N/P1N} specimen. D, Whole-mount staining of the mesenterium from Cbfb\textsuperscript{2m/2m} newborn mice with anti–VCAM-1 Ab. The dark blue spots indicated with arrowheads represent the mesenteric LN (MLN) anlagen. Consistent with the presence of MLN in adult Cbfb\textsuperscript{2m/2m} mice, formation of MLN anlagen was not impaired by loss of Cbf\beta variant.
revealed a novel and essential function of Runx1/Cbfβ2 complexes in the development of the lymphoid tissues anlagen during embryogenesis.

Role of Cbfβ2 in the regulation of Rorγ expression

To further examine whether a scattered distribution of IL-7Rα+ cells is caused by a migratory defect or by a reduced number of LTi cells, we performed a flow cytometry analysis of 17.5 dpc fetal intestines. Whereas LTi cells were detected in Lin−/CD3, B220, CD11c, NK1.1, Gr-1, and Ter-119) CD45+ population as α4β7+ IL-7Rα+ cells in the control mice, this cell subset was almost absent in the Rorγ-deficient (Rorγtgfp/P1N) fetuses (Fig. 3A), as previously reported in histological analyses (18). In Runx1P1PIN/P1IN and Cbfβ2m/m fetuses, the proportions of Lin−α4β7+IL-7Rα+ cells were significantly reduced (Fig. 3A). In contrast, the expression level of CXCR5, which is required for the migration of LTi precursors to the appropriate place to initiate anlagen formation (30), was unaffected in the residual Lin−α4β7+IL-7Rα+ cells by the loss of either the P1-Runx1 or Cbfβ2 variant (Fig. 3B). This suggests that an impairment in differentiation, rather than in migration, of LTi cells is most likely responsible for the resulting impairment in anlagen formation.

Because GFP expression from the Rorγtgfp allele is a powerful genetic tool to mark LTi-lineage cells in embryos (18), we introduced the Rorγtgfp allele onto a Runx1P1PIN/P1IN or Cbfβ2m/m background. Interestingly, GFP expression levels in Lin−α4β7+IL-7Rα+ cells were lower in the Cbfβ2m/m fetuses than in the control or Runx1P1PIN/P1IN fetuses (Fig. 3C). This observation can be explained either by developmental inhibition at the transition from the Rorγlow to the Rorγhigh stage or by an inefficient upregulation of the Rorγ gene. To distinguish between these two possibilities, we examined other markers that might define the developmental stage of LTi cells at anlagen. Upregulation of CD4 expression in Lin−α4β7+IL-7Rα+ cells was accompanied by the upregulation of GFP from the Rorγtgfp locus in the control fetuses (Fig. 3D). Thus, there are two cell subsets, RorγmCD4low− and RorγhighCD4+, in the Lin−α4β7+IL-7Rα+ population at anlagen. In the Cbfβ2m/m fetuses, CD4 expression levels in Lin−α4β7+IL-7Rα+ cells expressing low amounts of GFP were similar to those observed in control Lin−α4β7+IL-7Rα+Rorγhigh cells (Fig. 3D). There were milder and severe reductions in a proportion of α4β7+IL-7Rα+ LTi cells in Runx1P1PIN/P1IN fetuses in anlagen for peripheral LNs (Supplemental Fig. 3), consistent with variation in the number of PPs and LNs. Importantly, low GFP expression in the α4β7+IL-7Rα+ cells was similarly observed in peripheral LN anlagen from the Cbfβ2m/m fetuses (Supplemental Fig. 3). These results suggest that Cbfβ2 is likely to influence Rorγ gene expression rather than inhibit the differentiation of RorγhighCD4+− cells into Rorγhigh CD4+ cells. However, GFP level in Lin− lamina propria cells prepared from adult gut was unaffected by loss of Cbfβ2, although the proportion of these cells was severely reduced (Fig. 3E; M. Tachibana, unpublished observations). Thus, the effect of Cbfβ2

