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Gata3 drives development of ROR γ t⁺ group 3 innate lymphoid cells

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Group 3 innate lymphoid cells (ILC3) include IL-22-producing NKp46⁺ cells and IL-17A/IL-22-producing CD4⁺ lymphoid tissue inducerlike cells that express ROR γ t and are implicated in protective immunity at mucosal surfaces. Whereas the transcription factor *Gata3* is essential for T cell and ILC2 development from hematopoietic stem cells (HSCs) and for IL-5 and IL-13 production by T cells and ILC2, the role for *Gata3* in the generation or function of other ILC subsets is not known. We found that abundant GATA-3 protein is expressed in mucosa-associated ILC3 subsets with levels intermediate between mature B cells and ILC2. Chimeric mice generated with *Gata3*-deficient fetal liver hematopoietic precursors lack all intestinal ROR γ t⁺ ILC3 subsets, and these mice show defective production of IL-22 early after infection with the intestinal pathogen *Citrobacter rodentium*, leading to impaired survival. Further analyses demonstrated that ILC3 development requires cell-intrinsic *Gata3* expression in fetal liver hematopoietic precursors. Our results demonstrate that *Gata3* plays a generalized role in ILC lineage determination and is critical for the development of gut ROR γ t⁺ ILC3 subsets that maintain mucosal barrier homeostasis. These results further extend the paradigm of *Gata3*-dependent regulation of diversified innate ILC and adaptive T cell subsets.

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Abbreviations used: CLP, common lymphoid progenitor; ILC, innate lymphoid cell; LI, large intestine; LTi, lymphoid tissue inducer; NK, natural killer; PP, Peyer's patch; SI, small intestine

Innate lymphoid cells (ILCs) represent a novel subset of hematopoietic effector cells that have rapid and potent cytokine production capacity and are enriched in mucosal tissues, including the gut, lung, and skin (Spits and Di Santo, 2011; Spits et al., 2013). ILCs have important roles in lymphoid tissue formation during both embryonic and adult life, play protective roles in immunity and during inflammation, and are implicated in tissue homeostasis and repair. Three groups of ILCs have been identified that show developmental and functional similarities with T helper subsets: group 1 ILC (ILC1) include NK cells and other innate cells that express the transcription factor T-bet (*Tbx21*) and produce the T_H1-associated cytokines IFN- γ (Townsend et al., 2004; Yu et al., 2007; Gordon et al., 2012; Bernink et al., 2013). ILC2 produce T_H2-associated cytokines (including IL-5 and IL-13, which are important for helminth resistance and are able to provoke airway inflammation;

Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Monticelli et al., 2011). The transcription factors *Rora*, *Gata3*, and *Tcf7* are important regulators of ILC2 development and homeostasis (Yang et al., 2011; Halim et al., 2012; Hoyler et al., 2012; Liang et al., 2012; Mjösberg et al., 2012; Wong et al., 2012; Klein Wolterink et al., 2013; Furusawa et al., 2013; Mielke et al., 2013; Yang et al., 2013). ILC3 include three ROR γ t⁺ mucosal subsets that produce T_H17-associated IL-17 and IL-22 (Romera-Hernandez et al., 2013). Lymphoid tissue inducer (LTi) cells are ROR γ t⁺ ILC3 that are critical for lymphoid tissue organogenesis during fetal life and are present in adult tissues where they promote lymphoid follicle formation; LTi cells can be further divided based on CD4 and CCR6 expression (Cupedo et al., 2009; Sawa et al., 2010;

N. Serafini and R.G.J. Klein Wolterink contributed equally to this paper.

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Klose et al., 2013). A second subset of $\text{ROR}\gamma^+$ ILC3 can be distinguished by the expression of the NK cell marker NKP46 (Sato-Takayama et al., 2008; Cella et al., 2009; Sanos et al., 2009; Sawa et al., 2010). NKP46^+ ILC3 reside in the small intestine (SI) lamina propria and are potent IL-22 producers. The transcription factor *Tbx21* is critical for the development of NKP46^+ ILC3 (Sciumé et al., 2012; Klose et al., 2013; Rankin et al., 2013) and both LT α and NKP46^+ ILC3 require signaling through the aryl hydrocarbon receptor (*Ahr*; Kiss et al., 2011; Lee et al., 2012; Qiu et al., 2012). Lastly, a heterogeneous population of $\text{NKP46}^- \text{CD4}^-$ ILC3 exists that has been implicated in intestinal inflammation and colonic tumor formation (Buonocore et al., 2010; Kirchberger et al., 2013).

Although several of the molecular pathways that generate diverse ILC subsets have been identified, the signals that regulate the earliest stages of ILC lineage development are less well understood. It has been suggested that all ILCs develop from a common ILC precursor based on the common dependence of all known ILC groups for inhibitor of DNA binding 2 (*Id2*) expression (Yokota et al., 1999; Moro et al., 2010; Sato-Takayama et al., 2010). In a current model (Vosshenrich and Di Santo, 2013), *Id2* expression serves to titrate E protein levels in hematopoietic precursors (including common lymphoid precursors [CLPs]) as these have been shown to have potential to give rise to ILC1, ILC2, and ILC3 subsets (Walker et al., 2013), thereby repressing B and T cell potential in favor of ILC potential. The transcription factors that regulate *Id2* are not known, but their deficiency would be expected to impact on the subsequent development of several (or all) ILC groups.

The zinc finger transcription factor *Gata3* is required at the earliest stages of T cell development to facilitate T cell specification and at later stages to promote differentiation to the $\text{T}_\text{H}2$ fate (Ansel et al., 2006; Rothenberg, 2012). Similarly, *Gata3* is required for generation of ILC2 from lymphoid precursors and to maintain effector functions in fully differentiated ILC2 (Liang et al., 2012; Hoyler et al., 2012; Furusawa et al., 2013; Klein Wolterink et al., 2013). Although *Gata3* plays a limited role in bone marrow NK cell development (Samson et al., 2003), *Gata3* is essential for the development of thymic NK cells (Vosshenrich et al., 2006) and has been reported as redundant for ILC3 function (Hoyler et al., 2012). In this report, we provide evidence that *Gata3* plays a critical role in the development and function of fetal liver hematopoietic cell-derived intestinal ILC3. These results suggest a broader role for *Gata3* in ILC lineage specification.

