Runx3 specifies lineage commitment of innate lymphoid cells

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Subsets of innate lymphoid cells (ILCs) reside in the mucosa and regulate immune responses to external pathogens. While ILCs can be phenotypically classified into ILC1, ILC2 and ILC3 subsets, the transcriptional control of commitment to each ILC lineage is incompletely understood. Here we report that the transcription factor Runx3 was essential for the normal development of ILC1 and ILC3 cells but not of ILC2 cells. Runx3 controlled the survival of ILC1 cells but not of ILC3 cells. Runx3 was required for expression of the transcription factor RORγt and its downstream target, the transcription factor AHR, in ILC3 cells. The absence of Runx3 in ILCs exacerbated infection with Citrobacter rodentium. Therefore, our data establish Runx3 as a key transcription factor in the lineage-specific differentiation of ILC1 and ILC3 cells.

Innate lymphoid cells (ILCs) reside in mucosal surfaces to facilitate immune responses, maintain mucosal integrity and promote lymphoid organogenesis1. They do not express rearranged antigen-specific receptors and are dependent on the common γ-chain of the cytokine receptor IL-2R for differentiation, and all ILCs in the intestine express the cytokine receptor chain IL-7Rα (CD127), which forms a heterodimer with common γ-chain of IL-2R. The ILC populations are classified into three groups, ILC1, ILC2 and ILC3, on the basis of their expression of specific cytokines, similar to the classification of T cell subsets1. ILC1 cells are characterized by their ability to produce the type 1 cytokine interferon γ (IFN-γ) in response to interleukin 12 (IL-12), IL-15 and IL-18. ILC2 cells respond to IL-25 and IL-33 and secrete a set of type 2 helper cell (TH2 cell) cytokines: IL-5, IL-9, IL-13 and amphiregulin. ILC3 cells share many features with the T117 and T122 subsets of helper cells and can be stimulated by IL-1β and IL-23 to elicit the production of IL-17 and IL-22. ILC3 cells are heterogeneous and can be further subcategorized into additional subsets by their expression of the co-receptor CD4 and the activating receptor NKP46 as follows: CD4+ ILC3 cells, NKP46+ ILC3 cells (also known as NK22 or ILC22 cells), and CD4−NKP46− (double-negative) ILC3 cells1. ILC3 cells in fetal intestine are CD4− or CD4+ lymphoid tissue–inducer (LTi) cells, which are necessary for the development of lymph nodes and Peyer’s patches (PPs)2. NKP46+ ILC3 cells specifically produce only IL-22, not IL-17 (refs. 1, 3, 4), and have the potential to differentiate into IFN-γ-producing ILC1 cells4,5. Thus, ILCs can be classified into different subsets that can be distinguished and that serve distinct roles in immune responses.

As for their differentiation and transcriptional regulation, all ILC lineages are derived from common lymphoid progenitors (CLPs), which also give rise to B cells and T cells1. The earliest progenitor cells specific to ILCs are CXCR6+ integrin αβ+–expressing CLPs (CXCR6+ αβLPs), which have the potential to differentiate into ILC1, ILC2, ILC3 and splenic natural killer (NK) cells. The transcription factor NFI13 (E4BP4) is essential for the differentiation of CXCR6+ αβLPs and all ILC lineages. The common progenitor to all helper-like ILC lineages (CHILPs) is defined by its Lin−CD127+Id2+CD25−αβ+ phenotype and gives rise to ILC1, ILC2 and ILC3 cells but not splenic NK cells. In this context, NK cells might be a different subset, distinct from ILC1 cells. The common precursor to ILCs (ILCP) is identified by expression of the transcription factor PLZF and can generate ILC1, ILC2 and ILC3 cells, although ILCPs do not differentiate into the CD4+ ILC3 subset or splenic NK cells. PLZF is expressed in a proportion of CHILPs, which suggests that these are precursors of ILCPs. However, the ILC lineage–specification process downstream of ILCPs remains to be completely elucidated.

The differentiation of each ILC subset requires specific transcription factors1. While ILC1 cells in the intestine are DX5− and do not express the transcription factor Eomes, splenic NK cells are DX5+Eomes+ and seem to be dependent on Eomes for full maturation1–5. Although both ILC1 cells and splenic NK cells express T-bet, a TH1 transcription factor, ILC1 cells in the intestine are highly dependent on T-bet, whereas splenic NK cells are only modestly affected by the absence of T-bet1,5,8. ILC2 cells require GATA-3, a TH2 transcription factor, and the transcription factor RORγt for their development9–11. The transcription factor RORγt is required for ILC3 cells, and deficiency in the

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transcription factor AHR affects all ILC3 subsets, which suggests a potential link between RORγt and AHR in ILC3 cells that has not been elucidated. Both RORγt and AHR are also indispensable for Tbet17 and Tγ22 cells. Of the ILC3 subsets, only NKp46+ ILC3 cells express and require T-bet. Although initially published studies suggested that GATA-3 is an ILC2-specific transcription factor, subsequent studies have indicated that ILC1 and ILC3 cells also have intermediate expression of GATA-3 and that it regulates these populations through the maintenance of CD127 expression. Thus, the requirements for transcription factors studied thus far for the specification of ILC subsets are generally similar to those for helper T cells.

Transcription factors of the Runx family, especially Runx1 and Runx3, have important roles in the development of various hematopoietic lineages, including T cells. Runx1 is essential for the emergence of hematopoietic stem cells from hemogenic endothelial cells and for the development of progenitors of lymphoid cells and dendritic cells, megakaryocytes, Foxp3+ regulatory T cells, and T17 cells. Runx3 is important for the differentiation of CD8+ T cells, T11 cells, and splenic NK cells. Most phenotypes that result from deficiency in Runx1 or Runx3 are more pronounced with deletion of Cbfβ, which encodes the common obligatory partner (CBF-β) of all Runx proteins; this suggests that members of the Runx family have overlapping roles in the development and function of cells of the immune system.

A study published before the identification of progenitors of ILCs reported that mice lacking the main transcript variant of Runx1 or CBF-β have fewer PPs and RORγt+ LTI cells in the fetal intestine than did their wild-type counterparts. Deficiency in Runx1 or CBF-β in hematopoietic cells affects Flt3+ progenitor cells, including CLPs17, which suggests that the lower number of RORγt+ LTI cells in the fetal intestine in the absence of Runx1 or CBF-β might be due to effects on CLPs, the precursors to all ILCs. Therefore, the requirements for Runx proteins in the differentiation of ILCs have not been clearly defined.