![FIGURE 3. Impaired differentiation of LTi at PP anlagen in Runx1P1PIN/P1IN and Cbfβ2m/m fetuses. A. Expression profiles of α4β7 and IL-7Rα in Lin− CD45+ gated fetal intestinal cells from 17.5 dpc fetuses, showing the impaired differentiation of αββ−IL-7Rα− LTi cells in Runx1P1PIN/P1IN, Cbfβ2m/m, and Rorγ-deficient Rorγtgfp/P1N fetuses. B. CXCR5 expression on Lin−αββ−IL-7Rα− fetal intestine cells from control (thin line) and Cbfβ2m/m (bold line) 17.5 dpc embryos. The shaded histogram represents staining with an isotype control Ab. Data shown are representative of two experiments. C. GFP expression from the Rorγtgfp allele in the Lin−αββ−IL-7Rα+ population in 17.5 dpc fetal intestines. The shaded histogram represents data from mice negative for the Rorγtgfp allele. The thin line, the dotted line, and the bold line are from control, Runx1P1PIN/P1IN, and Cbfβ2m/m fetuses harboring one Rorγtgfp allele, respectively. D. Expression profiles of CD4 and IL-7Rα in the Lin−αββ−IL-7Rα− populations in embryonic day 17.5 fetal intestines. The Lin−αββ−IL-7Rα− population was divided into GFPlow and GFPhigh populations in the control Rorγtgfp mice. CD4 expression correlated well with induction of GFP expression. CD4 upregulation was observed in Lin−αββ−IL-7Rα− cells expressing low GFP in Cbfβ2m/m fetuses. Data shown are representative of two experiments. E. GFP expression from the Rorγtgfp allele in Lin−αββ−IL-7Rα− cells prepared from adult intestines is shown as in C. The data are representative of five experiments.](http://www.jimmunol.org/ Downloaded from)
Differentiation of B-lymphoid lineage cells was not affected by loss of P1-Runx1. Expression detected two subsets in the Lin<sup>2</sup>7R<sup>a</sup> population. Therefore, we examined the early differentiation of LTi precursors in fetal liver. Based on the expression levels of IL-7R<sup>a</sup>, the Lin<sup>2</sup>7R<sup>a</sup> high subset was first detected at the transition from 12.5–13.5 dpc embryonic day and was accompanied by the initiation of Ror<sup>gt</sup> expression (Fig. 4A). Importantly, the emergence of αβ<sup>-</sup>IL-7R<sup>a</sup> high subset was observed only in the αβ<sup>-</sup>IL-7R<sup>a</sup> mid subset was also severely reduced. However, in contrast to Ror<sup>gt</sup> and Id2<sup>-/–</sup> fetuses, the percentage of the αβ<sup>-</sup>IL-7R<sup>a</sup> mid subset was also significantly reduced in the Runx1<sup>P1/P1</sup> and Cbf<sup>β<sub>2m</sub>/2m</sub> fetuses (Fig. 4A, 4B), demonstrating that development of αβ<sup>-</sup>IL-7R<sup>a</sup> mid cells depends on the function of Runx1/Cbfβ2 complexes, but not on Rorgt or Id2.

In the Runx1<sup>P1/P1</sup> fetuses, we also observed a severe reduction in the αβ<sup>-</sup>IL-7R<sup>a</sup> subset, which may contain multipotent hematopoietic progenitors. Given that Runx1/Cbfβ complexes are essential for generation of hematopoietic stem cells (22, 29), it is important to determine whether the reduction of the αβ<sup>-</sup>IL-7R<sup>a</sup> mid population in the Runx1<sup>P1/P1</sup> and Cbf<sup>β<sub>2m</sub>/2m</sub> fetuses is caused by a reduced number of hematopoietic progenitors, as observed in Ikaros<sup>–/–</sup> mice (31), or by the inhibition of progenitor differentiation into αβ<sup>-</sup>IL-7R<sup>a</sup> mid LTi precursors. Because differentiation potency toward both αβ<sup>-</sup>IL-7R<sup>a</sup>+ cells and B-lymphocyte lineages was detected in the Lin<sup>–</sup>αβ<sup>-</sup>IL-7R<sup>a</sup> fetal liver population in cell culture (13), we examined whether early B cell differentiation is impaired in the Runx1<sup>P1/P1</sup> fetuses. The development of B-lymphoid lineage cells in the 15.5 dpc fetal liver was detected as CD19<sup>+</sup>B220<sup>+</sup> cells in both Runx1<sup>P1/P1</sup> and control fetuses (Fig. 4D). Furthermore, in the Cbfβ<sup>β<sub>2m</sub>/2m</sub> fetuses.