RESULTS AND DISCUSSION

GATA-3 protein is expressed in diverse gut ILC3 subsets

We first analyzed GATA-3 protein expression in CD4^+ , NKP46^+ and $\text{CD4}^- \text{NKP46}^-$ ILC3 subsets (Fig. 1, A–C; and Fig. S1). As $\text{ROR}\gamma^+$ ILCs are enriched in mucosal sites, we focused our attention on ILC3 present in the lamina propria of the SI and large intestine (LI) and in the Peyer's patches (PP). ILC3s were identified as CD3^- cells that co-expressed CD90.2 (Thy1.2), CD127 ($\text{IL-7R}\alpha$), and $\text{ROR}\gamma$ as previously described (Sato-Takayama et al., 2008; Sawa et al., 2010).

Intestinal ILC3s are the most abundant ILC group in the SI and PP, and they clearly expressed GATA-3 at levels exceeding those found in B cells, although at reduced levels (roughly fivefold) compared with intestinal ILC2 (Fig. 1 B). Interestingly, GATA-3 was not homogeneously expressed in the gut: ILC3 in the LI expressed about twofold higher levels of GATA-3 compared with ILC3 in the SI or PP (Fig. 1 B). GATA-3 levels were similar within intestinal ILC3 subsets that differentially expressed NKP46 and/or CD4 (Fig. 1 C). The fact that all intestinal ILC3 subsets were GATA-3 $^+$ raise the possibility that GATA-3 could play a role in ILC3 differentiation, as has recently been shown for ILC2 (Furusawa et al., 2013; Hoyler et al., 2012; Klein Wolterink et al., 2013; Mjösberg et al., 2012).

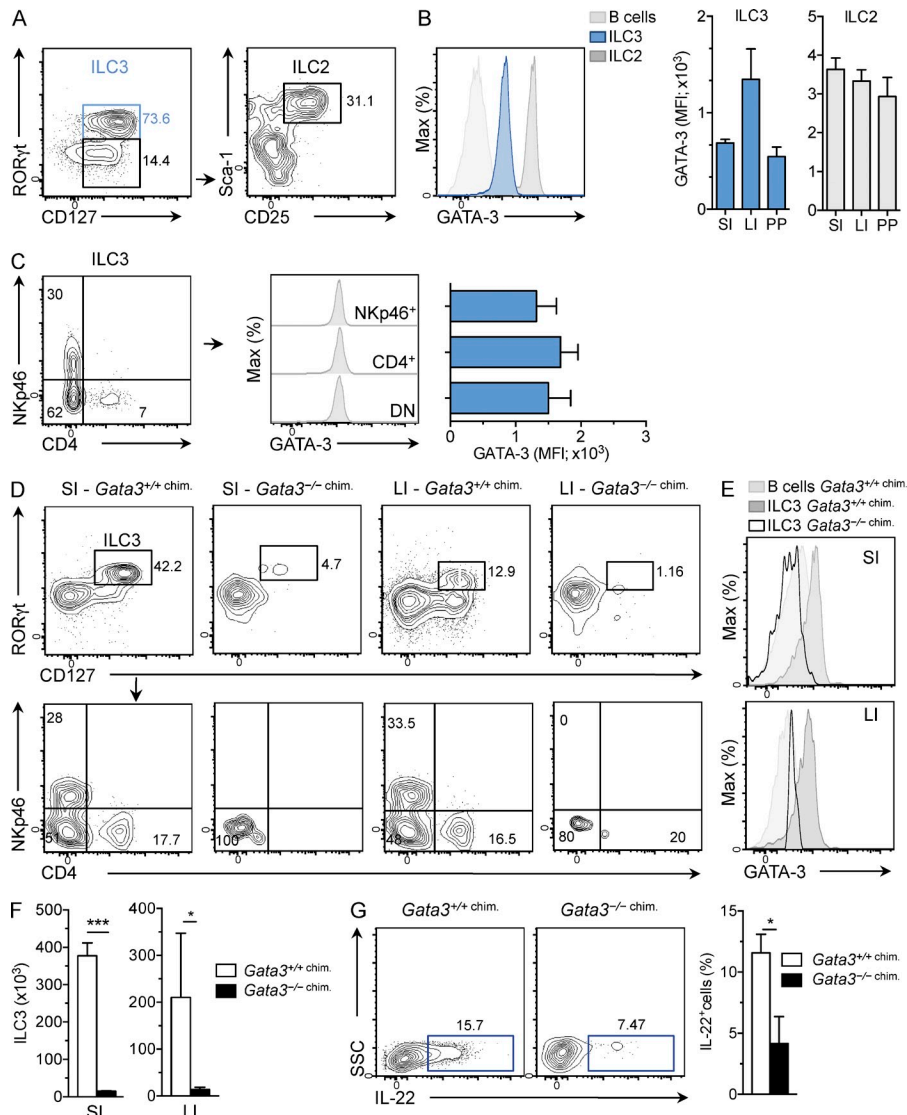
Gata3 expression required for ILC3 subset development in vivo

Previous studies demonstrated that deletion of *Gata3* leads to lethality during mouse embryogenesis (Pandolfi et al., 1995; Ting et al., 1996). To elucidate the in vivo requirement for *Gata3* in ILC3 development, we analyzed mice engrafted with *Gata3*-deficient (*Gata3* $^{-/-}$) hematopoietic precursor cells from rare E12.5–13.5 embryos that could be rescued by pharmacological treatment with β -adrenergic agonists (Kaufman et al., 2003). *Rag2* $^{-/-} \text{Il2rg}^{-/-}$ mice were recipients for the adoptive transfer as these hosts lack endogenous ILC (Moro et al., 2010; Neill et al., 2010; Price et al., 2010). As such, ILC present in these chimeric mice are donor derived (Klein Wolterink et al., 2013). *Gata3* $^{+/+}$ and *Gata3* $^{-/-}$ hematopoietic precursors engrafted to a similar extent in *Rag2* $^{-/-} \text{Il2rg}^{-/-}$ mice, leading to robust B, NK cell, and myeloid cell reconstitution as previously described (Samson et al., 2003; Vosshenrich et al., 2006; García-Ojeda et al., 2013).

