Group 2 innate lymphoid cells (ILC2s; also called nuocytes, innate helper cells, or natural helper cells) provide protective immunity during helminth infection and play an important role in influenza-induced and allergic airway hyperreactivity. Whereas the transcription factor GATA binding protein 3 (Gata3) is important for the production of IL-5 and -13 by ILC2s in response to IL-33 or -25 stimulation, it is not known whether Gata3 is required for ILC2 development from hematopoietic stem cells. Here, we show that chimeric mice generated with hematopoietic stem cells fail to develop systemically dispersed ILC2s. In these chimeric mice, in vivo administration of IL-33 or -25 fails to expand ILC2 numbers or to induce characteristic ILC2-dependent inflammatory responses. Moreover, cell-intrinsic Gata3 expression is required for ILC2 development in vitro and in vivo. Using mutant and transgenic mice in which Gata3 gene copy number is altered, we show that ILC2 generation from common lymphoid progenitors, as well as ILC2 homeostasis and cytokine production, is regulated by Gata3 expression levels in a dose-dependent fashion. Collectively, these results identify Gata3 as a critical early regulator of ILC2 development, thereby extending the paradigm of Gata3-dependent control of type 2 immunity to include both innate and adaptive lymphocytes.

Essential, dose-dependent role for the transcription factor Gata3 in the development of IL-5+ and IL-13+ type 2 innate lymphoid cells

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Group 2 innate lymphoid cells (ILC2s; also known as natural helper cells, nuocytes, innate helper cells, or natural helper cells) provide protective immunity during helminth infection and play an important role in influenza-induced and allergic airway hyperreactivity. Whereas the transcription factor GATA binding protein 3 (Gata3) is important for the production of IL-5 and -13 by ILC2s in response to IL-33 or -25 stimulation, it is not known whether Gata3 is required for ILC2 development from hematopoietic stem cells. Here, we show that chimeric mice generated with hematopoietic stem cells fail to develop systemically dispersed ILC2s. In these chimeric mice, in vivo administration of IL-33 or -25 fails to expand ILC2 numbers or to induce characteristic ILC2-dependent inflammatory responses. Moreover, cell-intrinsic Gata3 expression is required for ILC2 development in vitro and in vivo. Using mutant and transgenic mice in which Gata3 gene copy number is altered, we show that ILC2 generation from common lymphoid progenitors, as well as ILC2 homeostasis and cytokine production, is regulated by Gata3 expression levels in a dose-dependent fashion. Collectively, these results identify Gata3 as a critical early regulator of ILC2 development, thereby extending the paradigm of Gata3-dependent control of type 2 immunity to include both innate and adaptive lymphocytes.

asthma | innate immunity

ILCs Express High Levels of Gata3 Protein and Require Gata3 Expression for Development in Vivo. We first analyzed Gata3 protein expression in ILC2s present in different tissue sites (Fig. 1A). ILC2s were identified as lineage-negative cells that coexpress IL-5 and -13. These IL-25– and IL-33–responsive innate cells have a broad distribution (1, 2), and were shown to be essential for the innate response to the helminth parasite Nippostrongylus brasiliensis (2–4). ILC2s can be distinguished by coexpression of CD25, CD127, IL-33R, and IL-25R. ILC2s in the respiratory tract play a crucial role in virus-induced airway hyperreactivity and in maintenance of epithelial barrier integrity during influenza infection (5, 6). Damaged epithelial cells or infected myeloid cells release inflammatory cytokines, including IL-25 and -33, that can potently expand and activate ILC2s in mucosal tissues (7–10). Human ILC2s have been found in the lung and are associated with chronic rhinosinusitis (5, 11). In addition, we and others have shown that ILC2s are major IL-5 and -13 producers in various murine allergic asthma models (7–10, 12). ILC2s belong to an emerging family of innate lymphoid cells (ILCs) that includes ILC1 [natural killer (NK) cells and other innate IFN-γ–producing cells] as well as a distinct group 3 ILC (ILC3) that includes subsets expressing the orphan transcription factor retinoid-related orphan receptor γ (ROR-γ) and producing IL-17 or -22 or having lymphoid tissue inducer function (reviewed by refs. 13, 14). The transcriptional regulator inhibitor of differentiation 2 (Id2) has been identified as a common factor required for all ILC subsets (13, 14); however, the developmental stages from common lymphoid progenitors (CLPs) to fully mature ILC subsets have not been elucidated. Transient Notch signals appear critical for generation of ILC2s and ILC3s from CLPs (15–19). Nevertheless, the regulatory factors that orchestrate this process are poorly understood, although recently the transcription factor ROR-α was identified as an essential factor for efficient ILC2 development and in particular for IL-25–mediated expansion of the ILC2 compartment (10, 17).