Here we found that among the three members of the Runx family, Runx3 derived from its distal promoter was specifically expressed in ILC1 and ILC3 cells but not in ILC2 cells. Specific deletion of Cbfβ or Runx3 in Nkp46-expressing cells resulted in a substantial reduction in ILC1 cells and Nkp46+ ILC3 cells in the intestine, which led to poor control of infection with Citrobacter rodentium. Deletion of Runx3 in hematopoietic cells did not affect the number of CLPs, αLIPs, CHILPs or ILC3s but abrogated RORγt expression and subsequent AHR expression by all ILC3 subsets in the intestine. Thus, our data reveal a non-redundant role for Runx3 in the differentiation of ILC1 and ILC3 cells.

RESULTS

Expression of distal Runx3 in ILC1 and ILC3 cells not ILC2 cells

We first assessed the expression of Runx1, Runx2, Runx3 and Cbfβ in ILC1 cells from the epithelium of mouse small intestine, in ILC2 cells and in all ILC3 subsets from the lamina propria of mouse small intestine. Among transcripts encoding members of the Runx family, Runx3 transcripts were expressed mainly in all ILCs. All members of the Runx family, including Runx3, can be expressed in two forms that originate from a proximal promoter (‘proximal Runx3’) or distal promoter (‘distal Runx3’), with distal Runx3 transcripts being dominant in lymphocytes. Although proximal Runx3 transcripts are poorly translated into protein in CD4+ T cells that express only proximal Runx3, CD8+ T cells use the distal Runx3 promoter for the expression of Runx3 protein. To discriminate between use of the proximal promoter and use of the distal promoter in ILCs, we further assessed Runx3 expression in ILCs by RT-PCR and in ‘Runx3dYFP mice’, which express membrane-bound yellow fluorescent protein (YFP) from the distal Runx3 promoter (called ‘Runx3d’ in this designation). Whereas ILC1 and ILC3 cells expressed only distal Runx3, expression of Runx3 in ILC2 cells was driven only by the proximal promoter (Fig. 1b,c). Splenic NK cells expressed both Runx3 transcripts but less distal Runx3 than proximal Runx3 (Fig. 1b,c), as observed before. The expression of distal Runx3 was highest in ILC1 cells, moderate in ILC3 cells, low in splenic NK cells and undetectable in ILC2 cells (Fig. 1b,c). NK cells in liver and skin are known to segregate into two subsets: DX5−CD49a+ tissue-resident NK cells and DX5−CD49a− conventional NK cells. The tissue-resident NK cells in the liver expressed more distal Runx3 than did DX5−CD49a− conventional NK cells (Supplementary Fig. 1a). In contrast, distal Runx3 expression by both NK cell populations in the skin was low (Supplementary Fig. 1a). While expression of the integrin CD49a (α5) and the natural killer cell marker DX5 on NK cells from the salivary glands has not been reported before and the tissue residency of these cells has not been determined, to our knowledge, their distal Runx3 expression was very high (Supplementary Fig. 1a). Thus, distal Runx3 was ‘preferentially’ expressed in the ILC1 and ILC3 subsets but not in ILC2 cells.

We next assessed progenitors of ILCs in adult bone marrow for their expression of distal Runx3 and other transcripts encoding Runx proteins by RT-PCR and reporter expression in Runx3dYFP mice. We isolated CLPs, αLIPs and CHILPs from the bone marrow of wild-type mice and isolated ILCPs from the bone marrow of mice that express a fusion of green fluorescent protein (GFP) and Cre recombinase from promoter of the gene encoding PLZF, in conjunction with PLZF protein. While expression of transcripts encoding all members of the Runx family was relatively low in CLPs, αLIPs and CHILPs from adult bone marrow, ILCPs had high expression of Runx1 and Runx3 transcripts (Fig. 1d). We also confirmed distal Runx3 expression in ILCPs isolated from Runx3dYFP mice (Fig. 1e). A small fraction of CHILPs in the bone marrow of Runx3dYFP mice were PLZF+, and these cells expressed YFP driven from distal Runx3 promoter (Fig. 1e,f), which suggested that PLZF+ ILC progenitor cells started expressing distal Runx3 before specification of the ILC lineage. Because ILC2 cells and their precursors (ILC2Ps) did not have substantial distal Runx3 expression, as indicated by the lack of YFP expression in these cells in Runx3dYFP mice (Fig. 1e,c), the apparent loss of distal Runx3 expression seemed to be an ILC2–specific event. However, ILC1 cells maintained high expression of distal Runx3, and ILC3 cells downregulated distal Runx3 but still maintained moderate expression of distal Runx3. These data suggested that Runx3 might contribute to specification of the ILC lineages.

CBF-β is indispensable to NKP46+ ILC3 and ILC1 cells

Because members of the Runx family can bind to the same Runx-binding motif, other Runx proteins might potentially compensate for the deficiency in one member of the Runx family. To investigate the roles of the Runx family in ILC1 and ILC3 cells, we sought to abolish the function of Runx3 and other members of the Runx family by deleting their binding partner, CBF-β. We generated mice with intrinsic deletion of Cbfβ in Nkp46+ ILC1 and ILC3 cells by crossing mice withloxP-flanked Cbfβ alleles (CbfβΔ) with mice that express Cre recombinase from the gene encoding NKP46 (NKP46-Cre). CbfβΔ/NKP46-Cre mice lacked NKP46+ ILC3 cells in the small intestine, colon and PPs (Fig. 2a,b and Supplementary Fig. 1b,c). In addition, CbfβΔ/NKP46-Cre mice had considerably lower numbers of other NKP46-expressing cells, including ILC1 cells in the intestine, and NK cells in the spleen, liver, salivary gland and skin, relative to
the number of these cells in Cbfbβ/NKp46-Cre mice (Fig. 2c, d and Supplementary Fig. 1d,e). In contrast, the differentiation of other ILC3 subsets was not affected by CBF-β deficiency (Fig. 2a, b). Thus, CBF-β expression in NKp46+ cells seemed to be required for development of the ILC1 and NKp46+ ILC3 lineages.

To address the possibility that NKp46+ ILC3 cells lacking CBF-β were not able to express NKp46, we assessed NKp46 expression in splenic NK cells and found that it was not changed by CBF-β deficiency (Supplementary Fig. 1f). The milder phenotype of the Cbfbβ/NKp46-Cre mice (Fig. 2c, d and Supplementary Fig. 1d, e) indicates that the deletion of CBF-β in NKp46+ cells is preferentially expressed in NKp46+ ILC3 cells but not in other cell subsets as in a (above plots) from Runx3y/GFP or wild-type mice (key).

Quantitative RT-PCR analysis of transcripts encoding Blimp-1 (encoding β-actin), ND, not detected. (b) RT-PCR analysis of transcripts from the distal and proximal promoters of Runx3 (left margin) in cells as in a. c. Flow cytometry analyzing the expression of YFP driven by distal Runx3 (distal Runx3-YFP) by cell subsets as in a (above plots) from Runx3y/GFP or wild-type mice (key).

