Group 3 innate lymphoid cells continuously require the transcription factor GATA-3 after commitment

Chao Zhong1, Kairong Cui2, Christoph Wilhelm3,4, Gangqing Hu2, Kairui Mao5, Yasmine Belkaid3, Keji Zhao2 & Jinfang Zhu1

The transcription factor GATA-3 is indispensable for the development of all innate lymphoid cells (ILCs) that express the interleukin 7 receptor α-chain (IL-7Rα). However, the function of low GATA-3 expression in committed group 3 ILCs (ILC3 cells) has not been identified. We found that GATA-3 regulated the homeostasis of ILC3 cells by controlling IL-7Rα expression. In addition, GATA-3 served a critical function in the development of the NKP46+ ILC3 subset by regulating the balance between the transcription factors T-bet and RORγt. Among NKP46+ ILC3 cells, although GATA-3 positively regulated genes specific to the NKP46+ ILC3 subset, it negatively regulated genes specific to lymphoid tissue–inducer (LTi) or LTi-like ILC3 cells. Furthermore, GATA-3 was required for IL-22 production in both ILC3 subsets. Thus, despite its low expression, GATA-3 was critical for the homeostasis, development and function of ILC3 subsets.

Innate lymphoid cells (ILCs) that express CD127 (the interleukin 7 receptor α-chain (IL-7Rα)) are regarded as the innate counterpart of the adaptive immune system’s CD4+ helper T cells1. An especially strong parallel is the expression of master regulatory transcription factors and signature effector cytokines by distinct subsets of ILCs and helper T cells. For example, group 2 ILCs (ILC2 cells) express the transcription factor GATA-3 (refs. 2–5), which is the master regulator of the T helper cell lineage 20. Some CCR6− ILC3 cells express a natural cytotoxicity–triggering receptor (NCR): NKp46 (encoded by NCR1)21. CCR6− ILC3 cells represent a separate ILC3 lineage that can eventually develop into NCR+ ILC3 cells20. Unlike LTi cells, CCR6− ILC3 cells are derived from PLZF+ progenitor cells22, and they proliferate substantially in mice, mainly after birth to 3–4 weeks of age23. Although both CCR6+ ILC3 cells and NCR+ ILC3 cells are able to produce IL-22 (refs. 9,11), a key cytokine that is essential for a protective immune response to intracellular bacteria such as Citrobacter rodentium, the unique function of NCR+ ILC3 cells is still unclear.

In T cells, GATA-3 not only regulates T helper cell development24. Similar to its function in the development of CD4+ T cells, GATA-3 is indispensable for the development of all IL-7Rα-expressing ILCs3, including ILC3 cells25. GATA-3 has high expression in ILC2 cells, but it also has low expression in mature ILC1 cells and ILC3 cells. Although its expression is low, GATA-3 is also required for the maintenance of ILC1 homeostasis12. However, it is unclear whether GATA-3 has any function in mature ILC3 cells.

Here we found that deletion of Gata3 in already committed ILC3 cells through the deletion of loxp-flanked Gata3 alleles (Gata3fl/fl) by Cre recombinase expressed from Rorc (Rorc-Cre) blocked further development of CCR6−NCR− ILC3 cells into NCR+ ILC3 cells. This arrest was partly due to upregulation of RORγt expression upon removal of GATA-3. Furthermore, we found that GATA-3 affected the homeostasis of ILC3 cells and their function by positively regulating the expression of IL-7Rα and that of IL-22, respectively. As a consequence, mice with deletion of Gata3 specifically in ILC3 cells succumbed to infection with C. rodentium, which highlights the importance of maintaining GATA-3 expression in the ILC3 lineages.

RESULTS

GATA-3 affects ILC3 homeostasis by regulating IL-7Rα

To study the function of GATA-3 in already developed ILC3 cells, we crossed Gata3fl/fl mice26 with Rorc-Cre mice27 to generate mice with

1Molecular and Cellular Immunoregulation Unit, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA. 2Systems Biology Center, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA. 3Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA. 4Institute of Clinical Chemistry and Clinical Pharmacology, University Hospital Bonn, University of Bonn, Bonn, Germany. 5Laboratory of System Biology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA. Correspondence should be addressed to J.Z. (jfzhu@niaid.nih.gov).

Received 12 August; accepted 9 October; published online 23 November 2015; corrected online 8 December 2015 (details online); doi:10.1038/ni.3318
conditional GATA-3 deficiency only in cells that had expressed and/or were expressing RORγt, including ILC3 cells (*Gata3*Δfl/flRorc-Cre mice; called ‘*Gata3*Δfl/flCre mice’ here). Unlike *Gata3*Δfl/flVav-Cre mice, which do not have lymph node structure, *Gata3*Δfl/flCre mice developed lymph nodes (data not shown), which suggested that their ILt cells were intact in terms of inducing lymphoid tissue development. Presumably because RORγt is also expressed during T cell development at the immature CD4+CD8+ double-positive stage in the thymus, and GATA-3 is involved in CD4+ T cell development, we observed slightly fewer CD4+ T cells in *Gata3*Δfl/flCre mice than in wild-type mice (data not shown).

Since RORγt is not expressed in progenitors of ILCs, such as PLZF+ common ILC progenitors, the conditional deletion of GATA3 in *Gata3*Δfl/flCre mice should not have directly affected the development of ILC populations other than ILC3 cells. Indeed, GATA-3 was abolished only in ILC3 cells but not in ILC2 cells (Supplementary Fig. 1a,b). ILC3 cells from the small intestine lamina propria (siLP) of *Gata3*Δfl/flCre mice had lower expression of CD127 (IL-7Rα) than their *Gata3*Δfl/fl counterparts had (Fig. 1a,b). CD127 expression was reduced in both CCR6+ ILC3 subsets and CCR6− ILC3 subsets after deletion of GATA3, but we noted no substantial change in expression of the alloantigen CD90 (Thy-1), the cell-surface marker c-Kit, the lineage marker Sca-1, the T cell-activation marker CD25 or the activation and memory marker CD44 (Supplementary Fig. 2a). We also studied mice with global deletion of loxP-flanked *Gata3* alleles via Cre expressed from the tamoxifen-inducible Cre-ER(T2) transgene (*Gata3*Δfl/flCre-ER(T2) mice). At 1 week after treating *Gata3*Δfl/flCre-ER(T2) mice with tamoxifen, we detected a reduction in the expression of CD127 in ILC3 cells similar to that in ILC3 cells from *Gata3*Δfl/flCre mice (Fig. 1c), which indicated that GATA-3 was constantly required for the regulation of IL-7Rα expression in ILC3 cells. We then performed high-throughput whole-transcriptome sequencing (RNA-Seq) analysis comparing the gene-expression profiles of *Gata3*Δfl/fl ILC3 cells and *Gata3*Δfl/flCre ILC3 cells from siLP. IL-7r (which encodes IL-7Rα) was among the genes whose expression was lower in *Gata3*Δfl/flCre ILC3 cells than in *Gata3*Δfl/fl ILC3 cells (Supplementary Fig. 2b).