Like Th2 cells, ILC2s promptly produce IL-5, -13, and, to a lesser extent, IL-4 (1–4). Regulation of Il4, Il5, and Il13 expression is complex and in Th2 cells requires the zinc-finger transcription factor GATA binding protein 3 (Gata3), which is selectively up-regulated during Th2 differentiation (refs. 20 and 21 and reviewed in ref. 22). Conditional deletion of the Gata3 gene in established Th2 cells demonstrated that Gata3 is critical for IL-5 and -13, but not IL-4, production by Th2 cells (23). Likewise, deletion of the Gata3 gene in ILC2s abolished their IL-5 and -13 production (24). In addition to its role in regulation of Th2 cytokine expression in Th2 cells and ILC2s, Gata3 is required at multiple stages of T-cell development and has an essential role in the specification of the earliest T-cell progenitors in the thymus (25–27). In this work, hematopoietic chimeras, as well as mutant and transgenic mice in which Gata3 gene copy number is altered, are used to address the essential, dose-dependent role for Gata3 in ILC2 differentiation from lympho-hematopoietic precursors.
(see below) and had similar Gata3 levels as lung ILC2s (Fig. 1A). Gata3+ ILC2s were also identified in spleen, lymph nodes (LNs), and intestine (see below).

To assess the requirement for Gata3 in the development of ILC2s in vivo, we analyzed chimeric mice generated by using Gata3-deficient hematopoietic precursors cells as described (28, 29). To control for the resultant T-cell deficiency in these chimeras (30), we generated T-cell–deficient, Gata3-proficient chimeras using Cd3e−/− hematopoietic precursors. Both Gata3+/+ and Gata3−/− hematopoietic precursors engrafted to a similar extent, leading to robust B, NK, and myeloid cell reconstitution (Fig. S1A and ref. 28).

Because Rag2−/−Il2rg−/− hosts lack ILC2s (3), all ILC2s in the chimeric mice were donor-derived, as expected (Fig. S1B). Both BM and lung ILC2s developed in Gata3+/+ chimeras (Fig. 1B and C), and these ILC2s normally expressed CD127, CD25, and IL-33R, as expected (5, 31). In Gata3−/− chimeras, we found a similar number of BM Lin−CD127+ cells, although these cells

Fig. 1. GATA-3 is expressed by ILC2s and is required for ILC2 development in vivo. (A) Gating strategy for analysis of BM and lung ILC2s. Histograms show intracellular expression of GATA-3 of ILC2s (bold line) compared with CD3+ CD4+ T cells (black line) and CD19+ B lymphocytes (gray area). Numbers indicate relative frequencies of gated populations. Shown are representative results from three independent experiments (n ≥ 6 total mice analyzed). (B and C) Analysis of lymphocytes from BM (B) and lung (C) of Rag2−/−Il2rg−/− hosts transplanted with Gata3+/+ and Gata3−/− precursors for expression of the indicated surface markers. Plots show gating strategy for ILC2s. Bar graphs show quantification of the indicated donor-derived populations in chimeras. (D) Analysis of pulmonary Lin− lymphocytes for CD127 and intracellular IL-5 expression. Plots show gating strategy for IL-5+ lymphocytes and ILC2s. Bar graphs show quantification of the indicated populations in Gata3+/+ and Gata3−/− chimeras. (B–D) Shown are representative results from two independent experiments with n = 3 (Gata3+/+) or 5 (Gata3−/−) total mice analyzed per genotype. ns, not significant. *P < 0.05; ***P < 0.001.
failed to express characteristic surface markers of ILC2s (Fig. 1B). In the lung of Gata3−/− chimeras, Lin−CD127+ cells were severely reduced, and ILC2s were essentially absent (Fig. 1C).

Analysis of IL-5 production by Lin− cells in the lung and BM of Gata3+/+ chimeras showed that all cells that produced IL-5 were ILC2s (Fig. 1D). In contrast, IL-5 production was undetectable in lung and BM cell preparations from Gata3−/− chimeras (Fig. 1D), consistent with the absence of ILC2 in these tissues (Fig. 1B and C).

GATA-3 Is Required for Characteristic Innate Responses to Exogenous IL-33 or -25. Intranasal (i.n.) administration of IL-33 in vivo results in a dramatic increase in the absolute numbers of ILC2s in lung and broncho-alveolar lavage (BAL) fluid (2, 7–10, 32). i.n. IL-33 (Fig. 2A) triggered recruitment of Siglec-F+ eosinophils and Gr-1+ neutrophils in the BAL fluid in Gata3+/+, but not in Gata3−/−, chimeras (Fig. 2B). This inflammatory reaction was accompanied by a dramatic increase in ILC2 numbers within the lung tissue and BAL fluid of Gata3+/+, but not Gata3−/−, chimeras (Fig. 2C and D). Gata3+/+ ILC2s in the lung promptly produced IL-5 and -13 following stimulation, whereas cytokine production was not detected in Lin− cells from Gata3−/− chimeras (Fig. S2). We conclude that lung-resident IL-33-responsive ILC2s require Gata3 for their generation and/or function.