We then investigated whether deletion of Runx3 in NKp46-expressing cells recapitulated the phenotype of Cbfbβ/NKp46-Cre mice. The number and frequency of NKp46+ ILC3 cells were selectively lower among ILC3 subsets in the PPs and among LPLs of Runx3y/NKp46-Cre mice than among those of Runx3β/NKp46-Cre control mice (Fig. 3a). No RORγt+ ILC3 subsets were detectable in the remaining PPs or populations of lamina propria lymphocytes (LPLs) in Runx3y/Vav1-Cre mice (Fig. 3b, c). These data suggested a defect in ILC3 development in the setting of Runx3 deficiency.

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Runx3 regulates RORγt expression in ILC3 cells

Published reports have indicated that ILC3s express very little RORγt but express GATA-3, which is important for all ILC differentiation in the intestine5-7. We next investigated the hypothesis that Runx3 regulates RORγt expression during ILC3 differentiation (i.e., from ILCPs to RORγt+ ILC3 cells). We were able to sort Lin−CD127+NK1.1− LPLs into two main populations distinguished by expression of GATA-3 and RORγt. GATA-3hiRORγt+ ILC2 cells and GATA-3loRORγt+ ILC3 cells (Fig. 3d), as reported before8,15. The number of GATA-3hi ILC2 cells was unaffected in Runx3y/Vav1-Cre mice (Fig. 3d, e), which suggested that ILC2 differentiation was not abrogated by the lack of Runx3.
Runx3 in hematopoietic cells. The overall frequency and absolute number of GATA-3± LPLs in the large intestine were similar in adult Runx3floxed/Vav1-Cre mice and adult Runx3floxed (control) mice (Fig. 3d,e). However, in contrast to the RORγt expression of such cells from Runx3floxed (control) mice, the GATA-3±LPLs in Runx3floxed/Vav1-Cre mice did not express RORγt (Fig. 3d,e). These Lin−CD127±NK1.1−GATA-3±RORγt±LPLs (called ‘CD127±ILC3LCLN cells’ here) accumulated more in the large intestine of adult Runx3floxed/Vav1-Cre mice than in that of adult Runx3floxed (control) mice (Fig. 3d,e), and were not ILCPs, because they did not express αβ or PLZF (Fig. 3d).

Next we investigated whether the CD127±ILC3LCLN cells in the intestine of adult mice had the potential to become RORγt±ILC3 cells and if Runx3 was necessary for this process. Because KLRG1 can be used as another marker for ILC2 cells among the Lin−CD127±GATA-3hi cells in LPL populations, we sorted Lin−CD127±αβ−NK1.1−KLRG1− RORγt−GFP± LPLs (Runx3-sufficient CD127±ILC3LCLN cells) from the small and large intestine of adult Runx3±Vav1-Cre mice without excluding RORγt± cells, because we did not detect RORγt± ILC3 cells among LPLs from Runx3±Vav1-Cre mice. We injected sorted Runx3-sufficient or Runx3-deficient cells (H-2b) into non-lethally irradiated host mice (H-2b) deficient in recombination-activating gene 2 and the common γ-chain (Rag2−/−Il2rgc−/− mice). At 3 months after this injection, Runx3-sufficient donor CD127±ILC3LCLN cells had differentiated into intestinal Lin−CD127±GATA-3±RORγt±ILC3 cells, but Runx3-deficient CD127±ILC3LCLN cells had not (Fig. 4b). Runx3-sufficient CD127±ILC3LCLN cells also differentiated into ILC1 cells among LPLs (Lin−CD127±NKp46−RORγt−) and among intraepithelial lymphocytes (IELs) (Lin−CD127±NK1.1−NKp46+), while the ILC1 differentiation of Runx3-deficient CD127±ILC3LCLN cells was impaired, although less so than their ILC3 differentiation (Fig. 4b), possibly due to compensatory effects by other members of the Runx family, as noted above. In contrast, no ILC2 cells (Lin−CD127±GATA-3hi) were derived from Runx3-sufficient CD127±ILC3LCLN cells or Runx3-deficient CD127±ILC3LCLN cells (Fig. 4b), which suggested that CD127±ILC3LCLN cells sorted as described above were progenitors of ILC1 and ILC3 cells and that Runx3 deficiency in these precursors dampened mainly differentiation into RORγt-expressing ILC3 cells.

To further confirm the findings reported above, we sorted Runx3-sufficient CD127±ILC3LCLN cells from Runx3±/GFP mice and Runx3-deficient CD127±ILC3LCLN cells from Runx3±Vav1-Cre mice and cultured the cells in vitro on OP9 stromal cells expressing the Delta-like ligand (OP9-DL1 cells) with IL-2, IL-7 and stem-cell factor, as described. On day 14, wells seeded with Runx3±/GFP (Runx3-sufficient) CD127±ILC3LCLN cells included cells that expressed RORγt-GFP (Fig. 4c), and we were able to detect expression of RORγt protein in these cells by staining with antibody to RORγt (Fig. 4d). These cells produced IL-22 in response to IL-23 and IL-1β (Fig. 4d). However, we did not detect IL-22-producing RORγt± cells in wells seeded with Runx3-deficient CD127±ILC3LCLN cells (Fig. 4d). Together these results indicated that expression of Runx3 was required for the differentiation of CD127±ILC3LCLN cells into RORγt-expressing, IL-22-producing ILC2s.

We then sought to investigate how Runx3 regulated RORγt expression in ILC3 cells. A putative Runx-binding site in the Rorc gene promoter has been reported, and Runx1 regulates RORγt expression in Th17 cells. To investigate whether Runx3 can directly affect RORγt expression by interacting with the putative Runx-binding site in any ILC, we performed a luciferase assay with the transfectable human
NK cell line NK-92 that expresses mainly Runx3, among members of the Runx family. Transfer of the mouse Runx3 (gt) promoter led to greater luciferase activity than did transfaction of a control vector without that promoter (Fig. 4e). Deletion of a Runx-binding motif in the Runx3 (gt) promoter abrogated the enhancement in luciferase activity (Fig. 4e). Transfer of vector encoding mouse Runx3 into NK-92 cells further increased the luciferase activity of the intact Runx3 promoter in NK-92 cells (Fig. 4f), which indicated that endogenous human Runx3 and ectopically expressed mouse Runx3 were able to promote RORγ expression in a manner specific to the Runx3-binding motif. Furthermore, by chromatin immunoprecipitation, we detected binding of Runx3 to the T-box (gt) promoter in ILC3 cells (Fig. 4f), as shown before for CD8+ T cells.47 These data suggested that Runx3 directly regulated RORγ expression in ILC3 cells.

