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The transcription factor GATA3 is critical for the development of all IL-7Rα-expressing innate lymphoid cells

Ryoji Yagi[1,#,$], **Chao Zhong**[1,#], **Daniel L. Northrup**[2,#], **Fang Yu**[1], **Nicolas Bouladoux**[3], **Sean Spencer**[3], **Gangqing Hu**[2], **Luke Barron**[3], **Suveena Sharma**[1], **Toshinori Nakayama**[4], **Yasmine Belkaid**[3], **Keji Zhao**[2], and **Jinfang Zhu**[1,*]

[1]Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA

[2]Systems Biology Center, National Heart, Lung and Blood Institute, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA

[3]Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA

[4]Department of Immunology, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan

SUMMARY

Innate lymphoid cells (ILCs) are critical in innate immune responses to pathogens and lymphoid organ development. IL-7Rα $^{+}$ ILC subsets, similar to CD4^{+}T helper (Th) cell subsets, produce distinct sets of effector cytokines. However, the molecular control of IL-7Rα $^{+}$ ILC development and maintenance is unclear. Here we report that GATA3 was indispensable for the development of all IL-7Rα $^{+}$ ILC subsets in addition to T cells, but not required for the development of classical NK cells. *Gata3* conditional deficient mice had no lymph nodes and were susceptible to *Citrobactor rodentium* infection. After the ILCs have fully developed, GATA3 remained important for the maintenance and functions of ILC2s. Genome-wide gene expression analyses indicated that GATA3 regulated a similar set of cytokines and receptors in Th2 cells and ILC2s, but not in ILC3s. Thus, GATA3 plays parallel roles in regulating the development and functions of CD4^{+} T cells and IL-7Rα $^{+}$ ILCs.

*Correspondence: jfzhu@niaid.nih.gov, Phone: 301-402-6662, Fax: 301-480-7352.

#These authors contributed equally to this work.

$Current address: Department of Immunology, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan.

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INTRODUCTION

$CD4^+$ T helper (Th) cells are central in orchestrating adaptive immune responses; distinct Th subsets are involved in protective immune responses to a variety of pathogens (Kanno et al., 2012; Zhu et al., 2010). For example, type 1 T helper (Th1) cells are critical for eradicating intracellular bacteria and viruses, whereas type 2 T helper (Th2) cells are indispensable for the expulsion of helminths. Interleukin-17 (IL-17)-producing Th (also known as Th17) cells are critical for defending against extracellular bacterial and fungal infections.

It usually takes several (5–10) days for antigen-specific $CD4^+$ T cells to expand from rare precursors in the naïve population and reach a meaningful number to execute host defense functions. Therefore, many innate effector cells including natural killer (NK) cells are responsible for early control of invading pathogens. Recently, a new class of innate effector cells, whose development relies on signaling through the IL-2 receptor (IL-2R) common γ chain and IL-7Rα, has drawn much attention. These cells, together with classical NK cells, are often referred to as innate lymphoid cells (ILCs) (Sonnenberg and Artis, 2012; Spits and Cupedo, 2012; Spits and Di Santo, 2011). Because distinct subsets of ILCs are capable of making the same characteristic effector cytokines as produced by different T helper cell subsets, they are similarly classified into type 1 innate lymphoid cells (ILC1s) including classical NK cells that produce interferon-γ (IFN-γ), type 2 innate lymphoid cells (ILC2s) that produce IL-5 and IL-13, and type 3 ILCs including lymphoid tissue inducer (LTi) cells that produce IL-17 and IL-22(Spits et al., 2013; Walker et al., 2013).

By producing Th2 cell effector cytokines such as IL-13, ILC2s play an important role during early immune responses to helminth infection (Fallon et al., 2006; Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Saenz et al., 2010). Mice with dysfunctional ILC2s have a significant delay in worm expulsion in *Nippostrongylus brasiliensis* infection whereas expanding the number of ILC2s by IL-25 injection can eliminate the need for Th2 cells in effective resistance to helminth infection. ILC2s are also important for allergen-induced airway inflammation and lung tissue repair in animal models (Chang et al., 2011; Halim et al., 2012a; Monticelli et al., 2011) and human cells corresponding to the ILC2s found in mice have been identified (Mjosberg et al., 2011). The ILCs that produce IL-17 and IL-22 also participate in the early phase of responses to infections and in inflammatory disorders (Buonocore et al., 2010; Lee et al., 2012; Powell et al., 2012; Satoh-Takayama et al., 2008). Thus, understanding the molecular mechanisms controlling the development and functions of ILCs is essential to develop strategies to control responses to pathogens and autoimmunity.

GATA3 is the key transcription factor for Th2 cell differentiation (Yagi et al., 2011). GATA3 expression is indispensable for proper induction of Th2 cytokines including IL-4, IL-5 and IL-13 both in vitro and in vivo (Zhu et al., 2004). Interestingly, GATA3 is critical not only for regulating Th2 cell differentiation, but also for $CD4^+$T cell development in the thymus at multiple stages (Ho et al., 2009; Pai et al., 2003; Ting et al., 1996).

It has been reported that GATA3 is highly expressed by ILC2 cells (Moro et al., 2010; Price et al., 2010). Conditional in activation of the *Gata3* gene with a transgenic Cre whose

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expression is driven by the *Il13* locus completely eliminated IL-13-producing ILC2 cells (Liang et al., 2012). GATA3 has been shown to be critical for the maintenance of ILC2 cell number and IL-13 production by these cells both in mice and in humans (Furusawa et al., 2013; Hoyler et al., 2012; Klein Wolterink et al., 2013; Mjosberg et al., 2012; Yang et al., 2013). However, because GATA3 affects ILC2 cell number, IL-13 regulation by GATA3 in the previous studies requires more careful assessment, i.e. at a single cell level with proper controls. Furthermore, other important target genes that are regulated by GATA3 in ILC2s are largely unknown. Finally, whether other ILC subsets require GATA3 to develop remains unclear.