Because the expression of the IL-33R component T1/ST2 is regulated by GATA-3 (21), the failure of Gata3−/− chimeras to respond to IL-33 in vivo may in part be explained by the lack of receptor expression by Gata3−/− ILC2s (Figs. 1 and 2). Systemically administered IL-25 can trigger an inflammatory response from ILC2s that provokes multisystem myeloid cell recruitment and mucus production (33). IL-25 injection resulted in the expansion of ILC2s in the BM and lung of Gata3+/+, but not

Fig. 2. GATA-3 is required for in vivo responses mediated by i.n. IL-33 and systemic IL-25 challenge. (A) Protocol of i.n. administration of recombinant IL-33 on the 3 d indicated with arrows. Mice were analyzed (A) on day 6. (B) Flow cytometric analysis and quantification of BAL fluid granulocytes for expression of indicated markers in IL-33-challenged Rag2−/−Il2rg−/− hosts transplanted with Gata3+/+ and Gata3−/− precursors. (C and D) Flow cytometric identification (contour plots) and quantification (bar graphs) of ILC2s in donor-derived lymphocytes in lung (C) and BAL fluid (D) of challenged chimeras. (E) Gata3+/+ and Gata3−/− chimeric mice were challenged with recombinant IL-25 protein as outlined in the protocol. Bar graphs show quantification of donor-derived ILC2s of the indicated tissues 1 d after the last challenge. Shown are representative results from two independent experiments with n ≥ 6 total mice analyzed per genotype. *P < 0.05; ***P < 0.001.
Gata3<sup>−/−</sup>, chimeras (Fig. 2E and Fig. S3A). Although we found that ILC2s were the major source of IL-5 produced by lung Lin<sup>−</sup> lymphocytes, IL-5 was not detected in these cells in the absence of Gata3 (Fig. S3B). Collectively, these results demonstrate that characteristic inflammatory responses following triggering of ILC2s by IL-25 and -33 are not elicited in chimeric mice made with Gata3<sup>−/−</sup> hematopoietic precursors.

**Failure of ILC2 Differentiation from Gata3<sup>−/−</sup> Hematopoietic Precursors in Vitro.** Notch-triggered differentiation of ILC2s from CLPs can be achieved in vitro by using stromal cells expressing delta-like ligand 4 in the presence of IL-7 and IL-33 (19). We found that cytokine production in BM ILC2s (Fig. S4A). RT-PCR analysis of these FL cultures showed that in the absence of Gata3, Id2 and Rom expression were not induced (Fig. S5); these transcription factors are highly expressed in ILC2s and are critical for their development (3, 17, 34). Moreover, we could not detect IL-5 or -13 production in these ILC2 Gata3<sup>−/−</sup> cultures, whereas ILC2 cultures of Gata3<sup>−/+</sup> fetal liver hematopoietic precursors gave rise to cells with characteristic IL-5 and -13 production (Fig. S4B).

**Cell-Intrinsic Gata3 Is Required for ILC2 Development.** To exclude the possibility that cell-extrinsic effects caused by Gata3 deficiency underlie the observed defects in ILC2 development, we generated and analyzed BM chimeras generated by using 9:1 mixtures of CD45.2<sup>−</sup> Gata3<sup>−/−</sup> and CD45.1<sup>−</sup> wild-type (WT) BM to generate hematopoietic precursors. Mice injected with 9:1 mixtures of CD45.2<sup>−</sup> Gata3<sup>−/−</sup> and CD45.1<sup>−</sup> Gata3<sup>−/−</sup> precursors served as controls (Fig. 3A). Both sets of mixed chimeras reconstituted to a similar extent (Fig. S6A). We confirmed that B and NK cell development occurred normally from both Gata3<sup>−/+</sup> and Gata3<sup>−/−</sup> precursors (Fig. 3B and Fig. S6B), and phenotypically mature ILC2s were generated in BM, lung, spleen, and small intestine (Fig. 3C and Fig. S6C). Further analysis revealed that all ILC2s in Gata3<sup>−/−</sup> mixed BM chimeras expressed CD45.1<sup>+</sup> (originating from Gata3<sup>−/−</sup> precursors), whereas ILC2s in Gata3<sup>−/+</sup> mixed chimeras originated from both CD45.2<sup>−</sup> Gata3<sup>−/−</sup> and CD45.1<sup>−</sup> Gata3<sup>−/+</sup> precursors (Fig. 3C and Fig. S6D). This result demonstrates that Gata3 is required in a cell-intrinsic fashion for ILC2 development. Interestingly, although analysis of the origin of B and NK cells recapitulated the ratio in which precursors were injected (i.e., 86–87% of the B and NK cells were CD45.2<sup>−</sup> independent of Gata3 genotype), ILC2s derived from Gata3<sup>−/−</sup> precursors were underrepresented relative to those derived from their Gata3<sup>−/+</sup> counterparts in control chimeras (Fig. 3C). This result suggests that the Gata3 gene dosage may influence overall ILC2 development.