To investigate whether RORγ deficiency caused the accumulation of CD127+ ILC3 cells in large intestine of adult mice, as observed for Runx3f/fVav1-Cre mice, we analyzed intestinal LPLs from Runx3f/fVav1-Cre (RORγ-deficient) mice. However, for reasons as yet unclear, the frequency of CD127+ ILC3 cells among intestinal LPLs was similar in wild-type mice and RORγ-deficient mice (Supplementary Fig. 4a). It is possible that complete deletion of RORγ in CD127+ ILC3 cells did not allow the cells to survive and the low residual amount of RORγt might have been required for the accumulation of the CD127+ ILC3 cells in Runx3f/fVav1-Cre mice.

Because RORγt is also required for the emergence and function of LTi cells in fetal intestine,28–30 we assessed the effect of Runx3 deficiency on these cells. The frequency and number of ‘LTi4 cells’ (CD3−CD11c−CD127+CD4+) and ‘LTi4 cells’ (CD3−CD11c−CD127+CD4+)30 were similar in Runx3f/fVav1-Cre cells and Runx3f/fVav1-Cre mice (Supplementary Fig. 4b). However, RORγt expression was lower in LTi cells from Runx3f/fVav1-Cre cells than in Runx3f/fVav1-Cre cells (Supplementary Fig. 4b), which might explain the reduction in PPs in the intestine of adult Runx3f/fVav1-Cre mice. These observations suggested that RORγt expression might be regulated differentially in fetal LTi cells versus ILC3 cells from adult mice.

T-bet is also involved in differentiation of ILC3 cells as well as ILC1 cells, because CD4−NKp46− (double-negative) ILC3 cells give rise to NKp46+ ILC3 cells in a T-bet-dependent manner.13,24 We investigated whether T-bet expression was altered by Runx3 deficiency in NKp46+ ILC3 cells from Cbfbf/fNKp46-Cre mice, which we used instead of Runx3f/fVav1-Cre mice here to exclude the possibility of compensatory effects by other members of the Runx family. We were unable to obtain sufficient RORγt−/−ILC3 cells from Cbfbf/fNKp46-Cre mice for analysis. However, some Lin−CD127−NK1.1+GATA-3− Runx3−/− RORγt−/− LPLs from Cbfbf/fNKp46-Cre mice were NKp46+ (Supplementary Fig. 5a), which suggested that Cbfb-deficiency might abrogate RORγt expression in NKp46+ ILC3 cells. T-bet expression in these GATA-3−RORγt−/−NKp46+ cells from Cbfbf/fNKp46-Cre mice was similar to that in NKp46− ILC3 cells from Cbfbf/fNKp46-Cre (control) mice (Supplementary Fig. 5b). In addition, the few residual ILC1 cells in Cbfbf/fNKp46-Cre mice had normal expression of T-bet (Supplementary Fig. 5b), which suggested that Runx3 was necessary for ILC1 and NKp46+ ILC3 cells through a T-bet-independent mechanism. Thus, Runx3 regulated ILC3 differentiation by inducing RORγt.
AHR expression is downstream of Runx3 in ILC3 cells

Because AHR is a transcription factor that critically regulates ILC3 differentiation in the intestine\textsuperscript{12,13}, we next investigated whether Runx3 deficiency affected AHR expression in Lin\textsuperscript{−}CD127\textsuperscript{−}NK1.1\textsuperscript{−} LPL populations, which include ILC2, ILC3 and CD127\textsuperscript{+} ILCN cells in Runx3\textsuperscript{gt}\textsuperscript{−} mice, and ILC2 and CD127\textsuperscript{+} ILCN cells in Runx3\textsuperscript{gt}\textsuperscript{−}Ahr mice. We did not detect expression of Ahr mRNA or Rorc\textsuperscript{gt} mRNA in Lin\textsuperscript{−}CD127\textsuperscript{−}NK1.1\textsuperscript{−} LPL populations isolated from Runx3\textsuperscript{gt}\textsuperscript{+}Vav1-Cre mice, which included ILC2 cells and CD127\textsuperscript{+} ILCN cells (Fig. 4g). In contrast, Lin\textsuperscript{−}CD127\textsuperscript{−}NK1.1\textsuperscript{−} cells from Runx3\textsuperscript{gt}\textsuperscript{−}Vav1-Cre mice had higher expression of Gata3 than did cells from Runx3\textsuperscript{gt}\textsuperscript{+}Vav1-Cre (control) mice (Fig. 4g), similar to results obtained for Runx3 deficiency in CD8\textsuperscript{+} T cells\textsuperscript{13}. These data suggested that AHR was downstream of Runx3 in ILC3 development.

To investigate whether Runx3 controlled AHR expression directly or indirectly, we investigated the expression of Ahr mRNA in Lin\textsuperscript{−}CD127\textsuperscript{−}NK1.1\textsuperscript{−} LPLs from the small and large intestine of Rorc\textsuperscript{gt}\textsuperscript{−} mice, which have heterozygous Rorc\textsuperscript{gt} expression. RT-PCR analysis showed 50% lower expression of Ahr mRNA in Rorc\textsuperscript{gt}\textsuperscript{−} than in their wild-type counterparts (Supplementary Fig. 6a), which suggested that AHR was downstream of ROR\textsuperscript{γ} in ILC3 cells. To determine if ROR\textsuperscript{γ} was able to bind to either the Ahr promoter or Ahr enhancers, we used published data analyzing ROR\textsuperscript{γ} by chromatin immunoprecipitation followed by deep sequencing\textsuperscript{12}, as well as the histone acetyltransferase p300 and histone H3K4–dimethylation profiles of T\textsubscript{H}17 cells (Supplementary Fig. 6b), which have expression of ROR\textsuperscript{γ} and AHR similar to that of ILC3 cells. The alignment of these data sets indicated that ROR\textsuperscript{γ} bound to at least three sites in the vicinity of the Ahr transcription start site, which, according to their H3K4me2 modification and p300 binding, might be Ahr enhancer regions (Supplementary Fig. 6b). These data were consistent with the possibility that ROR\textsuperscript{γ} might directly regulate Ahr via enhancer interactions.

To assess the possibility of direct regulation, we investigated the binding of Runx3 to the Ahr promoter in splenic NK cells, which express both Runx3 and AHR\textsuperscript{18,33}. Published data obtained by chromatin immunoprecipitation followed by deep sequencing\textsuperscript{18} have shown that active promoter or enhancer regions of Ahr promoter marked by monomethylation of H3K4 in splenic NK cells were almost identical to those marked by dimethylation of H3K4 in T\textsubscript{H}17 cells (Supplementary Fig. 6b). However, we did not detect binding of Runx3 to the Ahr promoter or enhancers in splenic NK cells (Supplementary Fig. 6b), which indicated that Runx3 did not regulate AHR in NK cells; these results could be taken as an indication that Ahr was also not directly regulated by Runx3 in ILC3 cells. Collectively, these data suggested that AHR expression was regulated by ROR\textsuperscript{γ}, whose expression was under direct control of Runx3 in ILC3 cells.