Here we report that GATA3 is not only critical for T cell development, but also indispensable for the development of all the IL-7Rα^{+} ILC lineages including ILC2s, lymphoid tissue inducer (LTi) cells, IL-7Rα-expressing NK cells, Nkp46^{+}RORγt^{+}, and Nkp46^{+}RORγt^{-} ILCs. Both ILC2 progenitors and LTi progenitors were diminished in the absence of GATA3. Genome-wide analysis of GATA3-regulated genes in ILC2s and ILC3s suggests that GATA3 function during ILC development seems to be independent of many known key transcription factors including Id2, RORα, RORγt and Tcf7. GATA3 regulates many critical genes including *Il5*, *Il13*, *Il1rl1*, *Il2ra*, *Il9r* and *Ccr8*, but not *Klrg1*in ILC2s. Comparing to GATA3-regulated genes in Th2 cells, we found that while many genes, such as *Pth, Cysltr1, Htr1b and Tph1*, are regulated by GATA3 in a cell type specific manner, most of the key type 2 immune response related genes are regulated by GATA3 in both ILC2s and Th2 cells. These results demonstrate that GATA3 plays parallel roles in regulating the development and functions of CD4^{+} T cells and IL-7Rα^{+} ILCs, that is, the development of both CD4^{+}T cells and all IL-7Rα^{+} ILCs requires GATA3, and GATA3 is especially critical for the maintenance and functions of ILC2s, similar to its role in Th2 cells.

All ILCs express GATA3 at different amounts

To determine the tissue distribution of ILCs, and ILC2s in particular, we assessed lineage negative (Lin^{-}) CD127 (IL-7Rα) positive cells by flow cytometry analysis of many lymphoid tissues and organs. Lin^{-}CD127^{+} cells represented <1% of the total cells in spleen, mesenteric lymph nodes and in the lung, but somewhat enriched in the small intestine lamina propria (siLP, FigureS1A). Among these ILCs, some are ILC2s, identified by expression of T1/ST2 (IL-33R)(Moro et al., 2010; Neill et al., 2010). ILC2s (T1/ST2^{+}Lin^{-}CD127^{+}) constituted the largest fraction of recovered ILCs in the lung-derived cells (> 80% of total Lin^{-}CD127^{+} cells, Figure S1B). In contrast, only ~16% of Lin^{-}CD127^{+} cells in the spleen were T1/ST2^{+}. In the mesenteric lymph nodes, ~40% of the Lin^{-}CD127^{+} cells were T1/ST2^{+}.

To assess GATA3 expression in the various subsets of ILCs, we performed ex vivo GATA3 intracellular staining of Lin^{-}CD127^{+} cells harvested from different tissues. Most ILC2s expressed high levels of GATA3 whether they were from spleen, mesenteric lymph node, lung or siLP (Figure S1B). Interestingly, other ILCs (T1/ST2^{-}Lin^{-}CD127^{+}) expressed intermediate amounts of GATA3. In the siLP, most of the Lin^{-}CD127^{+}GATA3^{int} cells in the siLP are RORγt^{+} ILCs and all Lin^{-}CD127^{+}GATA3^{hi} cells express another ILC2

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marker, KLRG-1, but not RORγt (Figure S1C). Thus, all IL-7Rα ILCs express GATA3 albeit at different amounts.

GATA3 is indispensable for the development of all IL-7Rα$^+$ ILCs

To study the role of GATA3 in ILC development in vivo, we generated *Gata3*$^{fl/fl}$-Vav-Cre mice in which *Gata3* exon 4 is deleted in hematopoietic stem cells. As expected, no T cells were detected in *Gata3*$^{fl/fl}$-Vav-Cre mice (Figure 1A), confirming a previous report on the critical role of GATA3 in T cell development using germline *Gata3* deletion (Ting et al., 1996). In contrast, there was a modest but significant increase of B cells in *Gata3*$^{fl/fl}$-Vav-Cre mice (Figure 1A), arguing against a loss of stem cell function. In line with a previous report showing that GATA3 is critical for the development of IL-7Rα$^+$ NK cells (Vosshenrich et al., 2006), IL-7Rα$^+$NK1.1$^+$ cells including CD3ε$^-$IL-7Rα$^+$NK1.1$^+$ were absent in the spleen of *Gata3*$^{fl/fl}$-Vav-Cre mice, whereas classical IL-7Rα $^-$NK1.1$^+$ cells were not significantly affected (Figure 1A). Staining of cells from the lung of *Gata3*$^{fl/fl}$-Vav-Cre mice showed complete absence of ILC2s (Figure 1B). In the bone marrow of wild-type mice, T1/ST2$^+$ cells were detectable among the Lin$^-$CD127$^+$ population; these cells have been regarded as the progenitors of ILC2s and they were all GATA3$^+$ (Figure 1C). However, this population was absent in the bone marrow of *Gata3*$^{fl/fl}$-Vav-Cre mice (Figure 1C). Overall, our data indicate that GATA3 is indispensable for the development of ILC2s and T cells but not B cells or IL-7Rα-negative NK cells.