IL-7 signaling is critical for the survival and proliferation of lymphocytes, including ILCs. Indeed, the ratio of ILC3 cells to ILC2 cells was significantly lower in *Gata3*Δfl/flCre mice than in *Gata3*Δfl/fl mice (Fig. 1d). Furthermore, we found that the frequency as well as the absolute number of ILC3 cells was also reduced in *Gata3*Δfl/flCre mice (Fig. 1e,f). Experiments with chimeras generated by reconstitution of irradiated lymphocyte-deficient Rag2−/−Il2rg−/− recipient mice with a mixture of *Gata3*Δfl/flCre bone marrow and wild-type bone marrow showed that although B cells of either donor genotype populated the recipient mice normally, *Gata3*Δfl/flCre ILC3 cells were unable to efficiently populate the host mice, in contrast to wild-type ILC3 cells (Supplementary Fig. 3a–c). These results indicated that the reduction in the number of ILC3 cells in *Gata3*-deficient mice was a cell-intrinsic effect.

We restored IL-7Rα expression in *Gata3*Δfl/flCre ILC3 by crossing the *Gata3*Δfl/flCre mouse strain with a transgenic mouse line that carries an transgene encoding IL-7Rα (called ‘Ill7ra’ here) driven by the promoter of the human gene encoding the activation-costimulation molecule CD2, which is expressed by lymphoid lineages (CD2-Ill7ra mice). In mixed–bone marrow chimera experiments similar to those described above, CD127 expression in ILC3 cells of either donor origin in the chimera mice was identical to the CD127 expression in ILC3 cells in their respective donors, as expected (Fig. 1g,h). The Ill7ra transgene efficiently restored CD127 expression in *Gata3*Δfl/flCre ILC3 cells and ‘rescued’ the number of ILC3 cells to that observed in chimeras that received wild-type bone marrow (Fig. 1i). Therefore, GATA-3 affected the homeostasis of ILC3 cells by regulating IL-7Rα expression.

We further performed chromat immunoprecipitation with antibody to GATA-3 (anti-GATA-3), followed by high-throughput sequencing (ChIP-Seq), with ILC3 cells, ILC2 cells and Tbet2 cells. A common GATA-3-binding peak located in intron 2 of Ill7ra was identified in all three cell types (Fig. 1j). Three GATAA motifs were present within the GATA-3-binding region. These results indicated that direct regulation of Ill7ra expression by GATA-3 might be a shared mechanism for GATA-3-mediated homeostatic regulation of both innate lymphocytes and adaptive lymphocytes.

**GATA-3 is indispensable for the development of NKp46+ ILC3 cells**

The expression of Ncr1 (which encodes the NCR NKp46) was much lower in *Gata3*Δfl/flCre ILC3 cells than in *Gata3*Δfl/fl ILC3 cells (Supplementary Fig. 2b). This suggested that GATA-3 might have an important role during the development of NKp46+ ILC3 cells. Indeed, although both the CCR6+ ILC3 lineage and CCR6− ILC3 lineage were present among RORγt+ ILC3 cells in *Gata3*Δfl/flCre mice, the NKp46+ ILC3 population was much smaller in *Gata3*Δfl/flCre mice than in *Gata3*Δfl/fl mice (Fig. 1a).

We then further divided the CCR6− ILC3 lineage into three stages on the basis of expression of T-bet and NKp46: NKp46+ T-bet−, NKp46+ T-bet+ and NKp46− T-bet+. Both the NKp46+ T-bet− CCR6+ ILC3 population and the NKp46+ T-bet+ CCR6− ILC3 population were reduced only modestly in number in *Gata3*Δfl/fl ILC3 mice, similar to the reduction of CCR6+ ILC3 cells in these mice, compared with the abundance of these populations in *Gata3*Δfl/flCre mice (Fig. 2b); this was probably due to the decreased expression of IL-7Rα. NKp46+ ILC3 cells were considerably fewer in *Gata3*Δfl/flCre ILC3 cells than in *Gata3*Δfl/fl mice (Fig. 2b,c). Furthermore, NKp46 expression was also lower in the remaining *Gata3*-deficient NKp46+ ILC3 cells than in their *Gata3*Δfl/fl counterparts (Fig. 2d). ILC1 cells positive for RORγt ‘fate mapping’ (that is, ILC1 cells that formerly expressed RORγt; ‘ex-ILC1’ cells) were also less abundant in *Gata3*Δfl/flCre ILC3 mice than in *Gata3*Δfl/fl mice, while the abundance of ILC1 cells negative for RORγt ‘fate mapping’ (that is, ILC1 cells that had never expressed RORγt) was similar in *Gata3*-sufficient mice and *Gata3*Δfl/flCre ILC3 mice (Supplementary Fig. 4a,b).

The villus of the small intestine is the main area that shows enrichment for NKp46+ ILC3 cells. Thus, we assessed the distribution of NKp46+ ILC3 cells by imaging the sections of the small intestine of *Gata3*Δfl/fl and *Gata3*Δfl/flCre mice. There were only slightly fewer NKp46+ ILC3 cells in the villi of *Gata3*Δfl/flCre mice than in those of *Gata3*Δfl/fl mice, whereas NKp46+ ILC3 cells were nearly completely absent from *Gata3*Δfl/flCre mice (Fig. 2e). The number of NKp46+ ILC3 cells in tamoxifen-treated *Gata3*Δfl/flCre-ER(T2) mice gradually decreased over time after tamoxifen treatment (Fig. 2f–h). At 3 months after treatment of *Gata3*Δfl/flCre-ER(T2) mice with tamoxifen, the number of NKp46+ ILC3 cells in these mice was substantially reduced and was similar to that observed in *Gata3*Δfl/flCre ILC3 mice (Supplementary Fig. 5). Since ILC2 cells completely disappeared within 1 week of tamoxifen treatment, whereas NKp46+ ILC3 cells were still present in substantial numbers 3 weeks after deletion of GATA3, we concluded that GATA-3 had a minimal role in the maintenance of NKp46+ ILC3 cells and that the main function of GATA-3 was to regulate the development of NKp46+ ILC3 cells. Restoration of IL-7Rα in *Gata3*Δfl/flCre ILC3 cells via the Ill7ra transgene did not restore the development of NKp46+ ILC3 cells (Fig. 2i), which indicated that GATA-3 determined the
development of NKP46+ ILC3 cells through a mechanism independent of the regulation of IL-7Rα.

**Gata3 deficiency in ILC3 cells results in RORγt upregulation**

The development of NKP46+ ILC3 cells requires T-bet expression28,29. However, among NKP46+ CCR6- ILC3 cells, we did not detect notable changes in T-bet expression at the population level after deletion of Gata3 (Fig. 3a). Unlike the binding of GATA-3 to an intron of Tbx21 (which encodes T-bet) in ILC2 cells and T(H)2 cells, a mechanism through which GATA-3 might silence Tbx21 expression in these cells, GATA-3 did not bind to the Tbx21 locus in ILC3 cells (Fig. 3b), which might have allowed T-bet to be expressed in this lineage.