We could identify $\text{CD127}^+ \text{ROR}\gamma^+$ ILC3 in the SI and LI of *Gata3* $^{+/+}$ chimeras that expressed GATA-3 protein levels similar to that observed in ILC3 from control C57BL/6 mice (Figs. 1, D and E). In the *Gata3* $^{-/-}$ chimeras, total numbers of CD127^+ ILC expressing $\text{ROR}\gamma$ were dramatically reduced and the remaining $\text{ROR}\gamma^+$ ILC3 lacked GATA-3 expression and were depleted in NKP46^+ and CD4^+ cells (Fig. 1, D–F). Moreover, these residual *Gata3* $^{-/-}$ ILC3 were unable to normally produce IL-22 after ex vivo IL-23 stimulation (Fig. 1 G). Collectively, these results clearly demonstrate that *Gata3* is essential for normal generation of intestinal ILC3 subsets in fetal liver hematopoietic chimeras.

Cell-intrinsic *Gata3* is required for ILC3 development

To exclude the possibility that cell-extrinsic effects caused by *Gata3*-deficiency in non-ILC lineages underlie the observed defects in ILC3 development, we generated and analyzed BM chimeras generated using mixtures (90%/10%) of CD45.2^+ *Gata3* $^{-/-}$ and CD45.1^+ wild type C57BL/6 hematopoietic precursors, respectively into $\text{CD45.1}^+ \text{Rag2}^{-/-} \text{Il2rg}^{-/-}$ hosts. Recipient mice injected with the same mixtures of CD45.2^+ *Gata3* $^{+/+}$ and CD45.1^+ *Gata3* $^{+/+}$ precursors served as controls. We previously reported that these mixed *Gata3* chimeras



and *Gata3*^{-/-} chimeras. Bars correspond to the mean \pm SEM of the values obtained (3 independent experiments with $n = 6$ mice analyzed for each genotype). *, $P < 0.05$; ***, $P < 0.001$. (G) SI lamina propria cells were stimulated for 4 h with IL-23 and IL-22 expression in ILC3 (CD3⁻ CD127⁺ CD90.2⁺ cells) was assessed by intracellular cytokine staining and flow cytometry. Bar graph represents the percentage (mean \pm SEM; $n = 4$ for each genotype) of IL-22⁺ cells. Representative results from two independent experiments. *, $P < 0.05$.

reconstituted to a similar extent with normal B, myeloid and NK cell development from both *Gata3*^{-/-} and *Gata3*^{+/+} precursors at the expected frequencies (Klein Wolterink et al., 2013).

Both sets of mixed chimeras developed phenotypically mature NKp46⁺, CD4⁺, and CD4⁻ NKp46⁻ (here referred to as double negative [DN]) RORγt⁺ ILC3 subsets in the gut as well as the spleen (Figs. 2 A). However, the origin of these ILC3 subsets differed dramatically in these two sets of mice. In mixed chimeras generated with CD45.2⁺ *Gata3*^{+/+}/CD45.1⁺ *Gata3*^{+/+} precursors, RORγt⁺ ILC3 derived from both donors, whereas these cells were almost exclusively derived from CD45.1⁺ B6 (*Gata3*^{+/+}) precursors in CD45.2⁺ *Gata3*^{-/-}/CD45.1⁺ *Gata3*^{+/+} chimeras (Fig. 2, B and D). The generation of all ILC3 subsets (NKp46⁺, CD4⁺, or DN) was

affected by the absence of GATA-3 in mixed chimeras, resulting in a predominance of CD45.1⁺ WT cells in *Gata3*^{-/-} mixed chimeras (Fig. 2, E–G). These results demonstrate that *Gata3* is required in a cell-intrinsic fashion for ILC3 development.

GATA-3 is required for IL-22-dependent innate responses to *C. rodentium*

We next investigated the importance of *Gata3* for ILC3 function in the protection against infection by *C. rodentium*. In this model, the control of bacterial infection requires IL-23 signaling that promotes a sequential wave of IL-22 production, first by ILC and later by T cells that results in the clearance of *C. rodentium* (Zheng et al., 2008; Basu et al., 2012). RORγt⁺ ILC3 subsets are important producers of IL-22 in the intestinal