Runx3 is required for the survival of ILC1 cells

In splenic NK cells, Runx3 regulates the expression of genes encoding products related to cell survival and proliferation downstream of IL-15 signaling\textsuperscript{18}. Because IL-15 acts as a survival factor for splenic NK cells and intestinal ILC1 cells\textsuperscript{5,8,23}, we investigated whether Runx3 controlled the survival of ILC1 cells in the intestine. The residual intestinal ILC1 cells and liver-resident DX5\textsuperscript{−}CD49a\textsuperscript{+}...
Figure 5 Runx3 is essential for the survival of ILC1 cells but not of ILC3 cells. (a) Flow cytometry analyzing the apoptosis of CD3+CD19+ cells and ILC1 cells (CD45+CD3−CD19−NK1.1−NKp46+CD49a−) from the intestine of Cbfb−/NKp46-Cre, Cbfb−/NKp46-Cre, Runx3−/NKp46-Cre and Runx3−/NKp46-Cre mice. (b) Flow cytometry analyzing Ki67 expression in ILC1 cells from mice as in a. Numbers above bracketed lines indicate percent Ki67+ (proliferated) cells. (c) Flow cytometry analyzing Bcl-2 expression by intestinal ILC1 cells obtained from Cbfb−/ NKp46-Cre and Cbfb−/NKp46-Cre mice, then cultured for 24 h with IL-15 (20 ng/ml) (left); right, mean fluorescence intensity (MFI) of Bcl-2. Isotype, isotype-matched control antibody. (d) Flow cytometry of ILC1 cells as in b. Numbers adjacent to outlined areas indicate percent cells expressing active caspases. (e,f) Flow cytometry analyzing the apoptosis of CD3+CD19+ cells and CD45+CD3−CD19−CD127+NK1.1−GATA-3-int PP lymphocytes from Cbfb−/NKp46-Cre and Cbfb−/NKp46-Cre mice (e) or Runx3−/ and Runx3−/Vav1-Cre mice (f). *P < 0.05 (Student’s t-test). Data are representative of three independent experiments (mean ± s.d. of three mice in c).

NK cells in Cbfb−/NKp46-Cre mice and Runx3−/NKp46-Cre mice were more apoptotic (as assessed by staining with annexin V) and underwent greater proliferation (as assessed by staining with the proliferation marker Ki67) than those in Cbfb−/NKp46-Cre or Runx3−/NKp46-Cre (control) mice (Fig. 5a,b and Supplementary Fig. 7a,b). However, the effect of the deletion of Runx3 varied somewhat among various ILC1 subsets because of apparent tissue-dependent effects. For example, Runx3−/NKp46-Cre NK cells from the spleen and salivary glands had normal or only marginally greater apoptosis compared with that of Runx3−/NKp46-Cre (control) NK cells, while they showed greater proliferation than that of Runx3−/NKp46-Cre NK cells (Supplementary Fig. 7a,b).

Moreover, DX5+CD49a+ liver NK cells are thought to correspond to splenic NK cells, because they share the same gene-expression profiles, trafficking properties and transcription-factor dependence23. However, Cbfb−/NKp46-Cre and Runx3−/NKp46-Cre DX5+CD49a+ NK cells from the liver showed less pronounced annexin V staining than did their counterparts from the spleen (Supplementary Fig. 7a). Regardless of those findings, ILC1 cells from the intestine showed enhanced apoptosis in the absence of CBF-β and Runx3 (Fig. 5a,b).

Figure 6 Cell-intrinsic requirement for Runx3 in the development of ILC1 and ILC3 cells. (a) Chimerism of splenocytes, ILC1 cells (CD45+CD3−CD19−NK1.1−NKp46+CD49a−) from the small intestine, and ILC2 cells (CD45+CD3−CD19−CD127+GATA-3hi) and NKp46− or NKp46+ ILC3 cells (CD45+CD3−CD19−CD127+RORγt+) from the small and large intestine, of lethally irradiated (Ly5.1+) host mice reconstituted with a mixture (1:1) of bone marrow cells from wild-type (Ly5.1+) mice (WT) and Cbfb−/NKp46-Cre or Cbfb−/NKp46-Cre (Ly5.2+) mice, assessed by flow cytometry analyzing expression of the markers Ly5.1 and Ly5.2. (b) Chimerism of splenocytes, ILC1 cells (gated as in a), and ILC2 and ILC3 cells (gated as in a) from the PPs and among LPLs from the small and large intestine of lethally irradiated (Ly5.1+) host mice reconstituted with a mixture (1:1) of bone marrow cells from wild-type (Ly5.1+) mice and Runx3−/ or Runx3−Vav1-Cre (Ly5.2+) mice, assessed as in a. Numbers adjacent to outlined areas indicate percent Ly5.2+Ly5.1− (mutant donor) cells or Ly5.2−Ly5.1+ (wild-type) cells. Data are representative of two independent experiments.
To explore the mechanism of Runx-dependent apoptosis, we assessed the expression of regulators of apoptosis in Cbfb<sup>−/−</sup>NKp46<sup>-</sup>Cre ILC1 cells. The anti-apoptotic factor Bcl-2 is induced by stimulation with IL-22. In addition, deficiency in Cbfb<sup>−/−</sup> or Runx3 in ILC1 cells led to greater total caspase activity than that of Cbfb<sup>−/−</sup>NKp46<sup>-</sup>Cre or Runx3<sup>−/−</sup>NKp46<sup>-</sup>Cre (control) ILC1 cells (Fig. 5d). Therefore, Runx3 might control the survival of ILC1 cells through regulation of the expression of genes encoding anti-apoptotic factors, including Bcl-2.

To determine whether CBF-β and Runx3 also control the survival of ILC3 cells, we used PP lymphocytes instead of LPLs, because the collagenase treatment used to isolate LPLs caused cell death and interfered with the apoptosis assay. NKp46<sup>+</sup>Lin<sup>−</sup>CD127<sup>+</sup>NK1.1<sup>−</sup>GATA-3<sup>int</sup> ILC3 cells from Cbfb<sup>−/−</sup>NKp46<sup>-</sup>Cre mice, which had lost RORγt expression, had apoptosis rates similar to those of NKp46<sup>+</sup> ILC3 cells from Cbfb<sup>−/−</sup>NKp46<sup>-</sup>Cre mice (Fig. 5e). In addition, Runx3<sup>−/−</sup>Vav1<sup>-</sup>Cre Lin<sup>−</sup>CD127<sup>−</sup>NK1.1<sup>−</sup>GATA-3<sup>−</sup> ILC3 cells, which were unable to express RORγt because of their Runx3 deficiency, did not show greater apoptotic rates than those of Runx3<sup>+</sup>Vav1<sup>+</sup>Lin<sup>−</sup>CD127<sup>−</sup>NK1.1<sup>−</sup>GATA-3<sup>−</sup> ILC3 cells (Fig. 5f). These data indicated that CBF-β and Runx3 were crucial for the survival of ILC1 cells, but not that of ILC3 cells, in the intestine.