Consistent with the increase in Rorc mRNA after deletion of Gata3 (Supplementary Fig. 2b), flow cytometry showed an increase in the expression of RORγt protein in ILC3 cells in which Gata3 was deleted (Fig. 3c,d). This was true in both CCR6+ ILC3 cells and CCR6- ILC3 cells (Supplementary Fig. 6a). Moreover, at the fetal stage, the Gata3ΔILC3 LTi cells already had higher RORγt expression than that of Gata3fl/fl LTi cells (Supplementary Fig. 6b). By using mixed-bone marrow chimeras (transferring a mixture of wild-type and Gata3ΔILC3 bone marrow cells into Rag2-/-Il2rg-/- hosts), we confirmed that GATA-3-mediated suppression of RORγt expression was cell intrinsic (Supplementary Fig. 6c). Furthermore, we still detected higher RORγt expression in Gata3ΔILC3 ILC3 cells expressing the Il7ra transgene than in Gata3fl/fl ILC3 cells expressing the Il7ra transgene (Supplementary Fig. 6d), which suggested that GATA-3 suppressed RORγt expression independently of the regulation of IL-7Rα. At 1 week after tamoxifen treatment of the Gata3fl/flCre-ER22 mice, RORγt expression was higher in ILC3 cells from these treated mice than in ILC3 cells from their untreated counterparts (Fig. 3e), which indicated that GATA-3 constantly restrained RORγt expression in mature ILC3 cells.

GATA-3 directly binds to Rorc in T(H)2 cells and regulatory T cells30. We found that GATA-3 bound to that same site of the Rorc locus30 in T(H)2 cells and ILC2 cells (Fig. 3f). We also detected a smaller but notable GATA-3-binding peak at the same region in ILC3 cells, which suggested that GATA-3 directly limited RORγt expression in ILC3 cells.
Interplay among RORγt, GATA-3 and T-bet at different stages

To investigate whether a modest change in RORγt expression would affect the development of NKP46+ ILC3 cells, we analyzed mice in which one allele of Rorc was replaced with sequence encoding green fluorescent protein (GFP). Although such Rorcfgfp/+ mice have been widely used as RORγt reporter mice and are considered ‘wild-type’ animals, Rorcfgfp/+ mice had many more NKP46+ ILC3 cells than did their truly wild-type (Rorc+/+) littermates (Fig. 4a.b). Furthermore, Rorcfgfp/+ NKP46+ ILC3 cells also had higher expression of NKP46 and T-bet than did wild-type NKP46+ ILC3 cells (Fig. 4c). These results indicated that reducing RORγt expression by half promoted the development of NKP46+ ILC3 cells and expression of T-bet in these cells.

To further understand the relationship between T-bet and RORγt in NKP46+ ILC3 cells, we generated mice heterozygous for the development of NKP46+ ILC3 cells. (a) Flow cytometry of ILC3 cells (Lin−CD127+RORcγt+ cells or ‘ex-ILC3’ cells. For the control, the number of cells in various ILC3 subsets was measured, and the percent cells in each subset was calculated within the Lin−CD127+RORcγt+ population. (b) MFI of NKP46 in NKP46+ ILC3 cells as in a. (c) Imaging of villi in the small intestine of Gata3fl/fl and Gata3fl/lfl mice, showing the distribution of NKP46+ ILC3 cells (top), and absolute number of NKP46- or NKP46+ ILC3 cells (per villus area of 1 × 10^4 μm²) (bottom). Scale bars (top), 25 μm. Each symbol (bottom) represents one count of ILC3 cells in three to four neighboring villi; small horizontal lines indicate the mean (s.s.d.). (d) Flow cytometry of lymphocytes from the siLP of Gata3fl/flCre-ERγt/ mice (n = 3 per group) at 1 week, 2 weeks and 3 weeks after the first treatment with tamoxifen (given every other week) and in their untreated (control) counterparts (UT) (n = 3), analyzed as in a to identify various subsets of ILC3 cells (live Lin−CD127+RORcγt+). (e) Frequency of NKp46+ ILC3 cells among the CCR6−ILC3 lineage as in a. (f) MFI of NKP46 in NKP46+ CCR6− ILC3 cells (b, in mice as in f). (i) Flow cytometry of lymphocytes from siLP of Gata3fl/fl, Gata3fl/lfl, Gata3fl/flCD2-Il7ra− and Gata3fl/lflCD2-Il7ra− mice (n = 3 per group), stained as in a to identify various subsets of ILC3 cells (live Lin−CD127+RORcγt+). Data are representative of at least three (a,d) or two (e,i) independent experiments or one experiment (f-h) or are pooled from at least three experiments (b,c) (mean and s.d. in b,d,g,h).

Taking advantage of a transgenic bacterial artificial chromosome reporter mouse carrying sequence encoding the green fluorescent protein ZsGreen inserted into the gene encoding T-bet (T-bet–ZsGreen mice), we were able to analyze RORγt expression in CCR6−NKP46− T-bet–ZsGreen+ cells in the presence or absence of T-bet. Although T-bet was not expressed in Thb21+ T-bet–ZsGreen mice, due to the lack of Thb21, T-bet–ZsGreen expression was comparable in CCR6−NKP46− ILC3 cells from Thb21+ T-bet–ZsGreen mice and those from Thb21−/− T-bet–ZsGreen mice (Fig. 4g,h), which indicated that at this early phase, T-bet was dispensable for its own expression. Notably, the expression of RORγt was not increased in CCR6−NKP46− T-bet–ZsGreen+ cells in the absence of T-bet (Fig. 4i), which suggested that T-bet suppressed RORγt expression in the NKP46+ ILC3 cells, as reported before, it did not suppress RORγt expression in the CCR6−NKP46− ILC3 cells. Since RORγt expression was increased in Gata3-deficient cells, we concluded that RORγt was regulated by GATA-3 but not by T-bet at early developmental stages and that the interplay between GATA-3 and RORγt preceded the balance between T-bet and RORγt, both of which were critical for fate determination of the NKP46+ ILC3 lineage.

We restored RORγt expression in ILC3 cells from Gata3fl/lfl mice by crossing these mice with Rorcfgfp/+ mice. RORγt expression in ILC3 cells from the resultant Gata3fl/lflRorcfgfp/+ mice was comparable to that in ILC3 cells from Gata3fl/fl mice (Fig. 4j). More notably, we observed ‘rescue’ of the development of NKP46+ ILC3 cells in Gata3fl/lflRorcfgfp/+ mice (Fig. 4k,l). These results indicated that GATA-3-mediated repression of RORγt expression in ILC3 cells at
early stages was critical for setting up an environment in which the cross-regulation and balance between T-bet and RORγt took place during the development of NKp46+ ILC3 cells.

**Lineage-specific gene regulation by GATA-3 in ILC3 subsets**

We further performed an RNA-Seq analysis of distinct ILC3 subsets. To obtain pure ILC3 subsets, we first generated an RORγt reporter mouse strain carrying a transgenic bacterial artificial chromosome containing the Rorc locus in which sequence encoding the far-red fluorescent protein E2-Crimson was inserted at the start codon (ATG) of Rorc. In one of the transgenic lines, RORγt–E2-Crimson D9, E2-Crimson had high expression that faithfully reflected the expression of endogenous RORγt (Fig. 5a). Unlike Rorcγt−/− knock-in mice, these RORγt–E2-Crimson mice had normal distribution of ILC3 subsets (data not shown). We then bred RORγt–E2-Crimson mice with T-bet–ZsGreen mice to generate a double reporter strain (T-bet plus RORγt) to facilitate sorting of the ILC3 subsets. We also bred some of these double-reporter mice onto the Gata3ΔILC3 mice (Fig. 5b). Unlike Gata3−/− mice, these RORγt−Gata3−/− mice had normal distribution of ILC3 subsets, presented as read density (as in Fig. 1j).