We also noticed a defect in lymphoid organ development in the *Gata3*$^{fl/fl}$-Vav-Cre mice; these mice had no lymph nodes including the brachial and axillary lymph nodes similar to the phenotype of RORγt deficient mice. This finding suggested that GATA3 regulates the development of LTi cells, which are Lin$^-$CD4$^+$RORγt$^+$ and critical for the development of lymph node structure (Eberl et al., 2004a; Sun et al., 2000). Indeed, Lin$^-$CD4$^+$RORγt$^+$ LTi cells, which are found with a high frequency in the siLP of *Gata3*$^{fl/fl}$ mice, were markedly reduced in the *Gata3*$^{fl/fl}$-Vav-Cre mice (Figure 2A). Furthermore, total Lin$^-$RORγt$^+$ cells were reduced in the siLP of *Gata3*$^{fl/fl}$-Vav-Cre mice (Figure 2B). In fact, IL-7Rα$^+$ cells, whether they express RORγt or not, were abolished in *Gata3*$^{fl/fl}$-Vav-Cre mice suggesting GATA3 is critical for development of all IL-7Rα-expressing ILCs. The loss of RORγt$^+$ ILCs is not due to a possible Cre-mediated genotoxicity since all the *Gata3*$^{fl/+}$-Vav-Cre mice had normal lymph node structure and these mice developed RORγt$^+$ ILCs normally (Figure S2).

LTi progenitors have been identified in the mouse fetal liver (Cherrier et al., 2012; Possot et al., 2011). To assess the function of GATA3 in the development of LTi progenitors, we stained fetal liver cells from *Gata3*$^{fl/fl}$-Vav-Cre embryos. Lin$^-$CD127$^+$ cells were present at a similar frequency and in total cell number between the *Gata3*$^{fl/fl}$ and *Gata3*$^{fl/fl}$-Vav-Cre fetal livers (Figure 2C). However, LTi progenitors that are Flt3$^-$α4β7$^+$CD127^{hi} were greatly diminished in *Gata3*$^{fl/fl}$-Vav-Cre fetal livers suggesting GATA3 is critical for the development of such cells. Together with the finding that GATA3 is required for the generation of ILC2 progenitors in the bone marrow (Figure 1C), our data suggest that GATA3 may act in a common innate lymphoid progenitor (CILP) that gives rise to all IL-7Ra-expressing ILCs.

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IL-7Rα negative ILCs found in the compartment of intraepithelial lymphocytes (IELs) have been reported, and these cells express both NK1.1 and Nkp46 (Fuchs et al., 2013). Interestingly, these ILCs were significant increased in *Gata3*$^{fl/fl}$-Vav-Cre mice compared to those in *Gata3*$^{fl/fl}$ mice (Figure 2D), indicating GATA3 is critical for the development of IL-7Rα^{+} ILCs, but not IL-7Rα-negative ILCs.

Gata3$^{fl/fl}$-Vav-Cre mice are susceptible to *Citrobacter rodentium* infection

IL-7Rα^{+} ILCs also include IL-22-producing ILCs, some of which express Nkp46. These cells play a critical role in host defense against *Citrobacter rodentium* infection. Thus, we tested whether *Gata3*$^{fl/fl}$-Vav-Cre mice are susceptible to *Citrobacter rodentium* infection. On day 4 after infection, a substantial number of Lin^{-}IL-7Rα^{+} cells, many of which expressed Nkp46, were detected in the large intestine lamina propria (liLP) of littermate mice (Figures 3A and 3B); these cells were absent in the liLP of the *Gata3*$^{fl/fl}$-Vav-Cre mice. Accordingly, *Gata3*$^{fl/fl}$-Vav-Cre mice had much higher bacterial load than their littermates (Figure 3C). All the *Gata3*$^{fl/fl}$-Vav-Cre mice died after *C. rodentium* infection whereas all the control mice survived (Figure 3D). These data further establish the role of GATA3 in the development of IL-22-producing ILCs.

GATA3 effect on the development of IL-7Rα $^{+}$ ILCs is cell intrinsic

Vav-Cre deletes floxed genes specifically in hematopoietic stem cells; therefore, the failure of ILC development in *Gata3*$^{fl/fl}$-Vav-Cre mice is not due to a GATA3 function in non-hematopoietic cells. To further confirm this, we performed mixed bone marrow chimera experiments to directly examine whether the influence of GATA3 on ILC development is cell intrinsic. As expected, GATA3 sufficient and deficient bone marrow cells efficiently repopulated CD45^{+} cells in the spleen of *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (Figure 4). However, GATA3-deficient bone marrow cells failed to give rise to IL-7Rα^{+} ILCs in the mixed bone marrow chimeras. In contrast, B cells developed normally, where as T cells failed to develop from GATA3-deficient bone marrow progenitors. These results indicate that the effect of GATA3 on the development of IL-7Rα^{+} ILCs is cell intrinsic.

GATA3 is required for maintaining ILC2 cell number both in vivo and in vitro

At first consideration, our results contradict an earlier report showing that GATA3 is required for the development of ILC2 but not other ILCs (Hoyler et al., 2012). However, in that study, *Gata3* gene was deleted by inducible Cre driven by *Id2* locus after all the ILCs had fully developed. Therefore, it is likely that the survival of ILC2s is sensitive to *Gata3* deletion. To test this possibility, we generated *Gata3*$^{fl/fl}$-CreER^{T2} mice and treated these mice with tamoxifen. Three weeks after treatment, we observed that only KLRG1^{+} ILC2s but not RORγt^{+} ILCs in the siLP were diminished (Figure 5A), confirming that GATA3 is critical only for the maintenance of ILC2s in vivo.