**GATA-3 Gene Copy Positively Correlates with ILC2 Development, Homeostasis, and Function.** We next compared the effects of modifying GATA3 gene copy number on ILC2 development and function. We characterized ILC2 homeostasis and function in three mouse strains: C57BL/6 mice with two functional Gata3 alleles, Gata3<sup>−/+</sup> mice with one functional Gata3 allele (the second allele is disrupted by insertion of a beta-galactosidase reporter (35), and transgenic C57BL/6 mice that harbor a Gata3 transgene under the control of the human CD2 promoter (36). We found that Gata3 protein levels in BM and lung ILC2s were reduced in Gata3<sup>−/+</sup> mice compared with C57BL/6 mice, indicating that both Gata3 alleles are apparently active in ILC2s (Fig. S7A). In contrast, ILC2s from CD2–Gata3 Tg mice expressed the same high levels of Gata3 protein as WT C57BL/6 mice (Fig. S7A). Because we detected increased Gata3 RNA expression in BM ILC2s (Table S1), it is possible that the intracellular FACS assay is not sensitive enough to detect differences in Gata3<sup>−/+</sup> WT and CD2–Gata3 Tg ILC2s.

We next assessed the impact of alterations in Gata3 gene copy number on overall ILC2 homeostasis. Absolute numbers of mature ILC2s in the lung and BM were clearly reduced in Gata3<sup>−/+</sup> mice, whereas CD2–Gata3 Tg mice had an approximately three-fold increase in BM and lung ILC2s compared with Gata3<sup>−/+</sup> C57BL/6 mice (Fig. 4A). ILC2 function also positively correlated with Gata3<sup>−/+</sup> gene copy number: ILC2s from CD2–Gata3 Tg mice produced somewhat higher levels of IL-5 and -13 following stimulation compared with ILC2s from B6 WT mice, whereas cytokine production was reduced in ILC2s from Gata3<sup>−/+</sup> mice having only one functional Gata3 allele (Fig. 4A and Fig. S7B). Moreover, CD2–Gata3 Tg ILC2s showed higher surface expression of IL-25R and IL-33R (Fig. S7C).

These results suggest that Gata3 expression levels can modify ILC2 generation from hematopoietic precursors and thereby influence ILC2 homeostasis. Whereas absolute numbers of CLPs were normal in CD2–Gata3 Tg mice, we found that Gata3 levels in CLPs were modest but significantly increased in CLPs in CD2–Gata3 Tg mice compared with nontransgenic controls (Fig. 4B). This finding would be consistent with hCD2 Tg control elements being active in CLPs, a hypothesis that we confirmed using CD2–GFP mice (37) (Fig. 4C). To assess the influence of increased Gata3 levels in determining ILC2 potential from CLPs, we analyzed expression of ILC2-related genes (Rora, Id2, Il17rb, and Il17f) in CLPs from WT and CD2–Gata3 Tg mice. Whereas Gata3 expression was threefold increased, the levels of other ILC2-related genes were unaffected (Table S1). We next cultured single CLPs from Gata3<sup>−/+</sup>, Gata3<sup>−/−</sup>, or CD2–Gata3 Tg mice on stromal cells in the presence of IL-7 and -33. ILC2s developed from Gata3<sup>−/+</sup> CLPs at the expected frequency (f = 1/5) (19), whereas CD2–Gata3 Tg CLPs were almost twice as efficient in generating ILC2s (f = 1/2.6). In contrast, Gata3<sup>−/+</sup> CLPs showed a 2.6-fold reduction in ILC2 potential (f = 1/13) compared with their WT counterparts (Table 1, Fig. 4D, and Fig. S8). Collectively, these results suggest a model in which Gata3 levels in CLPs control initial commitment to the ILC2 lineage and confirm the important role for sustained Gata3 expression in maintenance of type 2 cytokine secretion from mature ILC2s (24).