**Figure 3** GATA-3 negatively regulates RORγt without affecting T-bet expression. (a) T-bet expression by ILC3 subsets (right) among lymphocytes from the siLP of Gata3γt−/− and Gata3ILC3 mice (n = 3 per genotype) (prepared and stained as in Fig. 2a), separated into various populations (left) by gating of live Lin− cells, or in CCR6+ ILC3 cells (negative control). Numbers adjacent to outlined areas (left) indicate percent NKp46+RORγt+ cells (top) or NKp46−RORγt− cells (bottom) (top row), or CCR6+ cells (top) or CCR6− cells (bottom) (bottom row). (b) ChiP-Seq analysis of the binding of GATA-3 around Tbx21 in ILC3, ILC2 and Th2 cells, presented as read density (as in Fig. 1j). (c) Flow cytometry analyzing RORγt in siLP ILC3 cells (c), and MFI of RORγt in siLP and clp ILC3 cells (d), among lymphocytes from the siLP and clp of the Gata3γt−/− and Gata3ILC3 mice (n = 5 per genotype), prepared and stained as in a. Numbers adjacent to outlined areas (e) indicate percent RORγt+ cells. Each symbol (d) represents an individual mouse; small horizontal lines indicate the mean (±s.d.). (e) RORγt expression by ILC3 cells among lymphocytes from the siLP of untreated Gata3γt−/−Cre-ERT2 mice (−) and tamoxifen-treated Gata3γt−/−Cre-ERT2 mice (+) (n = 3 mice per group) 1 week after the first injection of tamoxifen (protocol as in Fig. 2f, cells harvested and stained as in Fig. 1c). Numbers adjacent to outlined areas (left) indicate percent GATA-3+RORγt+ cells (top left) or GATA-3−RORγt− cells (bottom right). (f) ChiP-Seq analysis of the binding of GATA-3 around Rorc in ILC3, ILC2 and Th2 cells, presented as read density (as in Fig. 1j). Bottom, enlargement of peak area identified above, showing two putative GATA-3-binding motifs with a consensus GATA-3-binding sequence (red). *P < 0.001 (two-tailed unpaired Student’s t-test). Data are representative of at least three independent experiments (a,c,d) or one experiment (b,e,f).
Figure 4  The interplay among RORγt, GATA-3 and T-bet regulates the development of NKp46+ ILC3 cells at different stages. (a,d) Flow cytometry of lymphocytes from the siLP of Rorc−/− and Rorc+/+ mice (n = 3 per genotype) (a) or Rorc−/− and Rorc+/+ Tbx21-Cre mice (n = 2 per genotype) (d) stained as in Fig. 2a. (b,e) Frequency of NKp46+ ILC3 cells among CCR6+ ILC3 cells as in a (b) or d (e); bottom (b), total NKp46+ ILC3 cells among cells as above. (c,f) MF1 of T-bet and Nkp4+IILC3+ ILC3 cells among lymphocytes as in a (c) or d (f). (g) T-bet–ZsGreen reporter expression (bottom) in NKp46+ ILC3 cells among live Lin− cells (top; stained as in Fig. 2a) from the siLP of Tbx21−/− or Tbx21−/+ T-bet–ZsGreen mice (n = 3 per genotype). Numbers adjacent to outlined areas (top) indicate percent NKp46+RORγt+ cells (top) or NKp46+RORγt− cells (bottom). (h) Frequency of T-bet–ZsGreen− or T-bet–ZsGreen+ ILC3 cells in the CCR6−NKp46− population among lymphocytes as in g. (i) RORγt expression (top) and MF1 of RORγt (bottom) in ZsGreen− or ZsGreen+ CCR6−NKp46− ILC3 cells among lymphocytes as in g. (j,k) Flow cytometry analyzing RORγt (j) and subsets of ILC3 cells (k) among lymphocytes from the siLP of Gata3fl/fl and Gata3fl/flRorc−/+ mice (n ≥ 3 per group) (stained as in Fig. 2a). (l) Frequency of NKp46+ ILC3 cells in the CCR6−ILC3 lineage as in j,k. *P < 0.05 and **P < 0.001 (two-tailed unpaired Student’s t-test). Data are representative of at least three (a–c,e,k) or two (d–i) independent experiments or are pooled from four experiments (l) and s.d. in b,f,i).

background. We then sorted CCR6+ and NKp46+ ILC3 subsets, defined by expression of the reporters as RORγt−E2-Crimson+ T-bet–ZsGreen + CCCR6+, for the CCR6+ ILC3 population, and RORγt−E2-Crimson−T-bet–ZsGreen−NKp46+ for the NKp46+ ILC3 population (Fig. 5b), from Gata3fl/fl and Gata3−/− mice and subjected these cells to RNA-Seq analysis. Overall, GATA-3 positively or negatively regulated hundreds of genes (with a cutoff of a difference in expression (higher or lower) of over twofold in Gata3fl/fl cells relative to expression in Gata3−/− cells and with an RPKM (‘reads per kilobase of exon model per million mapped reads’) value of >5 in either sample) in CCR6+ ILC3 cells (462 positively regulated genes and 389 negatively regulated genes) and NKp46+ ILC3 cells (841 positively regulated genes and 751 negatively regulated genes) (Supplementary Table 1).

Using the same cutoff noted above, we also identified hundreds of genes that were ‘preferentially’ expressed by CCR6+ ILC3 cells (420 genes) or NKp46+ ILC3 cells (377 genes). To further identify genes that were expressed most differentially in CCR6+ ILC3 cells relative to their expression in NKp46+ ILC3 cells, we applied a more stringent cutoff of a difference in expression of over fourfold and an RPKM value of >10; this resulted in the identification of 78 CCR6+ ILC3–specific genes and 77 NKp46+ ILC3–specific genes (Fig. 5c and Supplementary Table 1). The CCR6+ ILC3–specific genes included the transcription factor–encoding genes Egr2, Fosx1, Id1 and Id3 and the cell surface molecule–encoding genes Cxcr5, H2-0a, Pdcd1, Ly6c1, Ly6c2, Nrp1 and Sip1, in addition to Ccr6. On the other hand, the NKp46+ ILC3–specific genes included the transcription factor–encoding genes Hey1, Ikf3, Ifo7 and Tbx21 and the cell surface molecule–encoding genes Ccr9, Cxcr3, Cxcr6, Fast, Icos, Ii12b1 and Libr41, in addition to Ncr1. CCCR6+ ILC3 cells ‘preferentially’ expressed Ifil7f, whereas NKp46+ ILC3 cells ‘preferentially’ expressed Csf2 and Areg, consistent with published findings32.

Among the 78 CCR6+ ILC3–specific genes and 77 NKp46+ ILC3–specific genes, 12 genes and 39 genes, respectively, were positively regulated by GATA-3 in these cells (Fig. 5d,e). 50 of the 78 CCR6+ ILC3–specific genes were negatively regulated by GATA-3 in NKp46+ cells, whereas 15 of the 77 NKp46+ ILC3–specific genes were negatively regulated by GATA-3 in CCR6+ cells (Fig. 5d,e). The finding that GATA-3 promoted more than half of the NKp46+ ILC3–specific genes, including Il2rb1, Libr41, Hey1, Ncr1, Ikf3 and Icos, while it inhibited more than half of the CCCR6+ ILC3–specific genes, including Cxcr5, Il3, Egr2, Ly6c1 and Ly6c2, in NKp46+ ILC3 cells, supported the proposal that GATA-3 expression was particularly important for specifying the fate of the NKp46+ lineage.