**Cell-intrinsic role for Runx3 for ILC1 and ILC3 cells in the intestine**

Because crosstalk between innate lymphocytes and CD4<sup>+</sup> T cells has been described<sup>35,36</sup> and commitment to the CD4<sup>+</sup> T lineage was also affected in Runx3<sup>−/−</sup>Vav1<sup>−</sup>Cre mice, we investigated whether Runx3 deficiency affected the number of ILCs via cell-intrinsic effects or cell-extrinsic effects. We generated competitive bone-marrow chimeras by transferring Cbfb<sup>−/−</sup>NKp46<sup>-</sup>Cre, Cbfb<sup>−/−</sup>NKp46<sup>-</sup>Cre, Runx3<sup>−/−</sup> or Runx3<sup>−/−</sup>Vav1<sup>−</sup> Cre bone marrow cells into lethally irradiated Ly5.2<sup>+</sup> mice, mixed at an equal ratio (1:1) with wild-type bone marrow cells (Ly5.2<sup>−</sup>), into lethally irradiated Ly5.1<sup>+</sup> congenic mice. We assessed donor chimerism at 8–10 weeks after injection in splenic and intestinal PPs, IELs and LPLs, on the basis of expression of the markers Ly5.1 and Ly5.2. Fewer ILC1 and NKp46<sup>+</sup> ILC3 cells were derived from Cbfb<sup>−/−</sup>NKp46<sup>-</sup>Cre donor cells (Fig. 6a) and fewer ILC1 and ILC3 cells were derived from Runx3<sup>−/−</sup>Vav1<sup>−</sup>Cre donor cells (Fig. 6b) than from the corresponding wild-type competitor cells, which demonstrated the importance of intrinsic Runx3 in the development or maintenance of ILC1 and ILC3 cells in the intestine. In particular, we recovered almost no ILC3 cells from Runx3<sup>−/−</sup>Vav1<sup>−</sup>Cre donor bone marrow in PPs (Fig. 6b). In contrast, ILC2 cells derived from Runx3<sup>−/−</sup>Vav1<sup>−</sup>Cre donor bone marrow repopulated the intestinal lamina propria and PPs to the same extent that wild-type cells did (Fig. 6b). Thus, Runx3 deficiency affected the development or maintenance of ILC1 and ILC3 cells in a cell-intrinsic manner but did not affect ILC2 cells.

**Runx3 in ILCs is essential for protection against C. rodentium**

We next investigated the physiological role of Runx3 in ILCs with a model of C. rodentium infection in Runx3<sup>−/−</sup>NKp46<sup>-</sup>Cre mice, given that CD4<sup>+</sup> T cells<sup>37</sup>, other hematopoietic cells and enterocytes<sup>38</sup> might also be affected in Runx3<sup>−/−</sup>Vav1<sup>−</sup> Cre mice. Runx3<sup>−/−</sup>NKp46<sup>-</sup>Cre mice...
infected with *C. rodentium* showed a degree of body weight loss and survival similar to that of *C. rodentium*-infected Runx3<sup>fl/fl</sup>NKp46-Cre mice (data not shown), similar to result obtained with T-bet-deficient mice, which do not have intestinal ILC1 cells or NKp46<sup>+</sup> ILC3 cells<sup>4,24</sup>. However, on day 8 after infection with *C. rodentium*, Runx3<sup>fl/fl</sup>NKp46-Cre mice had shorter colons and higher bacterial titers in the spleen than those of their Runx3<sup>fl/fl</sup>NKp46-Cre counterparts (Fig. 7a,b). There was no inflammation in Runx3<sup>fl/fl</sup>NKp46-Cre mice not infected with *C. rodentium*, similar to the lack of inflammation in uninfected Runx3<sup>fl/fl</sup>NKp46-Cre control mice (Fig. 7c). However, following infection with *C. rodentium*, Runx3<sup>fl/fl</sup>NKp46-Cre mice had more persistent intestinal damage, including features of greater epithelial injury, crypt hyperplasia and more infiltration of inflammatory cells, than that of their Runx3<sup>fl/fl</sup>NKp46-Cre counterparts (Fig. 7c,d). These observations suggested a specific role for ILC1 and NKp46<sup>+</sup> ILC3 cells in controlling infection with *C. rodentium*.

Because IL-22, especially that from ILC3 cells, and IFN-γ are required for the control of acute infection with *C. rodentium<sup>3,13,24,39</sup>, we assessed the production of these cytokines in *C. rodentium*-infected Runx3<sup>fl/fl</sup>NKp46-Cre mice. These mice had considerably fewer IL-22-producing ILC3 cells in the intestine than did *C. rodentium*-infected Runx3<sup>fl/fl</sup>NKp46-Cre mice (Fig. 7e). In addition, we detected fewer IFN-γ-producing ILC1 cells among IELs and LPLs in Runx3<sup>fl/fl</sup>NKp46-Cre mice after infection than in their Runx3<sup>fl/fl</sup>NKp46-Cre counterparts (Fig. 7g–i), which indicated that Runx3 expression in ILCs was critical for host immunity and cytokine production in response to infection with *C. rodentium*.

**DISCUSSION**

In this study, we showed that Runx3 regulated the development and/or maintenance of ILC1 and ILC3 cells in the intestine. Runx3 induced the transcriptional regulator RORγt and its downstream target AHR in ILC3 cells. Runx3 regulated the differentiation of ILC1- and ILC3-specific progenitor cells into ILC3 cells in the adult mouse intestine. As a maintenance factor, Runx3 was necessary for the survival of ILC1 cells but not that of ILC3 cells. Runx3 deficiency had a limited effect on ILC1 development, probably due to compensatory effects by other members of the Runx family at steady state. Studies of competitive bone-marrow chimeras revealed a cell-intrinsic contribution of Runx3 to the development of ILC1 and ILC3 cells but not that of ILC2 cells. Finally, a requirement for Runx3 in the maintenance of ILCs was evident after infection with *C. rodentium*. Thus, our results have established Runx3 as a key participant in ILC lineage-specific differentiation.

Together our data add substantial evidence in support of the proposal of parallel but somewhat distinct differentiation of ILCs and CD4<sup>+</sup> T cell subsets. CD4<sup>+</sup> T cells and ILCs share the same signature transcription factors, although all ILCs in the intestine expressed GATA-3 differentially. CD127<sup>+</sup> ILC1 and ILC3 cells also had intermediate expression of GATA-3, while ILC2 cells were characterized by high expression of GATA-3. T<sub>H</sub>1 cells and ILC1 cells were characterized by high expression of T-bet and Runx3. T<sub>H</sub>2 cells and ILC2 cells were identified as GATA-3<sup>+</sup> populations. T<sub>H</sub>17 cells used Runx1 to induce RORγt expression, while in ILC3 cells RORγt was driven by Runx3. Because members of the Runx family orchestrate CD4<sup>+</sup> T cell differentiation together with GATA-3 and T-bet, our findings offer additional insights into the mechanism of ILC-lineage differentiation.