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**Fig. 3.** Cell-intrinsic GATA-3 is required for ILC2 development. (A) Experimental protocol. Analysis was performed 5 wk after transplantation. (B) Analysis of B and NK cell development in chimeric mice. (C) ILC2 analysis in mixed chimeras. Numbers indicate relative frequencies of gated populations. Klein Wolterink et al. PNAS | June 18, 2013 | vol. 110 | no. 25 | 10243
Little is known about the mechanisms by which Gata3 promotes T or ILC2 specification in lymphocyte precursors. In contrast, Gata3 acts to direct cytokine production in differentiating Th2 cells (and by analogy in ILC2s) by binding directly to Il4, Il5, and Il13 promoters and the Th2 locus control region (38, 39) and through regulation of Il-33R expression (23, 24). Gata3 regulates fng expression through addition of H3K27me3 suppressive marks (40). Gata3 may promote early stages of T and ILC2 development through simultaneous gene activation and gene repression in a fashion similar to Gata1 (reviewed in ref. 41). Gata3-mediated gene regulation works in a context-dependent fashion that is strongly influenced by transcription factor coexpression. Specific targets of Gata3 in developing lymphocyte precursors are poorly defined. A recent analysis of Gata3-binding sites in early thymocyte progenitors (27) identified ROR-α as a Gata3 target potentially involved in T-cell specification. Because ROR-α is critical for ILC2 homeostasis (17, 34), it is possible that Gata3-mediated activation of ROR-α provides a mechanism to promote early ILC2 development. Indeed, analysis of FL cultures showed that the absence of Gata3, Rora expression was not induced (Fig. S5).

Among transcriptional activators, transient Notch signaling appears required for ILC2 and ILC3 development from lymphoid progenitors (16–18). The role for Notch in this process is not understood but could involve transient up-regulation of Gata3 or Rora that are essential for ILC2 and ILC3 generation, respectively. Sustained Notch signaling, however, seems detrimental to ILC2 and ILC3 development, because neither of these ILC subsets is generated to any appreciable degree within the thymus. One possible model is that common or dedicated ILC precursors are generated in adult mice in that following transient Notch triggering of CLPs; this process may be important for Gata3 up-regulation in CLPs that generate precursors with ILC2 potential. In CD2–Gata3 transgenic mice, these precursors might accumulate as the initiating event in ILC2 specification (Gata3 up-regulation) that is enforced in the BM. In contrast, in vivo overexpression of Gata3 in the presence of Notch signaling, in fetal thymocytes at the DN1 or DN2 stage, induced their redirection into the mast cell lineage in vivo (42). Although evidence for direct action of Notch at the Gata3 locus in lymphoid precursors is lacking, this mechanism has been amply demonstrated in Th2 cell differentiation (43, 44).

Recent studies reported on the role of Gata3 in mouse and human ILC2s (45, 46). Mjosberg et al. (45) showed that Gata3 is crucial for function of human ILC2s and that thymic stromal cell-derived lymphopoietin through activation of STAT5 can induce GATA-3 expression leading to cytokine production. Hoyler et al. (46) used an inducible Gata3 ablation strategy in mice to show that intestinal ILC2 development and homeostasis require constitutive Gata3 expression. Our results provide independent evidence that Gata3 is essential for development of intestinal as well as pulmonary ILC2s (5–9). Moreover, because our analysis involved generation of hematopoietic chimeras in hosts devoid of endogenous ILC2s (2, 3), we can conclude that Gata3 is strictly required for ILC2 development as we exclude the possibility that absence of Gata3-deficient ILC2s results from growth disadvantage compared with Gata3-proficient cells. Importantly, we demonstrate that Gata3 acts in a cell-intrinsic and dose-dependent fashion in vivo and in vitro to induce ILC2 development from isolated CLPs; Gata3 gene copy number directly correlated with subsequent ILC2 numbers and cytokine production capacity. This finding suggests that Gata3 expression levels in CLPs are decisive for induction of ILC2 fate.

Together, our results extend the understanding of the roles of GATA-3 in ILC2 development and function. We demonstrate a critical cell-intrinsic and dose-dependent role for Gata3 in early ILC2 development that mirrors the role played by this transcription

### Table 1. ILC2 fate decision from CLPs positively correlates with Gata3 copy number

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Frequency, 1/x</th>
<th>95% CI</th>
<th>χ² (P vs. WT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gata3lacZ/+</td>
<td>12.99</td>
<td>7.92–21.31</td>
<td>13.8 (&lt;0.001)</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>4.95</td>
<td>3.82–6.43</td>
<td>n/a</td>
</tr>
<tr>
<td>CD2–Gata3 Tg</td>
<td>2.63</td>
<td>2.05–3.38</td>
<td>12.4 (&lt;0.001)</td>
</tr>
</tbody>
</table>

ICL2 containing cultures 1 CLP → OP9Δβ + IL-7 + IL-33 (14–18 d). Single CLPs were cultured as described in Fig. 4 and analyzed for ILC2 development. Frequencies and 95% confidence interval are shown. Data from three pooled, independent experiments are shown. Frequencies were calculated by using an Extreme Limiting Dilution Assay. Statistical significance of differences between groups was determined by using a χ² test. χ² test for differences between any of the groups: χ² = 42.4 (P = 6.09 × 10⁻⁵), n/a, not applicable.
factor in early T-cell development in the thymus (25, 26). These observations provide another example of the remarkable similarities between the molecular mechanisms that regulate the differentiation of innate and adaptive lymphocytes (13).