GATA-3 regulates IL-22 production by ILC3 cells

Among the 122 genes and 45 genes that were positively regulated by GATA-3 (with a difference in expression of over fourfold and an RPKM value of >10) in CCCR6+ ILC3 cells and NKp46+ ILC3 cells, respectively, 9 genes, including Arg1 and Il22, were induced by GATA-3 in
both cell types (Fig. 6a and Supplementary Table 1). Furthermore, GATA-3 positively regulated the expression of Ahr (over twofold) in both CCR6+ ILC3 cells and NKp46+ ILC3 cells (Supplementary Table 1). Although IL-22 production by NKp46+ ILC3 cells has been correlated with resistance of mice to infection with C. rodentium, both CCR6+ ILC3 cells and NKp46+ ILC3 cells are able to produce IL-22. Indeed, our RNA-Seq results showed that both subsets expressed equivalent amounts of Il22 mRNA, but Il22 expression was reduced considerably when GATA-3 was absent in these cells (Fig. 6b). Our results obtained by ChIP-Seq with anti-GATA-3 showed that GATA-3 bound to the promoter of Il22 only in ILC3 cells, not in ILC2 cells or Tif12 cells (Fig. 6c). We identified a GATA-3-binding motif, GATAN, in the middle of the GATA-3-binding region (Fig. 6c). Thus, Il22 was probably a direct target of GATA-3 in both ILC3 subsets.

Gata3ΔILC3 ILC3 cells showed ~50% less IL-22 production compared with that of Gata3Ilfl/Il ILC3 cells (Fig. 6d). The Il7ra transgene restored IL-7Rα expression in Gata3ΔILC3 ILC3 cells (Fig. 1g), but it did not correct the IL-22-production defect of such cells (Fig. 6d). In vitro stimulation with IL-23 induced IL-22 production in both Gata3Ilfl/Il ILC3 cells and Gata3ΔILC3 ILC3 cells (Fig. 6e). However, IL-22 production was still substantially lower in Gata3ΔILC3 ILC3 cells than in Gata3Ilfl/Il ILC3 cells (Fig. 6e).

To further address the importance of GATA-3 expression in ILC3 cells during immune responses, we used C. rodentium–infection model. Since Gata3ΔILC3 mice had somewhat altered T cell development, as noted above, we bred these mice onto a background deficient in the RAG recombinase component RAG-1 (Rag1−/−) to generate Rag1−/−Gata3ΔILC3 and used Rag1−/−Gata3Ilfl/Il mice as controls. ILC3 cells from the Rag1−/−Gata3ΔILC3 mice expressed less IL-7Rα protein but more RORγt protein than did ILC3 cells from Rag1−/−Gata3Ilfl/Il mice, as expected (Supplementary Fig. 7a). We also observed a blockade in the development of NKp46+ ILC3 cells in the Rag1−/−Gata3ΔILC3 mice (Supplementary Fig. 7b). At 4 d after infection of mice with C. rodentium, we detected a much lower frequency of IL-22-expressing ILC3 cells in Rag1−/−Gata3ΔILC3 mice than in Rag1−/−Gata3Ilfl/Il mice (Fig. 6f). The total number of IL-22-producing ILC3 cells, as well as the mean fluorescence intensity of IL-22, were significantly lower in Rag1−/−Gata3ΔILC3 mice than in Rag1−/−Gata3Ilfl/Il mice (Fig. 6g). We also detected less IL-22, but not less IL-17A, in culture supernatants of cells obtained from the colonic lamina propria (CLP)
Figure 6 GATA-3 regulates IL-22 production by ILC3 cells and thus regulates the susceptibility of mice to infection with C. rodentium. (a) Overlap (middle) of GM positivity regulated by GATA-3 in CCR6+ ILC3 cells (left) and NKp46+ ILC3 cells (right); above, two of the nine genes in the overlap (Arg1 is known to be positively regulated by GATA-3; IL22 is studied further below). Numbers in plot indicate total genes in each set. (b) RNA-Seq analysis of IL22 mRNA in CCR6+ or NKp46+ ILC3 cells (horizontal axis) from Gata3fl/fl and Gata3∆ILC3 mice. (c) ChIP-Seq analysis of the binding of GATA-3 around IL22 in ILC3, IL2 and Tp2 cells, presented as read density (as in Fig. 1); below, GATA-3-binding sites (presented as in Fig. 3). (d,e) Expression of IL-22 by ILCs among lymphocytes obtained from the siLP of Gata3fl/fl, Gata3∆ILC3, Gata3∆ILC3CD2-Il7ra and Gata3∆ILC3CD2-Il7ra mice (n = 3 per group) and cultured in vitro for 3 h with medium alone (d) or with IL-23 (e), analyzed by flow cytometry (left), and frequency of IL-22+ ILC3 cells among RORγt+ cells (right). (f) Expression of IL-22 and T-bet by cLP ILC3 cells from Rag1−/−Gata3fl/fl mice (n = 4) and Rag1−/−Gata3∆ILC3 mice (n = 5), analyzed ex vivo 4 d after infection of mice with C. rodentium. (g) Total ILC3 cells (far left), frequency of IL-22-producing cells among ILC3 cells (middle left), MFI of IL-22 in IL-22+ ILC3 cells (middle right), and abundance of IL-22 (far right; arbitrary values) in mice as in f. (h) Multiplex cytokine assay of IL-22 and IL-17A in supernatants of cultured cLP cells as in f. (i,j) Weight (i) and survival (j) of Rag1−/−Gata3fl/fl mice (n = 6) and Rag1−/−Gata3∆ILC3 mice (n = 4) at various times (horizontal axis) after infection with C. rodentium. *P < 0.05, **P < 0.01 and ***P < 0.001 (two-tailed unpaired Student’s t-test (a,b,j) or one-way (i) or two-way (j) analysis of variance). Data are from one experiment (a,c,i,j), are representative of at least three (d,e) or two (h) independent experiments or are pooled from two experiments (g,h) (mean and s.d. in b,d,e,g,i).
of Rag1−/−Gata3ΔILC3 mice 4 d after infection with C. rodentium than in that of their similarly infected Rag1−/−Gata3ΔB mice (Fig. 6h). Consequently, Rag1−/−Gata3ΔILC3 mice experienced significant weight loss starting from day 9 after infection (Fig. 6i). Although the vast majority of Rag1−/−Gata3ΔB mice had survived the infection at the end of the experiments, all Rag1−/−Gata3ΔILC3 mice died by 12 d after infection (Fig. 6j).

DISCUSSION

In this study, we demonstrated that GATA-3 was critical for the homeostasis of ILC3 cells, the development of Nkp46+ ILC3 cells and IL-22 production by ILC3 cells. IL-7Rα signaling is required for the development, survival and proliferation of ILCs. We found that IL-7Rα expression in ILC3 cells needed continuous expression of GATA-3 and that GATA-3 bound to a region within intron 2 of Il7r. The binding pattern in ILC3 cells was very similar to the binding pattern in ILC2 cells and Tγδ2 cells, although the expression of GATA-3 is lower in ILC3 cells than in ILC2 cells and Tγδ2 cells. This would suggest that low expression of GATA-3 might be necessary and sufficient for the regulation of IL-7Rα expression and that GATA-3-mediated IL-7Rα expression might be a common mechanism through which GATA-3 regulates the homeostasis of innate and adaptive lymphocytes.