**Involvement of Runx1/Cbfβ complexes in early specification to the LTi lineage**

Normal Rorgt expression levels in Lin<sup>–</sup>αβ<sup>-</sup>IL-7R<sup>a</sup>+ LTi cells at anlage in the absence of the P1-Runx1 variant suggest that some mechanism other than Rorgt regulation is involved in reducing the number of LTi cells in the Runx1<sup>P1/P1</sup> fetuses. Therefore, we examined the early differentiation of LTi precursors in fetal liver. A combination analysis of Rorgt and IL-7R<sup>a</sup> expression detected two subsets in the Lin<sup>–</sup>αβ<sup>-</sup>IL-7R<sup>a</sup>+ population in the 13.5 dpc fetal liver. Based on the expression levels of IL-7R<sup>a</sup>, the Lin<sup>–</sup>αβ<sup>-</sup>IL-7R<sup>a</sup>+ subset could be subdivided into IL-7R<sup>a</sup> mid and IL-7R<sup>a</sup> high subset (Fig. 4A). In both Rorgt- and Id2<sup>-/–</sup> fetuses, the percentage of the αβ<sup>-</sup>IL-7R<sup>a</sup> mid subset was also severely reduced. However, in contrast to Ror<sup>gt</sup> and Id2<sup>-/–</sup> fetuses, the percentage of the αβ<sup>-</sup>IL-7R<sup>a</sup> high subset was also significantly reduced in the Runx1<sup>P1/P1</sup> and Cbfβ<sup>β<sub>2m</sub>/2m</sub> fetuses (Fig. 4A, 4B), demonstrating that development of αβ<sup>-</sup>IL-7R<sup>a</sup> mid cells depends on the function of Runx1/Cbfβ2 complexes, but not on Rorgt or Id2.

**Role of Runx1/Cbfβ complexes in differentiation of early LTi precursors in fetal liver.** A. Expression profiles of αβ<sup>-</sup> and IL-7R<sup>a</sup> in Lin<sup>–</sup> fetal liver cells from 13.5 dpc fetuses with the indicated genotypes. The numbers shown indicate the percentage of cells in the region. B. Statistical summary of the flow cytometric analyses of multiple mice shown in A. The numbers in each panel (1–5) represent the control, Runx1<sup>P1/P1</sup> Cbfβ<sup>β<sub>2m</sub>/2m</sub>, Ror<sup>gt</sup> and Id2<sup>-/–</sup> genotypes, respectively. C. Expression profiles of GFP from the Ror<sup>gt</sup> allele and IL-7R<sup>a</sup> in Lin<sup>–</sup> fetal liver cells from 12.5 and 13.5 dpc fetuses. D. Expression profiles of B220 and CD19 in fetal liver cells from Runx1<sup>P1/P1</sup> and Runx1<sup>P1/P1</sup>, 15.5 dpc embryo. E. Expression of c-Kit and Sca-1 in Lin<sup>–</sup> fetal liver cells and absolute numbers of these Lin<sup>–</sup>c-Kit<SUP>+</sup>Sca-1<sup>+</sup> cells. These results indicate that the generation of multipotent hematopoietic progenitors is unaffected by loss of Cbfβ2 variant. Data are representative of two independent experiments. *<i>p</i> < 0.01.
multipotent hematopoietic progenitors, defined as Lin<sup>−</sup> c-Kit<sup>−</sup>Sca-1<sup>+</sup> cells, were increased rather than decreased in 13.5 dpc fetal liver (Fig. 4E), followed by an increased percentage of the α4β7 IL-7Rα<sup>−</sup> subset. These results indicate that development of multipotent progenitors was unaffected by Cbfβ2 deficiency and support the inference that Runx1/Cbfβ2 complexes are involved in the early specification of cells in the α4β7 IL-7Rα<sup>−</sup> population to the LTi lineage to force the differentiation of α4β7<sup>+</sup>IL-7Rα<sup>mid</sup> cells.