Materials and Methods

Mice and Generation of Chimeric Mice. Rag2–/–Ig2r double-deficient on the C57BL/6 background, Cd3e-deficient, Gata3–/–, Cd2–/–Gata3, and Cd2–/–GFP Tg mice were maintained at the animal facilities of the Institut Pasteur, Erasmus University Medical Center, and the Faculdade de Medicina de Lisboa. C57BL/6 mice and BALB/c Rag2–/–Ig2r mice were obtained from Harlan and Charles River, respectively. Mice were analyzed at 8–16 wk of age. Five-gray-irradiated Rag2–/–Ig2r double-deficient animals as hosts for hematopoietic stem cell grafts and were analyzed 5–10 wk after transplantation. All animals were kept under specific pathogen-free conditions and provided with food and water ad libitum. All experiments were approved and performed in accordance with local regulations (Institutional Animal Care and Use Committee, Institut Pasteur, Paris).


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**Supporting Information**

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**SI Materials and Methods**

**Generation of Chimeric Mice.** Sublethally irradiated (5 Gy), 6- to 12-wk-old recombinase activating gene 2 (Rag2)−/−Il2rg−/− double-deficient mice were used as hosts to generate GATA binding protein 3 (Gata3−/) and Cd3ε-deficient chimeras. Gata3-deficient chimeras were generated by injection of Gata3-deficient fetal liver cells (embryonic day 15.5) obtained from noradrenaline-treated pregnant Gata3−/− dams mated to Gata3−/− males as described (1). The genotype of the embryos was confirmed by PCR. For Cd3ε-deficient chimeras, bone marrow (BM) cells from 4- to 6-wk-old mice were depleted of lineage-positive cells [CD4, CD8α, CD11b, CD11c, CD19, CD45R (B220) Ter-119, Gr-1, CD161 (NK1.1). Fc fragment of IgE, high affinity I, receptor for alpha polypeptide (FcRα), T-cell receptor β (TCR-β), and TCR-γδ] by using magnetic beads (Miltenyi), and 1–2 × 10^6 cells were injected into the immunodeficient hosts. For some experiments, Ly5.1+ Rag2−/−Il2rg mice were used as hosts, and CD45.1/CD45.2 were used as congenic markers. In other experiments, Rag2−/−Il2rg deficient hosts were on the BALB/c background (H2Dbαβ) and transplanted with cells on the C57BL/6 background (H2Ddβa). Mixed chimeras were generated by injection of a mix of Gata3−/− or Gata3−/+ fetal liver precursor cells with C57BL/6 lineage-depleted BM precursors. Levels of chimerism were verified by screening B lymphocytes, NK cells, and myeloid cells in spleen. Chimeric mice were analyzed 5–10 wk after transplantation.

**Antibodies for Extracellular Flow Cytometric Analysis.** Antibodies used for flow cytometry were against CD4 (GK1.5), CD8α (53-6.7), CD11b (M1/70), CD11c (N418), CD19 (1D3), CD25 (PC61.5), CD44 (IM7), CD45 (13/2.3), CD45.1 (A20), CD45.2 (104), CD117 (2B8), CD127 (A7R34), B220 (RA3-6B2), NK1.1 (PK136), FcRiRγt (MAR-1), Gr-1 (RB6-8C5), Ter-119 (TER-119), stem cell antigen-1 (Sca-1) (D7), IL-33R (D18; MD Bioproducts), H2Db (28-14-8), H2Dd (34-1-2S), Siglec-F (E50-2440), and immune costimulator ICOS (C398.4A). Different conjugated antibodies were purchased from eBioscience, BD Biosciences, or BioLegend, unless indicated otherwise. Biotin-conjugated antibodies were detected by using Pacific Blue (Invitrogen), eFluor 780, or PerCP/Cy5.5 (both eBioscience) conjugated streptavidin.