In addition to regulating the homeostasis of ILC3 cells, GATA-3 was critical for the development of Nkp46+ ILC3 cells from CCR6−Nkp46 T-bet+ ILC3 cells. Graded expression of T-bet is important for development of the Nkp46+ ILC3 lineage, and T-bet suppresses RORγt expression in Nkp46+ ILC3 cells20. Here we also found that RORγt suppressed T-bet expression in Nkp46+ ILC3 cells. Therefore, the balance between T-bet and RORγt might determine the fate of Nkp46+ ILC3 cells. Although initial induction of T-bet expression was not impaired in GATA-3-deficient ILC3 cells, RORγt expression was enhanced in the absence of GATA-3. Although T-bet deficiency abolished the development of Nkp46+ cells, RORγt expression did not increase after deletion of T-bet. These results indicated sequential involvement of GATA-3 and T-bet in suppressing RORγt expression during the development of Nkp46+ ILC3 cells.

RorcΔβ/− mice had a greater frequency of Nkp46+ ILC3 cells than did their wild-type (RorcΔβ/+) littermates. By restoring RORγt expression in Gata3-deficient ILC3 cells, we partially ‘rescued’ the development of Nkp46+ ILC3 cells. These results suggested that GATA-3 affected the balance between T-bet and RORγt particularly at the early stage of Nkp46+ ILC3 development and that sequential repression of RORγt first by GATA-3 and then by T-bet was necessary for the development and maturation of CCR6+ ILC3 cells into Nkp46+ cells. The delicate cross-regulation of T-bet, GATA-3 and RORγt at different stages offers a plausible explanation for the requirement for all three seemingly antagonistic transcription factors in the development of Nkp46+ ILC3 cells.

CCR6+ LTI ILC3 cells and Nkp46+ ILC3 cells are two separate ILC3 populations, and they develop from different progenitor cells22. Despite their developmental and functional differences, these cells’ global gene expression has been assessed by only one study so far22. Since ILC3 cells from RorcΔβ/− reporter mice were used in that study22, some of those findings might not truly reflect gene expression in wild-type ILC3 cells. Our results obtained with the RORγt−/− mice showed a similar pattern of expression of most genes similar to that published expression pattern22 but also detected dozens of lineage-specific genes not reported before. Thus, our data sets will be important for further understanding of the biology as well as the development of these two distinct ILC3 subsets.

Globally, GATA-3 regulated hundreds of genes, either positively or negatively, in each ILC3 subset. Although more than half of the Nkp46+ ILC3–specific genes were positively regulated by GATA-3 in Nkp46+ cells, the majority of the CCR6+ ILC3–specific genes were negatively regulated by GATA-3 in Nkp46+ cells. This suggested that GATA-3 might be an important switch in determining the fate of Nkp46+ ILC3 cells versus CCR6+ ILC3 cells, or possibly of CCR6+ ILCs versus CCR6- ILCs at earlier development stages. Interestingly, genes that were positively regulated by GATA-3 in one ILC3 lineage could be negatively regulated by GATA-3 in the other ILC3 lineage. All these results indicated that GATA-3 regulated the expression of many lineage-specific genes in these two ILC3 subsets in a cell context–dependent manner. GATA-3, together with other lineage-specific transcription factors, such as T-bet, Hey1, Ifi30, I3d, Egr2, etc., many of which were newly identified in our study here, might form distinct transcriptional regulatory networks that define ILC3 subsets.

In addition to its effects on the homeostasis of ILC3 cells and the development of Nkp46+ ILC3 subsets, GATA-3 also regulated their expression of the critical ILC3 effector cytokine IL-22. GATA-3 directly bound to the promoter of Il22 only in ILC3 cells, not in ILC2 cells or Tγδ2 cells. In addition to directly regulating Il22 transcription, GATA-3 might also indirectly affect Il22 expression by regulating the expression of Ahr, a transcription factor critical for IL-22 production16,33. The relative contributions of these two mechanisms to IL-22 production will require further investigation. Consistent with all these phenotypical changes, mice with Gata3 deficiency in ILC3 cells were susceptible to infection with C. rodentium. In summary, although GATA-3 had low expression in ILC3 cells, it served a critical role in modulating the homeostasis, development and function of the ILC3 subsets by directly regulating the expression of several hallmark genes, including Il7r, Rorc and Il22.

METHODS

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

Accession codes. GEO: RNA-Seq and ChIP-Seq data, GSE71198.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank D.R. Littman (New York University) for Rorc−Cre mice on the C57BL/6 background; A. Singer (National Cancer Institute) for Tg-NCD2-II7ra mice on the C57BL/6 background; C. Dulac (Harvard) for Tbx21−/Cre mice; D. Artis (University of Pennsylvania) for C. rodentium strain DRS100; A. Bhandoola, R. Germain, J. O’Shea, P. Schwartzberg and A. Sher for critical reading of our manuscript; L. Guo for suggestions and technical assistance in analyzing ILCs; K. Weng for assistance in cell sorting; L. Feigenbaum for assistance in generating RORγt−E2-Crimson mice; and the DNA Sequencing and Computational Biology Core of the National Heart, Lung, and Blood Institute for sequencing the RNA-Seq and ChIP-Seq libraries. Supported by the Intramural Research Program of the US National Institutes of Health, the National Institute of Allergy and Infectious Diseases and the National Heart, Lung, and Blood Institute.