Without Runx3, ILC3 development was arrested at the CD127<sup>+</sup> ILCLN stage. GATA-3 controls CD127 expression<sup>3</sup>, and only the PLZF<sup><sup>+</sup></sup> cells among Lin<sup>−</sup>CD127<sup>+</sup> bone marrow cells expressed distal Runx3. Although such findings suggest that Runx3 might be downstream of GATA-3, the inducible deletion of GATA-3 in ILC3 cells does not alter the expression of Runx1 or Runx3 (ref. 9). The interpretation that Runx3 is not regulated by GATA-3 in ILCs was also supported by the observation that ILC2 cells did not express distal Runx3 despite their high GATA-3 expression. GATA-3 expression was instead inversely correlated with distal Runx3 expression, because only GATA-3<sup>+</sup> ILC1 and ILC3 cells, but not GATA-3<sup>−</sup> ILC2 cells, expressed distal Runx3.

Runx3 promotes commitment to the T<sub>H</sub>1 lineage and represses GATA-3 through direct binding to GATA-3 (ref. 40). Conversely, GATA-3 blocks Runx3 function by direct interaction with Runx3 in T<sub>H</sub>2 cells<sup>41</sup>, which suggests that the balance between GATA-3 and Runx3 controls lineage determination toward T<sub>H</sub>1 cells or T<sub>H</sub>2 cells. The same transcriptional network might regulate ILC1 and ILC2 differentiation, given that ILCPs expressed both GATA-3 and Runx3. ILC1 cells had high expression of Runx3, which was apparently necessary to overcome their GATA-3 expression. In contrast, ILC2 cells acquired high GATA-3 expression and lost Runx3 expression, which seemed to be necessary for the GATA-3-dependent machinery to drive ILCPs to differentiate into ILC2 cells. Additionally, members of the Runx family interact with T-bet to control CD4<sup>+</sup> T cell differentiation. T-bet regulates Runx3 to enhance commitment to the T<sub>H</sub>1 lineage and attenuates skewing toward the T<sub>H</sub>17 lineage by inhibiting Runx1-mediated expression of RORγt through antagonistic binding to Runx1 (refs. 27, 42, 43). Possible interactions between T-bet and Runx3 during ‘determination’ of the ILC lineage seem to be very complex. During ILC differentiation, Runx3 expression is induced earlier than T-bet expression is induced, because ILCPs express Runx3 but not T-bet<sup>7</sup>. It is not still clear how T-bet is induced, associates with Runx3 and regulates two different subsets of T-bet-dependent ILCs (ILC1 cells and NKp46<sup>+</sup> ILC3 cells) differentially.

Published data have shown that deficiency in Runx1 or CBF-β is associated with fewer LTi cells in the fetal intestine<sup>19</sup>. However, it has been challenging to interpret those data in the absence of knowledge of progenitors of ILCs. Here we investigated the expression of members of the Runx family in progenitors of ILCs and all ILC subsets. Runx3, not Runx1, was expressed mainly by ILC1 and ILC3 cells and contributed to commitment of the ILC lineage to those two subsets. Although Runx1 expression is not very high in CLPs, Runx1 is critical for the differentiation of Flt3<sup>+</sup> progenitor cells, including CLPs<sup>17</sup>. Runx1 might also have a critical role in ILC differentiation along with Runx3, just before commitment to the ILC lineage, because ILCPs had high expression of both Runx1 and Runx3. Further studies will be needed to determine the function of Runx1 and Runx3 in ILCs.

RORγt expression was affected less severely in Runx3-deficient fetal LTi cells than in ILC3 cells from adult mice. This might have been due to different requirements for their development<sup>29,44,45</sup> and/or to different compensatory effects. For example, stimulation with IL-15 or IL-2 is needed to reveal proliferation defects in splenic Runx3-deficient NK cells<sup>19</sup>. Also, the lower abundance of ILC1 cells in Runx3-deficient mice was more evident in *C. rodentium*-infected Runx3<sup>fl/fl</sup>NKp46-Cre mice. Thus, differences in microbiota or the inflammatory milieu in fetal intestine versus adult intestine might affect the requirement for Runx3 in RORγt expression by ILC3 cells.

The CD127<sup>+</sup> ILCLN cells described here in Runx3<sup>fl/fl</sup>Vav1-Cre mice were α<sub>β</sub>γ<sup>+</sup> <sup>−</sup>. In contrast, some studies have found ILC progenitor cells in the Lin<sup>−</sup>CD127<sup>+</sup>α<sub>β</sub>γ<sup>+</sup> population from bone marrow, fetal liver and intestine<sup>5,7,44,46</sup>. However, we note that ILCs in the intestine of adult mice do not express α<sub>β</sub>γ<sup>+</sup> but do express CD127 (data not shown)<sup>2,10</sup>. Therefore, the CD127<sup>+</sup> ILCLN cells described here might have been ILC1- and ILC3-specific ILC progenitors that had already migrated to the intestine but were arrested in their ILC3 development because they required Runx3 and RORγt for further differentiation.
Runx3 dysfunction has been discussed for many years in the context of the pathogenesis of colitis\(^{17,47,48}\). Aberrant Th17 cells deficient in Runx3 are reported to be sufficient for the induction of colitis\(^{27}\). Here we demonstrated that Runx3 was indispensable for the development of ILC3 cells, which were the main source of IL-22 in the intestine for the maintenance of epithelial integrity. Thus, Runx3 mutations and loss of Runx3 function might diminish a host’s ability to protect the intestinal barrier against microbes, which would link Runx3 defects to the pathogenesis of intestinal inflammation.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

T.Eb. and W.M.Y. designed experiments and analyzed data; T.Eb., C.S., S.H.R., B.P.-D., L.Y., M.D.B. and T.S.S. performed the experiments; D.L. and Y.G. supplied reagents; T.Eg. analyzed data obtained by chromatin immunoprecipitation followed by deep sequencing; and T.Eb., T.G., T.S.S., M.C., T.Eg. and W.M.Y. wrote the paper.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. All the mice were bred and maintained at a specific pathogen–free facility of the Washington University School of Medicine, and all protocols were approved by the Washington University Animal Studies Committee. C57BL/6 mice and congenic Ly5.1 mice were obtained from the National Cancer Institute. Rorc(γt)(gt)/+ and Vav1-Cre mice were obtained from Jackson Laboratories. Cbfbf/f, Runx3f/f and Runx3d′/f′f′ mouse have been described and were provided by D.R. Littman (New York University). NKp46-Cre mice were provided by E. Vivier (Centre d’Immunologie de Marseille-Luminy). All Cre-expressing mice were heterozygous. Control mice were all littermates.