**Intracellular Flow Cytometric Analysis.** For measurement of intracellular cytokines, cells were stimulated for 4 h at 37 °C by using phorbol 12-myristate 13-acetate and ionomycin (Sigma-Aldrich) in the presence of GolgiStop (BD Biosciences). After extracellular staining, cells were fixed by using 2% (wt/vol) paraformaldehyde in PBS (Electron Microscopy Sciences) and stained for intracellular cytokines in PBS containing 5% saponin (Sigma-Aldrich). Antibodies against IL-4 (11B11), IL-5 (TRFK5), and IL-13 (eBio13A) were purchased from BD Biosciences and eBioscience. For Gata3 protein detection, cells were fixed and permeabilized by using the Foxp3 staining kit (eBioscience) according to the manufacturer’s protocol. PE-conjugated, Alexa Fluor 647-conjugated, or eFluor 660-conjugated antibodies against Gata3 were purchased from BD Biosciences or eBioscience.

**Flow Cytometric Analysis and Data Analysis.** After antibody staining, cells were analyzed by using an LSRII (three lasers) or LSRFortessa (four or five lasers) flow cytometer (both BD Biosciences) equipped with FACSDiva software. Sorting was performed by using FACSaria or FACSariaII (both three lasers; BD Biosciences) equipped with FACSDiva software. Data analysis was performed by using FlowJo (Version 8.8.7; Treestar).

**In Vitro Development of Group 2 Innate Lymphoid Cells from Hematopoietic Precursors.** Stroma cells expressing Notch ligand delta-like 4 (OP9Δ4) were grown in OptiMEM medium (Invitrogen) supplemented with 10% FCS (Thermo Scientific), 2-mercaptoethanol (Sigma or Invitrogen), and penicillin/streptomycin (Invitrogen) and seeded onto multiwell plates (BD Biosciences or TPP).

Hematopoietic cell precursors were cultured on OP9Δ4 cells in IL-7 (5 ng/mL; in-house hybridoma production) alone or in combination with IL-33 (20 ng/mL; Biolegend). For common lymphoid progenitor (CLP) cultures, single Lin−CD127+CD117lowSca1lowCD135+ cells were sorted directly into 96-well plates containing OP9Δ4 cells and IL-7/IL-33. Medium and cytokines were refreshed every 3 or 4 d, and cultures were split when necessary. Cultures were screened for growth by using an inverted phase microscope, and positive cultures were analyzed by flow cytometry on the indicated days.

**Gene Expression Analysis.** RNA was purified (RNaseasy Micro Kit; Qiagen) from OP9Δ4 stromal cell cultures at various time points, and cDNA was synthesized by using RevertAid H Minus Reverse Transcriptase and random hexamer primers in the presence of Ribolock RNase inhibitor (all from Thermo Scientific). Quantitative RT-PCR (qRT-PCR) was performed by using an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Life Technologies) using gene-specific primers (Invitrogen) combined with a Universal Probe Library amplicon-specific probe (Roche Diagnostics).

**Statistical Analysis.** Unpaired t tests were used for comparisons between two normally distributed groups. For groups that were not normally distributed, significance of difference between groups was determined by using Mann–Whitney U tests. For comparisons between multiple groups, one-way ANOVA was used, followed by a Dunnett posttest. Differences were considered significant at P < 0.05. All statistical analyses were performed by using Prism 4 and 5 (GraphPad). Significance levels of statistical tests are not significant (ns); *P < 0.05; **P < 0.01; or ***P < 0.001.

Fig. S1. Engraftment of Gata3$^{+/+}$ and Gata3$^{−/−}$ hematopoietic precursors. (A) Analysis of donor-derived (H2D$^b$) CD19$^+$ B cells and Gr-1$^+$ myeloid cells in spleen from hosts transplanted with Gata3$^{+/+}$ or Gata3$^{−/−}$ hematopoietic precursors. (B) Analysis of H2D$^b$ expression by Lin$^-$ CD127$^+$ CD25$^+$ Sca-1$^+$ T1/ST2$^+$ lung cells (Gata3$^{+/−}$ chimeric mouse). Shown are results from two independent experiments, each with $n = 3$ mice (Gata3$^{−/−}$) or 2 (Gata3$^{+/+}$) mice per group.