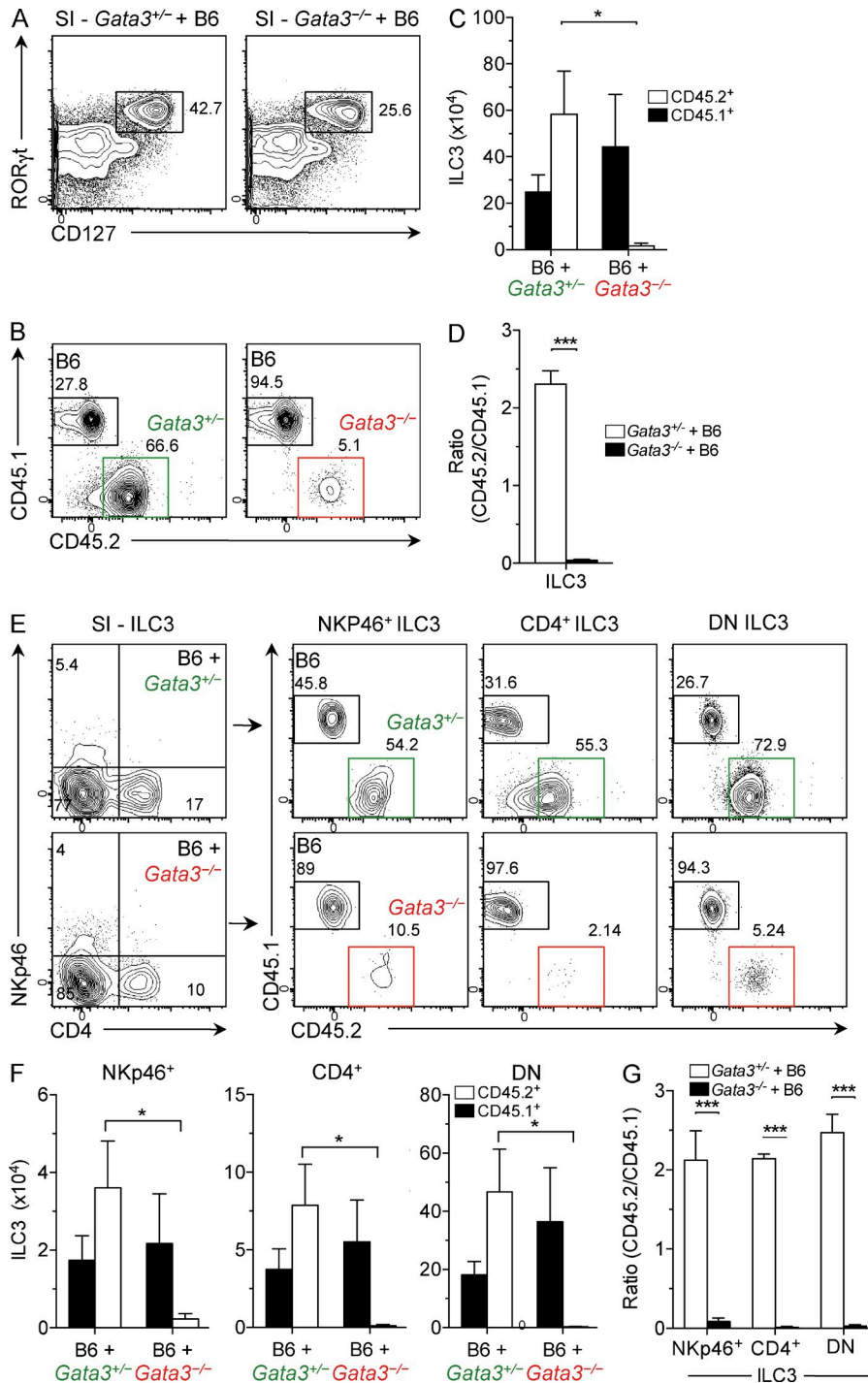


Figure 2. Cell-intrinsic role for *Gata3* in ILC3 development. Mixed chimeras were generated and analyzed as indicated in the Materials and methods. (A and B) ILC3 (CD3⁻CD127⁺CD90.2⁺ cells) in SI lamina propria were analyzed for CD45 allotypes using flow cytometry. Relative percentage of gated populations is indicated. Representative results from two independent experiments. (C) Absolute numbers of SI ILC3 were determined in *Gata3*^{+/+} and *Gata3*^{-/-} mixed chimeras. Bars correspond to the mean \pm SEM of the values obtained with five *Gata3*^{+/+} and four *Gata3*^{-/-} mixed chimeras (two independent experiments). *, $P < 0.05$. (D) Ratio of absolute numbers of CD45.2⁺ (*Gata3*^{+/+} or *Gata3*^{-/-}) to CD45.1⁺ (B6) ILC3 in SI. Each bar corresponds to the mean \pm SEM of the values obtained with five *Gata3*^{+/+} and four *Gata3*^{-/-} mixed chimeras (two independent experiments). ***, $P < 0.001$. (E) Representative FACS analysis of ILC3 subsets in SI of *Gata3*^{+/+} and *Gata3*^{-/-} mixed chimeras. ILC3 subsets were subsequently analyzed for expression of CD45 allotypes. (F) Absolute numbers of the indicated SI ILC3 subsets were determined in *Gata3*^{+/+} and *Gata3*^{-/-} mixed chimeras. Bars correspond to mean \pm SEM of the values obtained with five *Gata3*^{+/+} and four *Gata3*^{-/-} mixed chimeras (two independent experiments). *, $P < 0.05$. (G) Ratio of the absolute numbers of CD45.2⁺ (*Gata3*^{+/+} or *Gata3*^{-/-}) and CD45.1⁺ (B6) SI ILC3 subsets in mixed chimeras. Bars correspond to the mean \pm SEM of the values obtained with five *Gata3*^{+/+} and four *Gata3*^{-/-} mixed chimeras (two independent experiments). ***, $P < 0.001$.

tract, and genetic deficiencies in ILC3 development or antibody-mediated depletion of ILC render mice susceptible to *C. rodentium* dissemination and lethality (Satoh-Takayama et al., 2008; Cella et al., 2009; Qiu et al., 2012; Sonnenberg et al., 2011b). As *C. rodentium* induces IL-22-producing CD4⁺ T cells starting from day 8 after infection (Basu et al., 2012), we focused our attention on the early phase of *C. rodentium* infection to assess the innate immune response in *Gata3*^{+/+} and *Gata3*^{-/-} chimeric mice. This early protection against *C. rodentium* is present

in mice lacking adaptive immunity (*Rag2*^{-/-} mice) but not in mice lacking innate and adaptive lymphocytes (*Rag2*^{-/-}*Il2rg*^{-/-} mice; Zheng et al., 2008; Satoh-Takayama et al., 2008).

Mice were infected with bioluminescent *C. rodentium* to noninvasively monitor bacterial growth and dissemination (Wiles et al., 2006). Infected *Gata3*^{-/-} chimeric mice rapidly lost body weight, developed pathological symptoms (diarrhea and rectal bleeding) as indicated by the global clinical score and succumbed to infection (Fig. 3, A–C). Whole-body

in vivo imaging revealed that *Gata3*^{-/-} mice had higher *C. rodentium* loads, notably in LI, whereas bacterial growth was controlled in *Gata3*^{+/+} chimeras (Fig. 3, D and E). Intestinal IL-22 expression regulates antimicrobial peptides expression by epithelial cells that maintain barrier function (reviewed in (Sonnenberg et al., 2011a). IL-22 expression was clearly reduced in lamina propria cells of infected *Gata3*^{-/-} chimeras compared with their *Gata3*^{+/+} counterparts, and as a result, intestinal epithelial cell expression of the antimicrobial peptide RegIIIγ was reduced in absence of *Gata3* (Fig. 3 F). The enhanced

susceptibility of *Gata3*^{-/-} chimeras to *C. rodentium* infection was associated with the loss of intestinal ILC3 (Fig. 3, G and H) and their capacity to produce IL-22 (Fig. 3, I and J). Collectively, these results demonstrate that *Gata3* is required for innate immune responses that protect against *C. rodentium* infection.