AUTHOR CONTRIBUTIONS

C.Z. performed all experiments; K.C. helped with constructing libraries for RNA-Seq and ChIP-Seq; C.W. helped with C. rodentium–infection experiments; G.H. performed initial analyses of the RNA-Seq and ChIP-Seq data; K.M. performed imaging experiments; K.C., C.W., G.H., K.M., Y.B. and K.Z. offered suggestions on the project and edited the paper; and C.Z. and J.Z. conceived of the project, designed the experiments, analyzed the data and wrote the paper.
COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Spits, H. et al. Innate lymphoid cells—a proposal for uniform nomenclature. Nat. Rev. Immunol. 13, 145–149 (2013).
2. Hoyler, T. et al. The transcription factor GATA-3 controls cell fate and maintenance of type 2 innate lymphoid cells. Immunity 37, 634–648 (2012).
3. Yagi, R. et al. The transcription factor GATA-3 is critical for the development of all IL-7Rα+expressing innate lymphoid cells. Immunity 40, 378–388 (2014).
4. Klein Wolterink, R.G. et al. Essential, dose-dependent role for the transcription factor Gata3 in the development of IL-5+ and IL-13+ type 2 innate lymphoid cells. Proc. Natl. Acad. Sci. USA 110, 10240–10245 (2013).
5. Mjösberg, J. et al. The transcription factor GATA-3 is essential for the function of human type 2 innate lymphoid cells. Immunity 37, 649–659 (2012).
6. Moro, K. et al. Innate production of Tu2 cytokines by adipose tissue-associated c-Kit+Sca-1+ lymphoid cells. Nature 463, 540–544 (2010).
7. Neill, D.R. et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. Nature 464, 1367–1370 (2010).
8. Price, A.E. et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. Proc. Natl. Acad. Sci. USA 107, 11489–11494 (2010).
9. Satoh-Takayama, N. et al. Microbial flora drives interleukin 22 production in intestinal Nkp46+ cells that provide innate mucosal immune defense. Immunity 29, 958–970 (2008).
10. Buonocore, S. et al. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. Nature 464, 1371–1375 (2010).
11. Sonnenberg, G.F., Monticelli, L.A., EllTho, M.M., Fouser, L.A. & Artis, D. CD4+ lymphoid tissue-inducer cells promote innate immunity in the gut. Immunity 34, 122–134 (2011).
12. Klose, C.S. et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. Cell 157, 340–356 (2014).
13. Fuchs, A. et al. Intrathymic type 1 innate lymphoid cells are a unique subset of IL-12− and IL-15-responsive IFN-γ-producing cells. Immunity 38, 769–781 (2013).
14. Halim, T.Y. et al. Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. Immunity 40, 425–435 (2014).
15. Basu, R. et al. Th22 cells are an important source of IL-22 for host protection against enteropathogenic bacteria. Immunity 37, 1061–1075 (2012).
16. Qiu, J. et al. Group 3 innate lymphoid cells inhibit T-cell-mediated intestinal inflammation through aryl hydrocarbon receptor signaling and regulation of microflora. Immunity 39, 386–399 (2013).
17. Hepworth, M.R. et al. Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria. Nature 498, 113–117 (2013).
18. Ollmann, C.J. et al. MHCI-mediated dialog between group 2 innate lymphoid cells and CD4+ T cells potentiates type 2 immunity and promotes parasitic helminth expulsion. Immunity 41, 283–295 (2014).
19. Gasteiger, G. & Rudensky, A.Y. Interactions between innate and adaptive lymphocytes. Nat. Rev. Immunol. 14, 631–639 (2014).
20. Klose, C.S. et al. A T-bet gradient controls the fate and function of CCR6+RORγt+ innate lymphoid cells. Nature 494, 261–265 (2013).
21. Celli, M. et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. Nature 457, 722–725 (2009).
22. Constantinides, M.G., McDonald, B.D., Verhoef, P.A. & Bendelac, A. A committed precursor to innate lymphoid cells. Nature 508, 397–401 (2014).
23. Sawa, S. et al. Lineage relationship analysis of RORγt+ innate lymphoid cells. Science 330, 665–669 (2010).
24. Wang, L. et al. Distinct functions for the transcription factors Gata-3 and Thp10 during intrathymic differentiation of CD4+ T cells. Nat. Immunol. 9, 1122–1130 (2008).
25. Serafini, N. et al. Gata3 drives development of RORγt+ group 3 innate lymphoid cells. Immunity 37, 92–104 (2012).
26. Zhu, J. et al. Conditional deletion of Gata3 shows its essential function in Tu1-Tu2 responses. Nat. Immunol. 5, 1157–1165 (2004).
27. Eberl, G. & Littman, D.R. Thymic origin of intestinal eβ T cells revealed by fate mapping of RORγt− cells. Science 305, 248–251 (2004).
28. Sciumé, G. et al. Distinct requirements for T-bet in gut innate lymphoid cells. J. Exp. Med. 209, 2331–2338 (2012).
29. Rankin, I.C. et al. The transcription factor T-bet is essential for the development of Nkp46+ innate lymphocytes via the Notch pathway. Nat. Immunol. 14, 389–395 (2013).
30. Wei, G. et al. Genome-wide analyses of transcription factor Gata-3-mediated gene regulation in distinct T cell types. Immunity 35, 299–311 (2011).
31. Zhu, J. et al. Th22 cells. The transcription factor T-bet is induced by multiple pathways and prevents an endogenous Th2 cell program during Th1 cell responses. Immunity 37, 660–673 (2012).
32. Robinette, M.L. et al. Transcriptional programs define molecular characteristics of innate lymphoid cell classes and subsets. Nat. Immunol. 16, 306–317 (2015).
33. Qiu, J. et al. The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. Immunity 36, 92–104 (2012).
ONLINE METHODS

Mice. Gata3fl/fl and Gata3fl/fl-Cre-ERT2 mice on the C57BL/6 background have been described. For deletion of Gata3 in Gata3fl/fl-Cre-ERT2 mice, the mice were given intraperitoneal injection of 5 mg tamoxifen in corn oil every other day, three times, as described, and were analyzed at various times after the first injection. Gata3fl/fl mice were bred with RorγCre-mice on the C57BL/6 background (a gift originally from D.R. Littman (New York University), now also available as line 022791 from the Jackson Laboratory) for generation of the Gata3loxClox mouse strain. Some Gata3fl/fl and Gata3loxClox mice were further bred with Tg-hCD2-I2rra mice34 on the C57BL/6 background (provided by A. Singer (National Cancer Institute)) or Rag1−/− mice (line 146; Taconic) for the generation of either Gata3fl/fl and Gata3loxClox mice expressing the Il7ra transgene from human CD2 or Rag1−/− Gata3fl/fl and Rag1−/−Gata3loxClox mice, respectively. Some RorγCre- and Gata3loxClox mice were bred with the Rosa26Tdtomato reporter mice (line 007914;jax), in which a loxP–STOP codon–loxP–tdTomato cassette was targeted to the ubiquitously expressed Rosa26 locus. In the resultant mice, tdTomato+ cells represent cells that expressed and/or are expressing RORγt in wild-type and Gata3loxClox mice. Mice with sequence encoding GFP knocked into the Rorc locus (line 007571;jax), Rorcfl/fl mice (line 008771;jax) and Tbx21Cre mice (a gift originally from C. Dulac (Harvard), now also available as line 024507 from Jax) were purchased from Jackson Laboratory. CD45.1+ congenic mice (line 111; Taconic) were from the National Institute of Allergy and Infectious Diseases–Taconic repository. T-bet–ZsGreen reporter mice on a wild-type or Tbx21−/− background have been described. Some RORγt–E2-Crimson reporter mice were generated by a bacterial artificial chromosome (BAC) transgene strategy. Sequence encoding E2-Crimson was amplified by PCR from the vector plasmid pE2-Crimson-C1 (Clontech Laboratories) and was inserted at the ATG translation start site of the coding sequence of a truncated form of Roc in the BAC clone RP23-266131 by combination-mediated genetic engineering technology. The primer sequences were as follows: 5′-GC TGCTCTGGCTAATCCAGAAGAGGACAGGGCAATTTGCTCATG to human IL7Rα gene in ILC3 cells. All mice were bred and/or main -
tained in the National Institute of Allergy and Infectious Diseases specific-pathogen–free animal facility. All experiments used mice 6–16 weeks of age and protocols approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee.

C. rodentium infection. For infection with C. rodentium (formerly Citrobacter freundii, biotype 4280), C. rodentium strain DBS100 (provided by D. Artis (University of Pennsylvania)) was prepared by selection as a single colony and culture for 8 h in LB broth. Mice were inoculated with approximately 1 × 1010 colony-forming units of C. rodentium in 200 µl of PBS via oral gavage.