Based on the lack of both NK cells and LTi cells in the id2<sup>−/−</sup> mice, it was assumed that Id2 is necessary for the development of the common precursors for both NK and LTi lineages (15, 32). Because the impaired differentiation of LTi precursor cells by Cbfβ2 deficiency was already observed prior to the developmental stage at which Id2 would function, we examined whether the differentiation of NK cells is affected in the Cbfβ2<sup>−/−</sup> mice. NK cells, defined by their surface marker phenotype, were detected in the spleens and bone marrow of Cbfβ2<sup>−/−</sup> mice and control mice (Fig. 5A), although the expression of some NK markers such as CD11b was lower in the Cbfβ2<sup>−/−</sup> mice (Fig. 5B), as reported in mice expressing the dominant-negative form of Runx protein (33). Therefore, Cbfβ2 deficiency specifically inhibits the development of LTi cells but not NK cells.

**Discussion**

Hematopoietic LTi cells play an essential role in the development of second lymphoid tissue during embryogenesis. Albeit its unique function, an early differentiation pathway of LTi cells is not well understood. Results in this study revealed a novel function of Runx1/Cbfβ2 transcriptional factor complexes in lymphoid organogenesis in part via regulating LTi cell differentiation. There are two possible explanations why loss of P1-Runx1 and Cbfβ2 variant results in an impaired LTi development: reduced dosage of Runx1/Cbfβ expression or loss of variant-specific function. Although expression of both Cbfβ1 and Cbfβ2 transcripts are detected in many tissues at an almost similar ratio (25), expression of P1-Runx1 or P2-Runx1 was shown to be dominant in T-lineage cells or B-lineage and NK cells, respectively (24). Because we failed to examine expression of each Runx1 or Cbfβ variant protein due to a limited number of LTi cells in fetus tissues, dissection of these two possibilities waits for the future study. In both Runx1<sup>P1N,P1N</sup> and Cbfβ2<sup>−/−</sup> mice, the formation of PPs and LNs was impaired, but was not completely abolished. This observation might suggest that Runx1/Cbfβ complexes are important, but not absolutely essential, for LTi cell development. However, because function of Runx1/Cbfβ complexes was only attenuated in the Runx1<sup>P1N,P1N</sup> and Cbfβ2<sup>−/−</sup> mice, there remains a possibility that loss of entire Runx1/Cbfβ complex function completely inhibits LTi cell development, as was observed by the absence of the Rorγt or Id2 factor (15, 18).

Although the Rorγt and Id2 are known to be essential for the differentiation of LTi cells, the developmental stage at which these factors regulate LTi cell development is not well characterized. Our results show that Rorγt expression during LTi cell development is first detected in fetal liver cells of 13.5 dpc fetuses. Importantly, initiation of Rorγt expression correlates well with upregulation of the IL-7Rα expression. Thus, there are two cell subsets, Rorγt<sup>α4β7<sup>−</sup>IL-7Rα<sup>mid</sup></sup> and Rorγt<sup>α4β7<sup>−</sup>IL-7Rα<sup>high</sup></sup> cells, in the Lin<sup>−</sup> fetal liver population of 13.5 dpc fetuses. Together with the loss of the α4β7<sup>−</sup>IL-7Rα<sup>high</sup> subset in the Rorγt<sup><sup>−/−</sup></sup> mice and Id2<sup>−/−</sup> fetuses, Rorγt and Id2 factors potentially function at the same developmental stage to orchestrate differentiation of the α4β7<sup>−</sup>IL-7Rα<sup>mid</sup> cells into the α4β7<sup>−</sup>IL-7Rα<sup>high</sup> cells. Further studies are needed to confirm this developmental pathway and to understand how Rorγt and Id2 factors regulate development of the α4β7<sup>−</sup>IL-7Rα<sup>high</sup> subset. For instance, it is still unclear how upregulation of IL-7Rα is regulated at this transition. It is possible that Rorγt and Id2 factors are involved in regulating expression of the IL-7Rα gene. Alternatively, given that Runx1 is needed for efficient IL-7Rα expression during thymocyte differentiation (28), Runx1 may also play a role in the upregulation of IL-7Rα during LTi cell differentiation. However, the level of IL-7Rα expression on residual LTi cells is not impaired in the Runx1<sup>P1N,P1N</sup> and Cbfβ2<sup>−/−</sup> mice, suggesting that remaining P2-Runx1 and Cbfβ1 variant could be sufficient for regulation of IL-7Rα in these mice, respectively.