Fig. S2. Pulmonary IL-5 and -13 production by Lin$^-$ lymphocytes upon IL-33 challenge is group 2 innate lymphoid cell (ILC2)-derived. Lin-negative lymphocytes from lungs of IL-33-challenged chimeras were analyzed for the expression of CD127 and the indicated intracellular cytokines. Expression of the indicated ILC2 markers is shown for the boxed Gata3$^{+/+}$ population, and relative frequencies are indicated. Shown are representative results from two independent experiments with $n ≥ 6$ total mice analyzed per genotype.
Fig. S3. Cytokine production from ILC2s in IL-25-challenged chimeras. (A) Gating strategy for identification of ILC2s in BM and lung of IL-25-challenged chimeras. (B) Lin-negative lymphocytes from lungs were analyzed for IL-5 production. Cytokine-positive cells (boxed) were analyzed for expression of ILC2 markers. Shown are representative results from two independent experiments with n = 4–6 total mice analyzed per genotype.
Fig. S4. ILC2 differentiation in vitro requires GATA-3. (A) GATA-3–deficient or –competent fetal liver precursors were cocultured with OP9Δ4 stromal cells in the presence of IL-7 and -33 and Lin-negative lymphocytes were analyzed by flow cytometry for the indicated markers after 17 d. (B) Analysis of intracellular cytokine expression by ILC2s (Gata3-competent culture) or all lineage-negative lymphocytes (Gata3-deficient culture) after cultures as in A. Relative frequencies of the gated populations are indicated. Shown are representative plots of three independent experiments with n ≥ 2 samples per group.

Fig. S5. No induction of inhibitor of differentiation 2 (Id2) and Rora expression in fetal liver (FL) cultures in the absence of Gata3. GATA-3–deficient or –competent fetal liver precursors were cocultured with OP9Δ4 stromal cells in the presence of IL-7 and -33. Fractions of the cultures were analyzed at the indicated time points by using qRT-PCR for expression of the indicated genes. Expression levels were normalized to Gapdh expression levels. Gene expression levels in OP9Δ4 stroma cells are shown as negative control.
Cell-intrinsic GATA-3 is required for ILC2 development. (A) Cellularity of the various organs of mixed chimeric mice (90% Gata3+/− or Gata3−/− FL precursors supplemented with 10% B6 BM). (B) Levels of chimerism in reconstituted mice are shown as absolute number of CD4−CD8−CD19+ B and CD4−CD8−CD19−NKp46−CD11b+ NK cells in spleen. (C) Quantification of the total number of Lin−CD127−CD25−Sca1−T1/ST2+ ILC2s in the indicated organs of mixed chimeric mice. (D) Flow cytometric identification of ILC2s in the lineage-negative populations of spleen and small intestine lamina propria cells. ILC2s (boxed) were then probed for expression of allogenic markers.
Fig. S7. ILC2 cytokine production correlates with GATA-3 gene copy number. (A) Intracellular expression levels of GATA-3 were analyzed after fixation and permeabilization of ILC2s from lung and BM from the indicated mouse strains. GATA-3 levels were determined by using PE-conjugated or Alexa Fluor 660-conjugated antibodies as indicated. Mean fluorescence intensity for GATA-3 expression by ILC2s is indicated as mean ± SEM. One representative plot is shown from four independent experiments with n ≥ 2 mice per genotype. (B) Analysis of intracellular cytokine expression by lung and BM ILC2s gated as Lin−CD127+CD25+T1/ST2+ cells. Numbers in plots indicate percentages of cytokine+ ILC2s ± SEM. (C) Expression of IL-17BR and T1/ST2 by BM ILC2s gated as in B by WT and CD2–Gata3 Tg mice. B and C show representative plots of four independent experiments with n = 2–4 mice per group.

Fig. S8. Cytokine expression by in vitro CLP-derived ILC2s. Single Lin−CD127+Sca1lowCD117lowCD135+ CLPs were sorted from BM of mice with various Gata3 copy numbers and cocultured for 14–18 d with OP9Δ4 stroma cells in the presence of IL-7 and -33. Intracellular cytokine expression was analyzed for Lin−CD127+CD25+Sca1+T1/ST2+ ILC2s. One representative sample is shown with numbers indicating relative frequencies of gated ILC2s. Data from three independent experiments are shown with n ≥ 144 cultures in total.

Table S1. Effects of the CD2–Gata3 Tg on expression of ILC2-associated genes in BM CLPs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative expression in CLP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gata3</td>
<td>3.0†</td>
</tr>
<tr>
<td>Id2</td>
<td>0.69</td>
</tr>
<tr>
<td>Rora</td>
<td>0.84</td>
</tr>
<tr>
<td>Il17rb</td>
<td>0.89</td>
</tr>
<tr>
<td>Il1r1l</td>
<td>1.01</td>
</tr>
</tbody>
</table>

*CLPs were FACS sorted from BM as Lin−Sca-1lowc-kitlowFMS-like tyrosine kinase 3-positive cells.
†Relative expression values, as determined by qRT-PCR and normalized to Gapdh, of the indicated genes in CD2–Gata3 Tg CLPs relative to WT cells. For each gene, values in the WT were set to 1.0. These data show that Gata3 is expressed ~3.0-fold higher in CLPs from CD2–Gata3 Tg mice compared with WT mice. Other genes are not significantly increased. Data are from two experiments using sorted CLPs (pooled from three mice).