Since GATA3 is required for the development and maintenance of ILC2s in vivo, it is difficult to assess the function of GATA3 in ILC2s without obtaining a pure ILC2 population. ILC2s, although present at a low frequency, expand during type 2 immune responses, presumably as a result of stimulation by IL-25 and/or IL-33, which are produced by epithelial cells. Indeed, injection of IL-25 induced substantial expansion of ILC2s (Neill

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et al., 2010; Saenz et al., 2013). We also observed that IL-25 injection induced the expansion of ILC2s. Culturing sorted Lin^- cells harvested from mesenteric lymph nodes of IL-25-injected mice with IL-33, IL-25 and IL-7 in vitro further expanded these cells. After 5–7 days of culture of purified Lin^- cells, virtually all cells were $GATA3^{hi}$ (Figure S3A). These cells also expressed two cell-surface molecules, Sca-1 and KLRG1, which are highly expressed by ILC2 (Figures S3B and S3C). Therefore, we chose this approach in an effort to obtain large numbers of ILC2s at high purity; staining and/or sorting for T1/ST2 and IL-7Rα-expressing cells is not required until the end of the culture, alleviating the concern that antibody staining would affect IL-33 or IL-7 signaling.

To determine the function of GATA3 in ILC2s, we prepared ILC2s, as described in Fig. S3, from *Gata3*$^{fl/fl}$-CreER^{T2} or *Gata3*$^{fl/fl}$ mice. After 5 days of culture, samples were split into two groups and treated either with 4-hydroxytamoxifen (4-OHT), the active metabolite of tamoxifen that binds to CreER^{T2} to induce Cre activity, or with a vehicle control, ethanol (EtOH). To test whether GATA3 affects ILC2 maintenance in culture in a cell intrinsic manner, we mixed *Gata3*$^{fl/fl}$-CreER^{T2} or *Gata3*$^{fl/fl}$ ILC2s with ILC2s generated from CD45.1 congenic mice. After 4-OHT treatment, there was a progressive decrease in the percentage of *Gata3*-deficient ILC2s over a 6-day period (Figure 5B). To comprehensively profile the genes that are regulated by GATA3 in ILC2s at a genome-wide level, we sorted live ILC2s that had or had not undergone *Gata3* inactivation on day 2, a time point when no dramatic ILC2 loss was observed, and performed RNA-seq analyses. Interestingly, we found that many TNF and TNFR superfamily genes, such as *Tnfrsf9* (encoding 4–1BB which provides co-stimulation) and *Tnfsf21* (encoding DR6, which induces cell apoptosis), and $NF_{\kappa}b$ family members, including *Nfkb2* and *Relb*, showed altered expression patterns (Figure 5C). In addition, the cell cycle inhibitor, *Cdkn2b*, was upregulated upon *Gata3* inactivation (Figure 5C). These changes are consistent with and may contribute to the loss of *Gata3*-deficient ILC2s.

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GATA3 regulates many critical genes in ILC2s and in Th2 cells

RNA-seq result confirmed efficient *Gata3* inactivation by Cre-ERT2 after 4-OHT treatment since a nearly 100% reduction of the reads at the *Gata3* exon 4 was observed (Figure 6A). Interestingly, reads at the other exons of the *Gata3* gene were only modestly affected implying that GATA3 is not a major player in maintaining its own transcription in ILC2s (Figure 6A).

We then compared genes that are either positively or negatively regulated by GATA3 in ILC2s and in Th2 cells (Wei et al., 2011). A total of 32 genes that are positively regulated by GATA3 in Th2 cells were also affected in ILC2s upon *Gata3* inactivation (Figures 6B and 6C, **and** Table S1). *Il13* transcripts were dramatically reduced when GATA3 was absent in ILC2s (Figure 6C and Table S1). Other genes that are positively regulated by GATA3 in both ILC2s and Th2 cells include *Areg* (encoding Amphiregulin), *Il5*, *Ccr8* and *Lif* (Figure6Cand Table S1). Interestingly, *Il1rl1* (encoding IL-33R) and *Il9r* were expressed at much higher levels in ILC2s than in Th2 cells; their expression was dramatically reduced when *Gata3* gene was disrupted in ILC2s (Figure6Cand Table S1).

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We also identified 225 genes including *Il4, Maf*, *Pth* and *Ikzf3* (encoding Aiolos) that are positively regulated by GATA3 in Th2 cells but not affected by GATA3 in ILC2s (Figure6B). On the other hand, 130 genes are positively regulated by GATA3 in ILC2s but not in Th2 cells; they include *Icos, Il2ra, Kit, Il1r2, Cysltr1, Htr1b*and *Tph1*, many of which are cell surface markers of ILC2s (Figures 6B and 6D, **and** Table S1). These data indicate that GATA3 has unique functions in ILC2s and Th2 cells in addition to its common regulation of many type 2 effector cytokines.

Gata3 inactivation in Th2 cells results in de-repression of several Th1 cell-associated genes, such as *Fasl*, *Il12rb2* and *Stat4* (Table S1). However, *Gata3* inactivation in ILC2s did not alter the expression of these genes; instead, it de-repressed several other genes such as *Cd244* (encoding 2B4), *Lta* and *Tnf* (Figure 6E and Table S1) that are characteristic of type I ILCs and/or NK cells.

Gata3 inactivation did not reduce *Klrg1* transcription (Figure6F), suggesting that *Klrg1* is not a GATA3 target gene. Other cell surface molecules expressed by ILC2s include CD127, Sca-1 and Thy1. The expression of *Il7r* (encoding CD127) and *Ly6a/e* (encoding Sca-1) were reduced less than 2 fold, whereas *Thy1* expression was increased upon *Gata3* inactivation (Table S1). The expression of *Rora* and *Id2*, encoding two transcription factors critical for the development of ILC2s, were not reduced upon *Gata3* inactivation (Figure6F).