In contrast, our results show that Runx1/Cbfβ complexes are potentially involved in regulating expression of the Rorγt gene. We observed that the loss of Cbfβ2 variant results in a low level of GFP expression from the Rorγt<sup>tgfp/gfp</sup> allele in residual α4β7<sup>−</sup>IL-7Rα<sup>−</sup> LTi cells.

**FIGURE 5.** Presence of NK cells with downregulated NK markers in Cbfβ2<sup>−/−</sup> mice. A. The proportions of NK cells, defined by the NK1.1<sup>−</sup>CD3ε<sup>−</sup> surface phenotype, in the bone marrow (B.M.), spleen (Spl.), and liver (L iv.) were similar between littermate controls and Cbfβ2<sup>−/−</sup> mice. B. Expression profiles of CD11b and DX5 in NK1.1<sup>−</sup> cells showing the downregulation of CD11b with the loss of Cbfβ2 variant.
Because the development of both NK cells and LTI cells are defective in the Id2−/− mice, Id2 was first supposed to regulate development of the common precursors for both NK and LTI lineages (15, 32). However, a recent study has shown that Id2-deficient mice retain a normal number of NK cell progenitors in adult bone marrow, suggesting that Id2 plays a role in the maturation of NK cells (37). Our results demonstrated that loss of Runxl1/Cbfβ2 function impairs development of LTI cells before the developmental stage at which Id2 functions. However, we observed that development of NK cells is not significantly affected by loss of Cbfβ2. This result challenges the supposition that there exist common NK and LTI precursors for which development depends upon Id2. However, our results do not formally exclude the possibility that attenuation of Runxl1/Cbfβ2 complex function results in a skewed differentiation of a common precursor toward NK lineage, as is observed in the cell-fate conversion of MHC class I-selected thymocytes toward helper lineage in part via dysregulated ThPOK transcription factor expression by loss of Runxl1/Cbfβ complex function (20). However, considering that an essential requirement of Cbfβ for NK cells development was reported by using hypomorphic Cbfβ allele (38), skewed differentiation toward NK lineage by the loss of Cbfβ2 variant is unlikely. Nevertheless, because Runx/Cbfβ complexes function before Id2 during the differentiation of LTI precursors, Id2 is likely to differentially regulate the development of NK and LTI cells rather than play an essential role in the generation of common precursors for NK and LTI lineage.

In addition to the PPs, other types of mucosa-associated secondary lymphoid tissues, such as nasopharynx-associated lymphoid tissue and tear duct-associated lymphoid tissue, are identified. Interestingly, nasopharynx-associated lymphoid tissue organogenesis depends on Id2, but not Rorγt expression, (9, 39), whereas tear duct-associated lymphoid tissue formation is independent of both factors (40). Because results in this study revealed that Runxl1/Cbfβ2 complexes act earlier than Id2 and Rorγt during differentiation of the LTI lineage cells, it is interesting to examine in the future study whether formation of another mucosa-associated secondary lymphoid tissues is impaired by loss of Runxl1/Cbfβ2 complexes function.

Collectively, our results demonstrate novel and essential roles for the Runx transcription factor complexes in regulating LTI cell development and provide insights into transcriptional regulation during the early specification stage toward the LTI lineage (Supplemental Fig. 5). Further studies that seek for a deeper understanding of Runx-mediated specification to the LTI lineage would be beneficial for engineering artificial immune tissue.

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Disclosures

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References

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