Cell preparation. Lymphocytes were isolated from the small and large intestine as described with some modifications. Small and large intestines were dissected and fat tissues were removed. PPs were removed from the small intestine and were ‘smashed’ through a 70-µm strainer. Intestines were cut open longitudinally and washed with PBS, then were cut into pieces 5 mm in length, followed by incubation for 20 min at 37 °C with Hank’s balanced-salt solution buffer containing 10% FBS, 5 mM EDTA and 15 mM HEPES. After being vortexed for 20s, the dissociated cells were collected as IELs. For the isolation of LPLs, the rest of the intestinal fragments were washed twice with PBS, and 0.5 g of the tissues were digested for 30 min at 37 °C with 25 ml of RPMI-1640 medium containing 5 mg of Liberase TL (Roche Life Sciences), 5 mg of DNase I (Roche) and 50 mg of Dispase II (Roche). The digested tissues were passed through 100-µm and 70-µm strainers after being vigorously vortexed for 20s. Then, lymphocytes were isolated from the interface of 40% and 80% Percoll gradient after centrifugation at 1,800 r.p.m. for 20 min.

Antibodies and flow cytometry. The supernatant of hybridoma 2.4g2 (anti-body to CD16/32) was used for blockade of Fc receptors. Cells were stained with Fixable Viability Dye eFlour 506 (eBioscience) for the detection of dead cells before staining of cell surfaces. An Annexin V Apoptosis Detection kit, eFlour 450 (eBioscience) and Ki67 kit (BD Biosciences) were used according to the manufacturers’ protocols. Antibodies used for flow cytometry are in Supplementary Table 1. Pan-caspase activity was detected with a FAM-FLICA Poly Caspase Assay Kit according to the manufacturer’s protocol (ImmunoChemistry Technologies). For staining of intracellular cytokines and transcription factors, the Foxp3/Transcription Factor Staining Buffer Set was used according to the manufacturer’s protocol (eBioscience). IELs (1 × 106 cells per ml) and LPLs (1 × 106 cells per ml) were stimulated for 4 h at 37 °C with IL-23 (30 ng/ml) and IL-1β (10 ng/ml) with GolgiPlug for the induction of cytokine production or for 24 h at 37 °C with IL-15 (20 ng/ml) for analysis of Bcl-2 expression. Data were acquired on a FACSCanto II (BD Biosciences) and were analyzed with FlowJo software (TreeStar).

RT-PCR. ILC1 cells among IELs, ILC3 subsets among LPLs and CD45+CD3−CD19−CD127+NK1.1−LPLs were isolated from the small and large intestine and or 100 cells were cultured for 14 d in individual wells containing a monolayer of mitomycin C-treated OP9-DL1 cells with IL-7 (25 ng/ml), stem–cell factor (25 ng/ml) and IL-2 (10 ng/ml). Cultured cells were stimulated for 4 h at 37 °C with IL-23 (30 ng/ml) and IL-1β (30 ng/ml) with GolgiPlug for the induction of IL-22 production.

Luciferase assay. The promoter of the gene encoding ROByt encompassing a region from position −242 to position +109 relative to the transcription start site was inserted into the XhoI-HindIII site of plasmid pGL3. For mutation of the Runx-binding motif, TGGTGTTT (positions −174 to −168 from the transcription start site), was modified to TACATTC. NK-92 cells were obtained from American Type Culture Collection and were transfected through the use of an Amaxa cell line Nucleofector Kit R according to the manufacturer’s protocol (Lonza). 5 × 106 NK-92 cells were transfected by electroporation with 1 µg of vector pGL3 with 1 µg of empty vector pEFl or Runx3-encoding pEF vector. After 18 h of incubation at 37 °C and 5% CO2, the cells were harvested, counted for normalization and resuspended in Glo Lysis Buffer. Luciferase activity was measured with a 1,450 Micro Beta (Wallac).

Chromatin immunoprecipitation. ILC3 cells (1.5 × 105) were isolated from Rorc(γt)(gt)/+ mice. Cells were cross-linked with 1% paraformaldehyde, lysed and sonicated to generate 100–300–base pair fragments of DNA. DNA was prepared from 5% of sample before immunoprecipitation was used as input DNA. DNA-protein complexes were immunoprecipitated with 4 µl of rabbit antiserum to Runx3, developed elsewhere. After reverse-cross-linking and DNA purification, immunoprecipitated DNA fragments were analyzed by quantitative RT-PCR with SYBR Green (primers, Supplementary Table 2).

Generation of bone marrow chimeras. Congenic Ly5.1− mice were lethally irradiated with 950 rads and were reconstituted with 1 × 107 Ly5.1+ wild-type bone marrow cells and 1 × 107 Ly5.2+ bone marrow cells obtained from Cbfbf/fNKp46-Cre, Cbfbf/fNKp46-Cre, Runx3f/f or Runx3f/fVav1-Cre mice. After 8–10 weeks, the spleen, PPs, IELs and LPLs of the small and large intestine were collected for analysis.

C. rodentium infection. Runx3f/fNKp46-Cre or Runx3f/fNKp46-Cre mice were orally infected with 5 × 109 C. rodentium strain DBS100 (American Type Culture Collection) as described elsewhere. On day 8 after infection, the spleen, small intestine and colon were collected from the infected mice. Spleens were weighed and transferred to a 2-ml tube with 1 ml of PBS and a 5-mm metal bead (Qiagen) and were homogenized for 1 min with Mini-BeadBeater-8 (Biospec) at a medium speed. 100 µl of the homogenate was plated onto MacConkey agar plates, and C. rodentium colonies were counted after overnight incubation at 37 °C. The colon was fixed for 2 h at 4 °C with Methacarn, then for 1 h at 4 °C with 70% ethanol, then were incubated overnight with 20% sucrose in PBS and were embedded into OCT compound (Tissue-Tek) for staining with hematoxylin and eosin. Colons were used for the isolation of IELs and LPLs as described above. For analysis of colonic pathology based on a published system for assigning pathology scores, observers were ‘blinded’ to the experimental conditions. The pathological parameters included the following: the extent of inflammatory infiltration; the degree of epithelial hyperplasia; goblet cell depletion; and crypt abscess. Data are presented as the proportion of damaged area along the colonic tissue.

Statistical analyses. Data were analyzed by the two-tailed Student’s t-test with Graphpad Prism 4. P values of <0.05 were considered statistically significant. No randomization was used in animal studies. No deliberate attempt was made to study only selected mice except based on genotype. No blinding was used except for assignment of histological scores (noted above).

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