Cell preparation and stimulation. In the mixed–bone marrow chimera experiments, total bone marrow cells were recovered from CD45.1+ congenic mice (line 7; Taconic). Gata3fl/fl and Gata3loxClox mice or other donor mice and were adoptively transferred into sublethally irradiated (450 rads) Rag2−/− Il2rg−/− mice. For preparing and analyzing ILCs, we prepared splP and clp with the following steps. Small intestine and colon were harvested from euthanized mice and the contents were emptied. Peyer’s patches were removed from small intestine. Small intestine and colon were then opened longitudinally, cut into pieces 1 cm in length and shaken for 20 min at 37 °C in RPMI-1640 medium containing 3% FBS, 5 mM EDTA and 1 mM DTT, followed by vortexing three times with RPMI-1640 medium containing 2 mM EDTA for dissociation of epithelial cells. The remained fragments were minced with scissors and were digested at 37 °C for 30 min in RPMI-1640 medium containing 0.1 mg/ml Liberase and 0.01% DNase I. The digested tissues were passed through a 40-µm filter and centrifuged at 1,600 r.p.m. for 6 min and were resuspended in HBSS containing 3% FBS for further analysis. For analysis of cytokine production by ILC3 cells, isolated splP or clp lymphocytes were resuspended by RPMI medium containing 10% FBS and 2 ng/ml IL-7. In some experiments, cells were also stimulated with 2 ng/ml IL-23. The cells were incubated for 3 h at 37 °C in the presence of 2 mM monensin during the final 2 h.

Flow cytometry. Single-cell suspensions or stimulated cells were first incubated for 10 min with anti-CD16/32 (clone 2.4G2) for blockade of Fc receptors. Cell-surface molecules were then stained in HBSS with 3% FBS, followed by fixation. For intracellular staining of transcription factors, cells were fixed and permeabilized with a Foxp3 staining buffer set (eBioscience). For intracellular staining of cytokines, cells were fixed in 1% formalin saline and were permeabilized with 0.1% saponin buffer. Flow cytometry data were collected with an LSR II (BD Biosciences) and the results were analyzed with FlowJo software (Tree Star).

Antibodies specific to mouse CD3 (2C11), CD5 (55-7,3), CD19 (B93D1), Gr-1 (RB6-8C5), CD45R (RA3-6B2), CD4 (RM4-5), NKp46 (29A1.4), Sca-1 (C7), CD25 (eBio3C7), CD45.1 (A20), CD45.2 (104) and IL-22 (H22-IP) were purchased from eBioscience; antibodies specific to mouse CD127 (A7R34), CCR6 (29-2L17) and CD117 (2B8) were purchased from BioLegend; and antibodies specific to mouse RORγt (Q31-378), Gata3 (150-823) and T-bet (O4-46) were purchased from BD Biosciences.

Immunofluorescence staining. The ileum of the small intestine was prepared as a ‘Swiss roll’ and was treated for 12 h with fixation and permeabilization solution (554722; BD Bioscience), followed by dehydration in 30% sucrose, before being embedded in OCT freezing medium (Sakura Finetek). Sections 18 µm in thickness were cut on a CM3050S cryostat (Leica) and were made to adhere to Superfrost Plus slides (VWR). Frozen sections were permeabilized and then were blocked in PBS containing 0.1% Triton X-100 (Sigma) and 10% normal mouse serum (Jackson ImmunoResearch), followed by staining in PBS containing 0.1% Triton X-100 and 5% normal mouse serum. Mouse NKp46 was stained with polyclonal goat anti–mouse NKp46 serum (AF2225; R&D Systems), followed by Alexa Fluor 555–conjugated donkey antibody to goat (anti-goat; A21432; Molecular Probes). RORγt was stained with monoclonal antibody to human and/or mouse RORγt (AFK56-9; eBioscience), followed by Alexa Fluor 488–conjugated goat antibody to rat (anti-rat; A11080; Molecular Probes). Mouse epithelial cell adhesion molecule (EpCAM) and CD3 were stained with rat anti–mouse EpCAM (G8.8; BD Pharmingen) and rat anti-mouse CD3ε (17A2; BioLegend), respectively. After staining, slides are mounted with Fluormount G (Southern Biotech) and examined on a Leica TCS SP8 confocal microscope. Images were analyzed by Imaris software (Bitplane).

RNA-Seq. ChIP-Seq and data analysis. For RNA-Seq analysis of total ILC3 cells, live Lin− CD127+ Thy1+KLRG-1− cells were sorted from the splP of Gata3fl/fl and Gata3loxClox mice with a FACSAria. For RNA-Seq analysis of ILC3 subsets, distinct ILC subsets were sorted from the splP of Gata3fl/fl or Gata3loxClox RORγt–E2-Crimson and T-bet–ZsGreen dual-reporter mice. Total RNA was purified with the QIAzol lysis reagent and an miRNAsey Micro Kit (Qiagen). 10–20 ng of total RNA was amplified using an Ovation RNA-Seq System V2 (NuGEN). The double-stranded cDNA was then sonicated with a Bioruptor Pico (Diagenode) (30 s ‘on’ and 30 s ‘off’ for 20 cycles) to an average size of 200 base pairs. Indexed sequencing libraries were then generated from 250 ng sonicated cDNA with the KAPA LTP Library Preparation Kit (KAPA Biosystems) according to the manufacturer’s protocol, with minor modifications. Multiplex sequencing reads of 50 base pairs were generated by the NIHBI DNA Sequencing and Computational Biology Core. Sequencing reads were mapped to the mm9 assembly of the mouse genome (UCSC Genome Browser). Gene expression in each sample was calculated by RPMV values (reads per kilobase of exon per million total reads). Differentially expressed genes were identified by the edger software package with two cutoffs that
satisfied the following criteria: a change in expression of over fourfold (higher or lower) and an RPKM value of >10 in either sample, or a change in expression of over twofold (higher or lower) and an RPKM value of >5 in either sample.

For ChIP-Seq analysis, live Lin−CD127+Thy1hiKLRG-1−NK1.1− ILC3 cells were sorted from the siLP of Rag1−/− mice, live Lin−CD127+Thy1+KLRG-1+ ILC2 cells were sorted from mesenteric lymph nodes of Rag1−/− mice given injection of IL-25, and Th2 cells were prepared from naïve CD4+ T cells differentiated for three rounds as described. Cells were cross-linked with 1% formaldehyde for 10 min. Chromatin was prepared by sonication with Bioruptor Pico (30 s ‘on’ and 30 s ‘off’ for nine cycles) and was immunoprecipitated with magnetic beads coated with antibody to mouse immunoglobulin G (Diagenode) that had been pre-incubated with anti-GATA-3 (L50-823; BD Biosciences), through the use of iDeal ChIP-Seq Kit for transcription factors (Diagenode). DNA in the ChIP immunoprecipitates was made into an indexed library and was then sequenced as described above for RNA-Seq.

Statistics. Samples were compared with Prism 6 software (GraphPad) by a two-tailed unpaired Student’s t-test or one-way or two-way analysis of variance. A P value of <0.05 was considered significant.

34. Yu, Q., Erman, B., Park, J.H., Feigenbaum, L. & Singer, A. IL-7 receptor signals inhibit expression of transcription factors TCF-1, LEF-1, and RORγt: impact on thymocyte development. J. Exp. Med. 200, 797–803 (2004).