To determine whether GATA3 has a similar function in ILC2s at the steady state, we performed RNA-seq using ex vivo purified KLRG1^{+} ILC2s from tamoxifen-treated *Gata3*$^{fl/fl}$ or *Gata3*$^{fl/fl}$-Cre-ER^{T2} mice. The results indicate that among the 162 genes positively regulated by GATA3 in activated ILC2s, 57 genes were also positively regulated by GATA3 in “naïve” ILC2s (Figure 6G and Table S1). These include *Il5, Il13, Areg, Il1r1, Ccr8, Il2ra, Cysltr1, Tph1* and *Htr1b*. Interestingly, *Il10* and *Il24* were regulated by GATA3 in activated ILC2s but not in “naïve” ILC2s (Figure 6G) suggesting that GATA3-mediated regulation of *Il10* and *Il24* expression requires cofactors that are only present in activated ILC2s. *Il4* expression was affected by *Gata3* deletion in “naïve” ILC2s but not in activated ILC2s (Figure 6G), consistent with our previous finding that GATA3 is required for the expression of IL-4 in developing Th2 cells but not essential in fully differentiated Th2 cells.

To verify the RNA-seq data at the protein level, we performed flow cytometry analysis. Compared to control samples, GATA3 protein was lost in 4-OHT-treated *Gata3*$^{fl/fl}$-CreER^{T2} ILC2 culture as judged by intracellular staining (Figure S4A), implying that the deletion of the *Gata3* gene was efficient and the pre-existing GATA3 protein was degraded in ILC2s over a two-day period. By contrast, consistent with the RNA-seq data, two cell surface molecules highly expressed by ILC2s, KLRG1 and Sca-1, were only modestly, if at all affected, by inactivation of *Gata3* (Figures S4B and S4C), demonstrating that ILC2s maintain some features of their phenotype in the absence of GATA3. Consistent with the role of GATA3 in Th2 cells, IL-13 production by ILC2s in response to IL-33 and IL-7 stimulation was dramatically reduced upon *Gata3* inactivation (Figure S4D). In agreement with RNA-seq data, T1/ST2 surface expression was also abolished when *Gata3* was inactivated in ILC2s (Figure S4E). This result raised the question whether defective expression of IL-13 was due to a failure of the *Gata3*-deficient cells to respond to IL-33 or

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to a direct effect of GATA3 on *Il13*. Therefore, we stimulated these cells with PMA and ionomycin. Even with this potent stimulus, IL-13 production was dramatically decreased in *Gata3*-deficient cells (Figure S4F) suggesting that GATA3 directly regulates IL-13 production in ILC2s, independent of its role in maintaining IL-33R expression. This is consistent with the established capacity of GATA3 to directly bind to the promoter of the *Il13* gene in many T cell subsets (Wei et al., 2011).

GATA3 regulates common and unique sets of genes in ILC2s and ILC3s

To determine the GATA3 target genes in ILC3s, we performed RNA-seq on ILC3s with or without *Gata3* deletion. Ninety-eight and 81 genes were either positively or negatively regulated by GATA3 in ILC3s. Among the 98 genes that were positively regulated by GATA3 in ILC3s, 29 of them, including *Atf3*, *Ccr9*, *Ets2* and *Nr4a2*, were also positively regulated by GATA3 in ILC2s (Figure 7A and Table S1). Similarly, among the 81 genes that are negatively regulated byGATA3 in ILC3s, 17 of them are negatively regulated by GATA3 in ILC2s. The functions of these 46 genes that were commonly regulated by GATA3 in ILC2s and ILC3s require further investigation. There are many more genes regulated by GATA3 in ILC2s than in ILC3s possibly because GATA3 is expressed at much higher amounts in ILC2s than that in ILC3s. Some genes encoding cytokine receptors that were regulated by GATA3 in ILC2s, such as *Il1rl1*, *Il2ra* and *Il9r*, were not regulated by GATA3 in ILC3s. The expression of key transcription factors that were reported to be critical for the development of ILC3s, including *Rorc, Runx1, Runx3, Ahr, Id2* and *Tcf7*, was not affected by *Gata3* deletion in ILC3s (Figure 7B). Together with the finding that GATA3 does not affect the expression of *Id2* and *Rora* in ILC2s (Figure 6F), our data indicate that the mechanism of GATA3 in regulating the development of IL-7Rα-expressing ILCs is independent of, or in parallel with, all the known key transcription factors.

DISCUSSION

Research on the ILCs has exploded since the description and characterization of the ILC2s in 2010 by four different groups (Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Saenz et al., 2010) and it is now well accepted that ILCs play important roles in innate immunity by producing effector cytokines(Spits et al., 2013). Since these effector cytokines are virtually identical to those that are produced by T helper cells, ILCs can be divided into group 1, 2, and 3 ILCs representing innate versions of Th1, Th2, and Th17-like cells, respectively (Spits et al., 2013). There are also similarities in the development of ILCs and T helper cells because the key transcription factor that determines a particular Th cell fate is also critically involved in the development of its ILC counterpart (Bernink et al., 2013; Eberl et al., 2004b; Hoyler et al., 2012; Klose et al., 2013; Mjosberg et al., 2012; Sciume et al., 2012; Spits et al., 2013; Vonarbourg et al., 2010). However, much less is known about the early development of ILCs. Our observation, that the development of all IL-7Rα^{+} ILCs but not classical NK cells requires GATA3, suggests that all IL-7Rα^{+} ILC subsets may have a common innate lymphoid progenitor (CILP). Furthermore, by comparing gene expression in mature ILC2s with or without GATA3 expression at a genome-wide level, we have provided important data that are crucial for further understanding the development and functions of ILC2s during type 2 immune responses.