GATA-3 required for normal development of RORγt⁺ cells in the fetal liver

To gain insights into the stage at which *Gata3* controls ILC3 development, we analyzed RORγt⁺ fetal liver hematopoietic

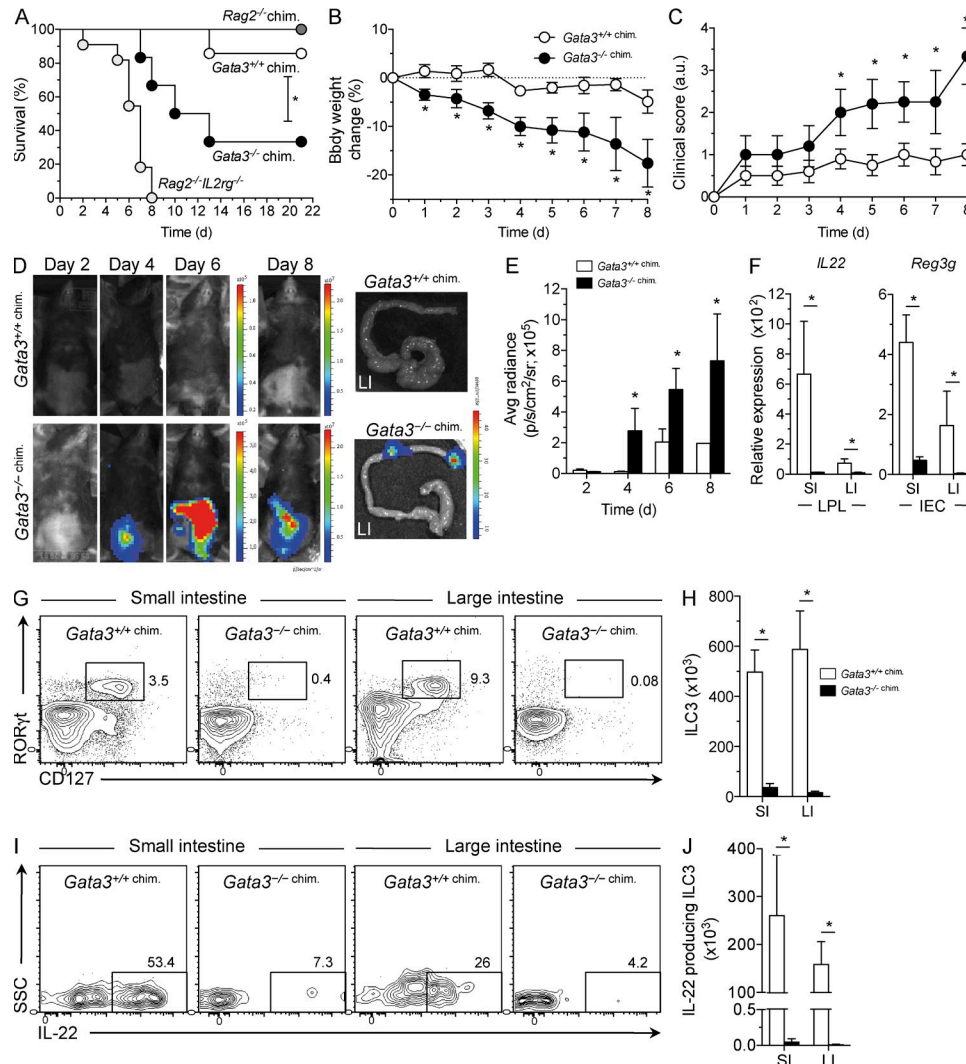
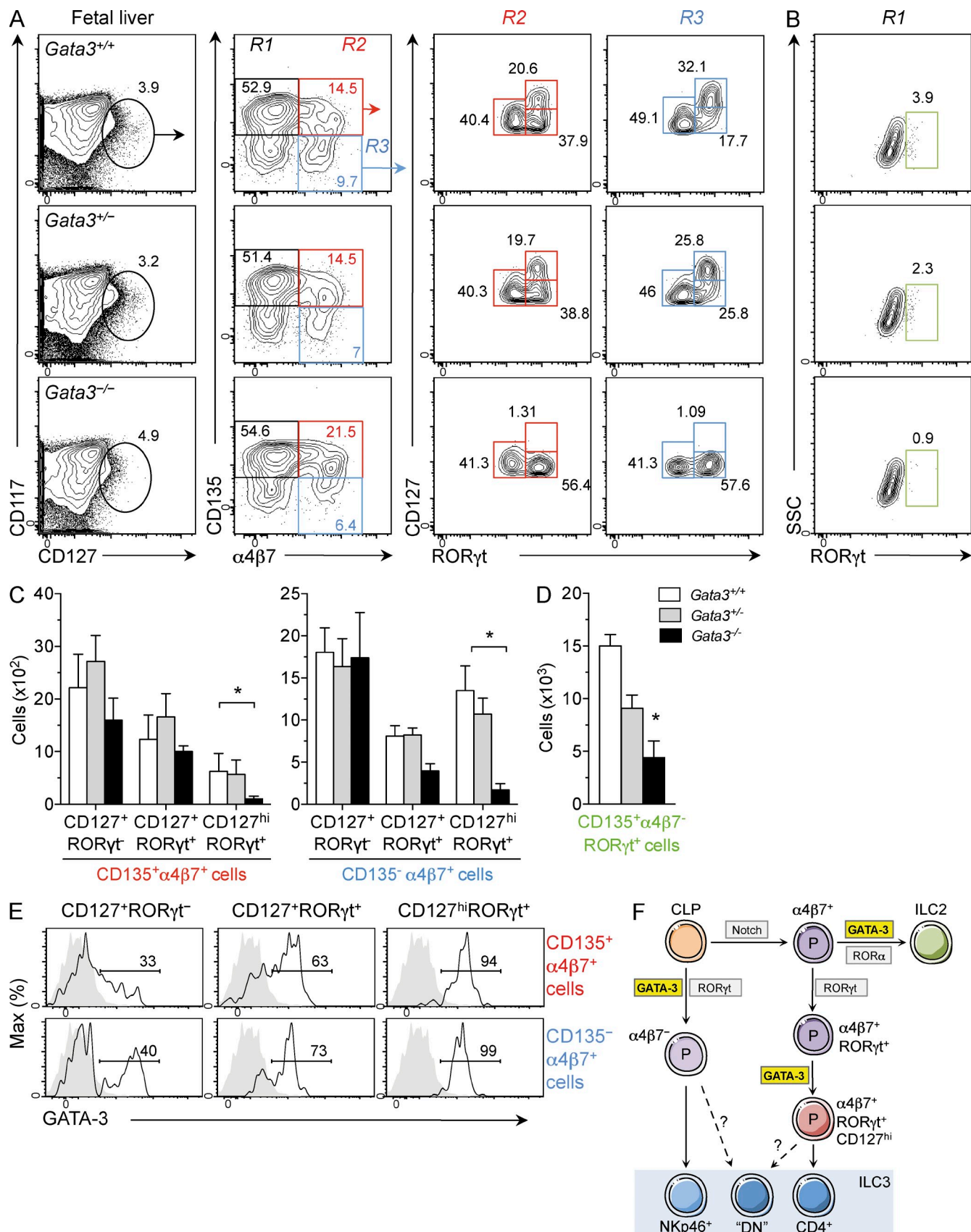


Figure 3. *Gata3* is required for resistance to *C. rodentium* infection. (A–D) *Gata3*^{+/+} (white circle; *n* = 10), *Gata3*^{-/-} (black circle; *n* = 6) chimeras, *Rag2*^{-/-} mice (dark gray circles; *n* = 8) and *Rag2*^{-/-}*IL2rg*^{-/-} mice (light gray circles; *n* = 8) were infected via oral gavage with 10⁹ bioluminescent *C. rodentium* (CR) and survival (A), body weight (B), clinical score (C), and in vivo CR growth dynamics (D) were assessed at the indicated times after infection. Composite results from two independent experiments are shown. In pseudocolor images, red represents the most intense light emission and blue corresponds to the weakest signal (scale bar, photon/s/cm²/sr). Representative noninvasive CR imaging at 6 d after infection in *Gata3*^{+/+} and *Gata3*^{-/-} chimera using Xenogen technology. (E) Relative growth of CR was determined in *Gata3*^{+/+} and *Gata3*^{-/-} chimeras (*n* = 6 for each time point). *, *P* < 0.05. (F) *IL22* and *Reg3g* expression was assessed by qRT-PCR in lamina propria cells (LPL) and intestinal epithelial cells (IEC), respectively, isolated from *Gata3*^{+/+} and *Gata3*^{-/-} chimeras 6 d after CR infection (*n* = 4 for each genotype from two independent experiments). Results are represented relative to *Gapdh* expression (mean ± SEM). *, *P* < 0.05. (G–J) RORγt⁺ ILC3 (G and H) and IL-22-producing ILC3 (I and J) were determined in SI and LI from *Gata3*^{+/+} and *Gata3*^{-/-} chimeras 6 d after CR infection. Relative percentage of gated populations is indicated. Bar graphs show quantification (mean ± SEM) of each population (H and J). *, *P* < 0.05. Representative results from two independent experiments (*n* = 4 for each genotype).



cells from *Gata3*^{+/+}, *Gata3*^{+/-}, and the rare *Gata3*^{-/-} embryos (ED12.5) that could be obtained after pharmacological rescue in utero (Kaufman et al., 2003). Previous studies have shown that CD135⁺ common lymphoid progenitors (CLPs) shed their B then T cell potential concomitant with acquisition of the integrin $\alpha 4\beta 7$ (Yoshida et al., 2001; Walker et al., 2013). Within the subset of fetal liver Lin⁻ $\alpha 4\beta 7$ ⁺ cells, a subset expressing *Rorc* transcripts and ROR γ t protein can give rise to CD4⁺ ILC3 cells in vitro (Sawa et al., 2010; Possot et al., 2011; Cherrier et al., 2012). In contrast, NKp46⁺ ROR γ t⁺ ILC3s derive from distinct $\alpha 4\beta 7$ ⁻ ROR γ t⁺ fetal liver precursors (Sawa et al., 2010). Using CD135 (Flk2/Flt3) and $\alpha 4\beta 7$, we identified these different ROR γ t⁺ subsets within fetal liver Lin⁻CD117⁺CD127⁺ cells (Fig. 4, A and B). $\alpha 4\beta 7$ ⁺ROR γ t⁺ cells included both CD135⁺ and CD135⁻ subsets with a distinct population of cells expressing higher levels of CD127 in *Gata3*-competent hosts. In contrast, this CD127^{hi} subset of $\alpha 4\beta 7$ ⁺ROR γ t⁺ cells was essentially absent from *Gata3*-deficient fetal liver cells, although ROR γ t⁺ cells with lower levels of CD127 were still present (Fig. 4, A and C). A small subset of CD135⁺ $\alpha 4\beta 7$ ⁻ cells expresses ROR γ t, and we found that these cells also require GATA-3 to develop (Fig. 4, B and D). This population corresponds to the previously described NKp46⁺ ILC3 precursor (Sawa et al., 2010), and its GATA-3 dependency could explain the NKp46⁺ ILC3 deficiency in our *Gata3*^{-/-} chimeras (Fig. 1 D and Fig. 2, F–H). Interestingly, GATA-3 protein levels varied within $\alpha 4\beta 7$ ⁺ cells with lowest levels in ROR γ t⁻ cells and progressively higher levels as cells acquire ROR γ t and increase CD127 expression (Fig. 4 E). These results suggest that GATA-3 is not essential for ROR γ t expression in the ILC3 subset that emerges from $\alpha 4\beta 7$ ⁺ fetal liver cells, but is required for normal homeostasis of CD127^{hi} ROR γ t⁺ ILC3; the latter subset has been previously shown to include CD4⁺ LTi cells that populate the intestine and promote lymphoid tissue organogenesis (Mebius et al., 1996; Sawa et al., 2011; Cherrier et al., 2012).