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Previous reports suggest that ILC2s can be identified by a combination of several cell surface markers, including T1/ST2 (IL-33R), IL-25R, ICOS, Kit, Sca-1 and KLRG-1 in addition to IL-2Rα and IL-7Rα (Halim et al., 2012b; Hoyler et al., 2012; Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Saenz et al., 2010; Wong et al., 2012). We have shown that GATA3 regulates most of these molecules. However, the expression of IL-7Rα and Sca-1 is only modestly reduced, and KLRG-1 is not affected by *Gata3* inactivation. In addition to known ILC2 cell surface markers, we also identified other cell surface molecules including IL-9R, CCR8, Cysteinyl leukotriene receptor 1 (Cysltr1), 5-hydroxytryptamine (serotonin) receptor 1B (Htr1b) and TNFR superfamily members 4-1BB and RANK, whose expression in ILC2s is GATA3 dependent. Cysltr1 is expressed by ILC2s and leukotriene D_4 is able to induce the expression of IL-5, IL-13 as well as IL-4(Doherty et al., 2013). Therefore, GATA3 may indirectly affect type 2 cytokine production in vivo by regulating two stimulating receptors, T1/ST2 and Cysltr1. Overall, our data indicate that while GATA3 regulates many of the distinguishing cell surface markers on ILC2s, other transcription factors are also involved. Identification of other factors that regulate KLRG1 expression may add important insights into the transcription factor networks controlling the development and maturation of ILC2s.

GATA3 plays critical roles in the differentiation and maintenance of both Th2 cells and ILC2s, and thus is important for mediating type 2 immune responses at different stages. Through genome-wide profiling of the genes that are regulated by GATA3 in ILC2s, we find a substantial overlap of the genes that are regulated by GATA3 in Th2 cells and in ILC2s. Most strikingly, these commonly regulated genes include many key effector cytokines and receptors, such as *Il13, Il5, Areg, Il10, Il24, Lif, Il1rl1, Il9r and Ccr8*. Except for *Il10* and *Il24*that are expressed only in activated ILC2s, all other key cytokines and receptors are also regulated by GATA3 in "naïve" ILC2s at the steady state. IL-4 is regulated by GATA3 in "naïve" ILC2s but not cultured ILC2s, consistent with our previous findings in T cells that GATA3 is important for the induction of IL-4 during Th2 cell differentiation but plays a minor role in IL-4 production in already developed Th2 cells. Thus, our data indicate that ILC2s resemble the innate counterpart of Th2 cells at a genomic scale and that GATA3 regulates the key functions shared by ILC2s and Th2 cells, possibly through a similar mechanism.

GATA3 is thought to be critical for controlling the cell fate of ILC2 but not other ILCs (Hoyler et al., 2012). The discrepancy between this report and our study can be explained by the timing of *Gata3* inactivation. In that study, Id2-driven Cre-ERT2 was used to conditionally delete *Gata3* by tamoxifen in otherwise normal mice. Because all the ILCs have already developed in these mice before *Gata3* inactivation and the half-life of ILCs may be longer than 3 weeks, the specific effect on ILC2s 3 weeks after GATA3 removal only confirms that GATA3 is critical for the maintenance of ILC2s but not other ILCs. Indeed, we were also able to show that tamoxifen-induced *Gata3* deletion in *Gata3*$^{fl/fl}$-CreER^{T2} mice abolished ILC2s but had no effect on RORγt^{+} ILCs once they had developed. Therefore, acute *Gata3* ablation after ILCs have developed obscures the role we have revealed here for GATA3 during development of ILCs. Vav-Cre-mediated gene

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inactivation disrupts *Gata3* gene before ILCs have developed, allowing us to conclude that GATA3 is critical for the development of all IL-7Rα$^+$ ILCs including ILC2s.

All the known subsets of ILCs and NK cells depend on Id2 expression for their development (Moro et al., 2010; Yokota et al., 1999). While E4BP4 (also known as NF-IL3) is critical for the development of NK cells (Gascoyne et al., 2009; Kamizono et al., 2009), no severe ILC development defect has been reported in mice deficient in E4BP4. For the development of IL-7Rα$^+$ ILC subsets, RORα is a transcription factor specifically required for inducing ILC2s(Halim et al., 2012b; Wong et al., 2012) but not RORγt-expressing ILCs(Halim et al., 2012b). On the other hand, RORγt is only needed for the development of RORγt-expressing ILCs(Eberl et al., 2004a; Halim et al., 2012b) but not ILC2s(Halim et al., 2012b; Moro et al., 2010). Up to now, the transcription factor critical for the development of all the IL-7Rα$^+$ ILC lineages but not NK cells has not been identified. Here we report that GATA3 is such a transcription factor. GATA3 does not seem to regulate Id2, RORa or ROR t, suggesting that GATA3 may collaborate with Id2 for ILC development in general, with RORa for ILC2 development, and with RORγt for ILC3 development. Our data indicate that different IL-7Rα$^+$ ILC subsets may develop through a GATA3-dependent mechanism from a common progenitor. Thus, a hematopoietic branch point, with E4BP4 committing progenitors to the NK cell lineage and GATA3 leading to the development of IL-7Rα$^+$ ILCs, may exist. Therefore, it is reasonable to consider that IL-7Rα$^+$ ILC subsets resemble innate version of CD4$^+$ T helper subsets, whereas conventional NK cells, which have higher cytotoxic activity than other IFNγ-expressing ILCs, represent innate version of CD8$^+$ T cells.

Our study show that there is an excellent symmetry in ILC and T helper cell development: while GATA3 is regarded as the master regulator for Th2 cells, it is also critical for the maintenance and functions of ILC2s; while GATA3 is critical for the development of all CD4$^+$ T cells(Ho et al., 2009), it is also indispensable for the development of all IL-7Rα$^+$ ILC subsets. GATA3, therefore, plays parallel roles in establishing and regulating both adaptive and innate lymphocyte populations.

Experimental Procedures

Mice

Gata3$^{fl/fl}$ mice on C57BL/6 background were previously described(Yagi et al., 2010). These mice were bred with either CreER^{T2}(Taconic line 10471) or Vav-Cre transgenic mice (JAX line 8610)on a C57BL/6 background to generate *Gata3*$^{fl/fl}$-CreER^{T2} or *Gata3*$^{fl/fl}$-Vav-Cre lines. C57BL/6 mice were ordered from Taconic. CD45.1 congenic mice (Line 7) and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (Line 4111) were from Taconic-NIAID contract. All the mice were bred and/or maintained in the NIAID specific pathogen free animal facility and the experiments were done when mice were 8 to 16 weeks of age under protocols approved by the NIAID Animal Care and Use Committee.

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Citrobacter rodentium infections

C. rodentium (formerly *Citrobacter freundii*, biotype 4280) strain DBS100 was prepared by selecting a single colony and culturing in LB broth for 8 hours. Mice were inoculated with approximately 1×10^{10} CFU in 200 μL of PBS via oral gavage. Cells from large intestinal lamina propria were prepared as previously described(Sun et al., 2007). CFU were determined via serial dilutions on MacConkey's agar from overnight cultures of homogenized fecal pellets.

Cell preparation

Single cell suspensions were prepared directly from fetal livers or different lymphoid organs of mice including lymph nodes, spleen and bone marrow. Cells from small intestinal lamina propria and intraepithelial lymphocytes (IELs) were prepared as previously described (Sun et al., 2007). To prepare cells from tissues such as lung, mice were perfused with PBS before organs were harvested. The lung was cut into small pieces and digested with DNaseI (Roche) and Liberase (Roche) for 30 min at 37°C. Single cell suspension from digested lung was subjected to Percoll-density gradient centrifugation. In some experiments, recombinant murine IL-25 (from either eBioscience or PeproTech, 0.4 ug per mouse) in PBS was injected intraperitoneally into mice for three consecutive days before mesenteric lymph nodes were harvested. In other experiments, tamoxifen (Sigma) was injected intraperitoneally into mice every other day for 5 times (5mg tamoxifen in 150 ul corn oil per mouse per injection). In mixed bone marrow chimera experiments, bone marrow cells from *Gata3*$^{fl/fl}$ or *Gata3*$^{fl/fl}$-Vav-Cre mice were mixed with bone marrow cells from CD45.1 congenic mice (Line 7) at 1:1 ratio and injected (10 million per mouse) into sublethally irradiated (450 Rad) *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (Line 4111). Cells were stained with a cocktail of antibodies to various lineage markers, including antibodies to CD3, CD5, CD45R, CD11b, CD11c, NK1.1, Gr-1, TER119, FcεRI and TCRγ/δ to identify lineage negative (Lin^{-}) cells. Lin^{-} cells were sorted using a FACS Aria and then cultured with IL-7 (10ng/ml), IL-33 (10ng/ml) and IL-25 (10ng/ml) for ~ 1 week. In some experiments, cells were treated with 100 nM 4-hydroxytamoxifen (4-OHT) after 5 days of culture to delete *Gata3* gene from *Gata3*$^{fl/fl}$-CreER^{T2} cells.

Flow cytometry (FACS) analysis

Cell surface molecules and cytokine intracellular staining was performed as previously described (Zhu et al., 2004). Staining for transcription factors was carried out with Foxp3 Staining Buffer Set (eBioscience) according to the manufacturer's instructions. Flow cytometry data were collected with LSR II (BD Biosciences) and results were analyzed using FlowJo software (Tree Star). Antibodies specific for mouse CD5 (53–7.3), CD45R (RA3-6B2), Gr-1 (RB6-8C5), NK1.1 (PK136), TCRγ/δ (UC7-13D5) and an erythroid cell marker (TER119), were purchased from BioLegend; antibodies specific for mouse CD3 (2C11), CD4 (RM4-5), CD11b (M1/70), CD11c (N418), CD127 (A7R34), CD45.1 (A20), CD45.2 (104), FcεRI (MAR-1), IL-13 (eBio13A), KLRG1 (2F1), NKp46 (29A1.4), RORγt (AFKJS-9), Sca-1 (D7), Flt3 (A2F10), α4β7 (DATK32) and c-Kit (2B8) were purchased from eBiosciences; antibodies specific for Thy1.2 (53–2.1) and GATA3 (L50-823) were

purchased from BD Biosciences; and antibodies for IL-33R (DJ8) were purchased from MD Bioproducts.