Concluding remarks

In this report, we show that the transcription factor *Gata3* is essential for the differentiation of ILC3 that express ROR γ t and can produce IL-17 and/or IL-22 (Spits and Di Santo, 2011; Spits et al., 2013). *Gata3* is required in a cell-intrinsic fashion for ILC3 development from fetal liver hematopoietic

precursors and is critical to promote innate immune responses in the gut that protects against intestinal pathogens. Conditional *Gata3* ablation in HSC from adult mice results in defects in ILC2 development (Furusawa et al., 2013); whether ILC3 differentiation is affected in this context will require further study. Previous studies suggested that *Gata3* was redundant for the homeostasis of Id2-expressing intestinal ILC3 as the frequency of ROR γ t⁺ ILC3 was unchanged when *Gata3* was deleted in Id2⁺ cells (Hoyler et al., 2012). Our results indicate that the one essential role for *Gata3* in ILC3 development (and by analogy in ILC2 development) may occur during the emergence of putative ILC precursors from CLP. Considering the important role for *Gata3* in the development of both ILC2 and ILC3, a common GATA-3⁺ progenitor shared between group 2 and group 3 ILCs may exist. Alternatively, *Gata3* may have an equivalent role in supporting the development, maintenance, or differentiation potential of distinct ILC2 and ILC3 progenitors. The absence of several ROR γ t⁺ ILC3 subsets in *Gata3*-deficient fetal liver cells demonstrates the critical role for this transcription factor in the earliest stages of ILC development (Fig. 4 F).

Does *Gata3* play a role as a generic promoter of ILC development? Whereas ILC2 (Hoyler et al., 2012; Liang et al., 2012; Klein Wolterink et al., 2013) and now ILC3 (this study) require *Gata3* for their development, previous works demonstrated that *Gata3* was not required for normal homeostasis of BM or splenic NK cells, although *Gata3* conditioned liver and thymic NK cell development (Samson et al., 2003; Vosshenrich et al., 2006). As such, *Gata3*-dependent and -independent pathways of NK cell development are possible, in contrast with ILC2 and ILC3. A human ILC1 subset that strongly produces IFN- γ but lacks classical NK cell markers and is not cytotoxic has recently been described that may represent an innate version of CD4⁺ T_H1 cells (Bernink et al., 2013). Although a mouse equivalent of this non-NK ILC1 has not been identified, it seems that ILC1 subset complexity is already substantial. Concerning the possible *Gata3*-dependent transcriptional targets, genes involved in the development of multiple ILC subsets (IL-7-dependent pathways, Id2) may be involved. The reduction in CD127⁺ ILC3 fetal liver cells that we observed in the absence of *Gata3* is consistent with this hypothesis. Further work will be required to understand how *Gata3* promotes the differentiation of distinct ILC subsets.

Figure 4. Characterization of ROR γ t⁺ cells in *Gata3*-deficient fetal liver cells. (A) Lymphohematopoietic precursors (CD3⁻CD19⁻GR-1⁻CD11c⁻Ter119⁻CD45.2⁺CD117⁺CD127⁺) in E12.5 fetal liver cells of *Gata3*^{+/+}, *Gata3*^{+/-}, and *Gata3*^{-/-} mice were analyzed by flow cytometry. CD117⁺CD127⁺ fetal liver cells were gated for CD135 and $\alpha 4\beta 7$ expression before analysis of CD127 and ROR γ t expression on $\alpha 4\beta 7$ ⁺ cells gated on CD135⁺ (R2; red box) or CD135⁻ (R3; blue box). Representative results from three independent experiments. (B) Expression of ROR γ t (green box) was analyzed in CD135⁺ $\alpha 4\beta 7$ ⁻ cells (R1; black box) in *Gata3*^{+/+}, *Gata3*^{+/-}, and *Gata3*^{-/-} embryos. Representative results from three independent experiments. (C and D) Bar graph shows the quantification for each precursor subset. Each bar corresponds to the mean \pm SEM of the values obtained from *Gata3*^{+/+}, *Gata3*^{+/-}, and *Gata3*^{-/-} embryos ($n = 7, 10$, and 4 , respectively). *, $P < 0.05$. (E) Representative histograms show intracellular expression of GATA-3 in ILC precursors (gray area for CD135⁺ $\alpha 4\beta 7$ ⁻ cells and black line for the others subsets) as indicated in the plots from E12.5 fetal liver. Representative results from three independent experiments. (F) Distinct roles for GATA-3 in ILC3 subset development. CLP generates ILC subsets under the influence of diverse transcription factors including ROR γ t, ROR α , Notch, and GATA-3 as shown.

Because *Gata3* is essential for T cell development (Rothenberg, 2012), it was logical to assume that *Gata3* would activate transcriptional targets involved in T cell specification and commitment. However, despite 20 yr of research, the *Gata3* targets that specifically promote T cell fate remain unknown. With our new results, an alternative role for *Gata3* function can be proposed, one in which *Gata3* is equally essential for both T cell and ILC development. This dual effect of *Gata3* could be achieved through repression of alternative (non-T/non-ILC) potentials in uncommitted lymphoid precursors. Recent results have shown that *Gata3* functions in this way to repress a latent B cell potential in early lymphoid progenitors in the BM and thymus (Banerjee et al., 2013; García-Ojeda et al., 2013). In this way, *Gata3* would globally (and equally) promote ILC and T cell fates from uncommitted precursors in primary lymphoid tissues.

MATERIALS AND METHODS

Generation of chimeric mice. *Rag2*^{-/-} and *Rag2*^{-/-}*Il2rg*^{-/-} mice on the CD45.1⁺ or CD45.2⁺ C57BL/6 background and *Gata3*-lacZ mice (Hendriks et al., 1999) were maintained at the animal facilities of the Institut Pasteur. C57BL/6 mice were obtained from Harlan. Chimeric mice were generated as previously described (Samson et al., 2003; Vossheerich et al., 2006; García-Ojeda et al., 2013) using 5 Gy irradiated recipients and E12.5–13.5 fetal liver cells from timed pregnant dams treated with L-phenylephrine (100 µg/ml), isoproterenol (100 µg/ml), and ascorbic acid (2 mg/ml; Sigma-Aldrich; Kaufman et al., 2003). This rescue technique did allow for recovery of viable E12.5–13.5 *Gata3*^{-/-} embryos but not at expected Mendelian ratios (only 8% were viable in contrast to the expected 25%). All mice were housed under specific pathogen-free condition at the Institut Pasteur, provided with food and water ad libitum, and analyzed 10–15 wk after transplantation. Experimental animal protocols were performed in accordance with guidelines of the Animal Care Use Committee at the Institut Pasteur and were approved by the French Research Ministry (project #2013-0033).

Cell isolation and FACS analysis. Fetal liver cells and lamina propria lymphocytes from small intestine, LI, and Peyer's patches were isolated and analyzed by FACS as previously described (Klein Wolterink et al., 2013; Satoh-Takayama et al., 2008). Antibodies used for analysis of cell surface phenotype, intracellular cytokines, and transcription factors are described in Table S1. Intracellular staining for cytokines and transcription factors was performed as previously described (Satoh-Takayama et al., 2009). Samples were acquired using an LSRFortessa (BD) and analyzed using FlowJo software (Tree Star).

***C. rodentium* infection and whole body imaging.** Log phase cultures of bioluminescent *C. rodentium* (Wiles et al., 2006) were washed in PBS and mice were orally gavaged with 10⁹ CFU as previously described. Mice were weighed daily and monitored for diarrhea and bloody stool. A clinical score was calculated as follows: normal stool and no blood (0), soft stool and no blood (+1), very soft stool and blood traces (+2), diarrhea and rectal bleeding (+3), normal weight (0), weight loss between 1 and 5% (+1), weight loss between 5 and 10% (+2), and weight loss between 10 and 20% (+3). For in vivo tracking of *C. rodentium*, mice were anesthetized with ketamine (Imalgene 1000) and xylazine (Rompun). Before BLI, the abdominal region was shaved and mice were placed in a confinement box (TEM SEGAs) and imaged using an IVIS 100 system (Caliper Life Sciences). Image acquisition and analysis were performed with the Living Image 3.2 software (Caliper Life Sciences). To quantify the amount of light emitted by bioluminescent bacteria in a specific organ, the region corresponding to this organ (ROI [region of interest]) was defined in the software and the average radiance of the defined region was calculated. For functional studies, infected mice were sacrificed and analyzed on day 5 after infection. Bacterial dissemination was confirmed by culture of livers and spleen suspensions from infected mice on kanamycin-containing agar plates.

RNA isolation and quantitative PCR. RNA was isolated from purified intestinal cell preparations with the RNeasy Mini kit (QIAGEN) and converted to cDNA with Transcriptor First strand cDNA synthesis kit (Roche). For quantitative PCR, reactions were performed using SYBR Green Jumpstart Taq ready mix (Sigma-Aldrich). Primers (EasyOligo; Sigma-Aldrich) used are listed in Table S2. Samples were normalized to *Gapdh* expression.

Statistical analysis. Experimental results are reported as mean ± SEM. Statistical differences between groups were determined using the unpaired Student's *t* test. *P* values <0.05 were considered significant. Significance for survival rates of Kaplan-Meier curves was calculated using the log-rank test. All data were analyzed with Prism v6 software (GraphPad).

Online supplemental material. Fig. S1 shows the gating strategy for FACS analysis of ILC. Table S1 shows antibodies used for analysis of cell surface phenotype, intracellular cytokines, and transcription factors. Table S2 lists primers used for quantitative PCR. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20131038/DC1>.

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SUPPLEMENTAL MATERIAL

Serafini et al., <http://www.jem.org/cgi/content/full/jem.20131038/DC1>

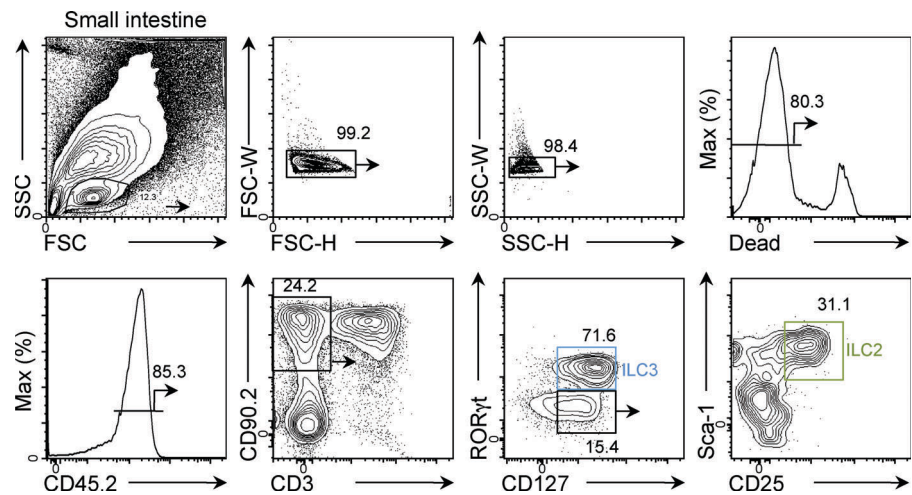


Figure S1. Representative FACS gating scheme for ILC2 and ILC3 analysis in the SI.

Table S1. Antibodies used for FACS analysis

Antibody	Clone	Manufacturer
CD3	145-211	BD
NK1.1	PK136	eBioscience
GATA-3	TWAJ	eBioscience
RORγt	AFKJS-9	eBioscience
CD127	A7R34	eBioscience
NKp46	29A1-4	eBioscience
CD4	RM4-5	eBioscience
CD19	ebio1D3	eBioscience
Sca-1	D7	eBioscience
CD25	PC61.5	eBioscience
CD45.1	A20	eBioscience
CD45.2	104	eBioscience
CD135	A2F10.1	eBioscience
α4β7	DATK32	eBioscience
CD117	2B8	eBioscience
CD11c	HL3	eBioscience
Te-r119	TER119	eBioscience
Gr-1	RB6 8C5	eBioscience
CD45.2	104	eBioscience
CD90.2	53-2.1	eBioscience

Table S2. Primers used for quantitative PCR

Gene name	Forward primers	Reverse primers
<i>Gapdh</i>	5'-AGGTCGGTCGTGAACGGATTTG-3'	5'-TGTAGACCATGTAGTTGAGGTCA-3'
<i>IL22</i>	5'-TTGAGGTCGTCCAACCTCCAGCA-3'	5'-AGCCGGACGTCGTGTTGTTA-3'
<i>Reg3g</i>	5'-CCGTGCCTATGGCTCCTATTG-3'	5'-GCACAGACACAAGATGTCCTG-3'