RNA-Seq and data analysis

Lin$^-$ cells were sorted from *Gata3*$^{fl/fl}$-CreER^{T2} mice that had been injected with IL-25 for 3 days. After 5 days culture in IL-7, IL-25 and IL-33, cells were either treated with 4-OHT or ethanol as a control for 2 days. *Gata3*-sufficient ILC2s were sorted from ethanol treated samples. *Gata3*-deficient cells were sorted from 4-OHT treated samples excluding residual T1/ST2-expressing cells. In other experiments, *Gata3*$^{fl/fl}$ or *Gata3*$^{fl/fl}$-CreER^{T2} mice were injected with tamoxifen (5mg/mouse). Two to three days later, Lin$^-$Thy1$^+$CD127$^+$KLRG1$^+$ (mostly ILC2s) and Lin$^-$Thy1$^+$CD127$^+$KLRG1$^-$ (mostly ILC3s) were sorted from small intestine lamina propria. RNA-Seq experiments were performed. Briefly, 100 ng of total RNA was amplified using the Ovation RNA-Seq system V2 kit (NuGEN). The resulting dsDNA was sonicated to 200–400 bp. 250 ng of sonicated DNA was used to prepare sequencing libraries for multiplex sequencing (Illumina) and 50 bp reads were generated by the NHLBI Sequencing and Genomics core. Sequencing reads were mapped to the mm9 genome. Gene expression levels were measured by RPKM (Reads Per Kilobase of exon per Million reads in library) (Mortazavi et al., 2008). Differentially expressed genes were identified by edgeR (Robinson et al., 2010) with criteria of false discovery rate (FDR)< 0.001, fold change (FC)> 2 and RPKM >3 in either *Gata3*-sufficient or deficient samples. Data are available in the Gene Expression Omnibus (GEO) database under the accession number GSE47851.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. GATA3 is critical for the development of all IL-7Rα-expressing ILCs.
2. *Gata3* conditional deficient mice fail to develop lymph node structure.
3. GATA3 is essential for the maintenance of ILC2s but not ILC3s.
4. Genome wide analysis indicates that GATA3 regulates Th2-related genes in ILC2s.

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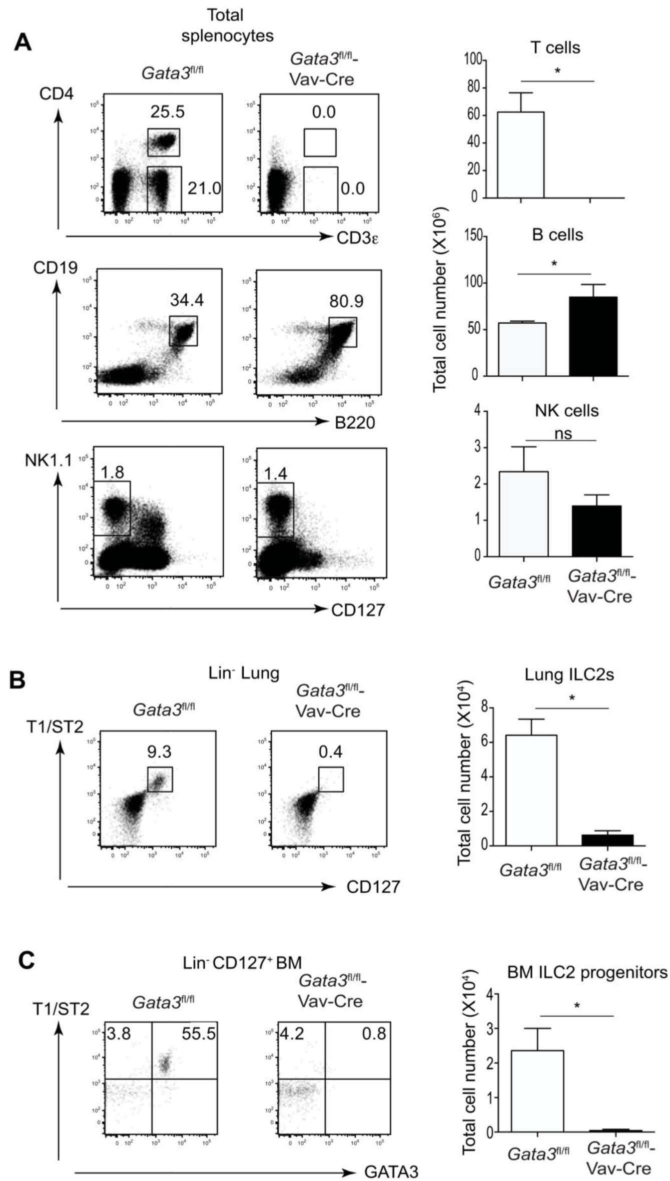

Figure 1. GATA3 is critical for the development of T cells and ILC2s, but not B cells and classical NK cells

(A) Total splenocytes from *Gata3*$^{fl/fl}$ or *Gata3*$^{fl/fl}$-Vav-Cre mice (3 mice per group) were stained for CD3ε, CD4, CD19, B220, NK1.1 and CD127. Dot plots (left panel) and total cell numbers of T, B and NK cells were shown (right panel).

(B) Cells prepared from the lungs of *Gata3*$^{fl/fl}$ or *Gata3*$^{fl/fl}$-Vav-Cre mice (3 mice per group) were stained with a cocktail of antibodies to various lineage markers, CD127 and T1/ST2. Dot plots gated on Lin$^-$ cells (left panel) and total cell numbers of lung ILC2s were shown (right panel).

(C) Cells prepared from bone marrows of *Gata3*$^{fl/fl}$ or *Gata3*$^{fl/fl}$-Vav-Cre mice (3 mice per group) were stained with a cocktail of antibodies to various lineage markers, CD127, T1/ST2 and GATA3. Dot plots gated on Lin$^-$ (left panel) and total cell numbers of ILC2 progenitors in the bone marrow were shown (right panel). Numbers indicate the percentages

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in each box or quadrant. Error bars represent Mean ± SD. “ns” indicates non-significant. “*” indicates $p<0.05$. Data are representative of three independent experiments.
See also Figure S1.

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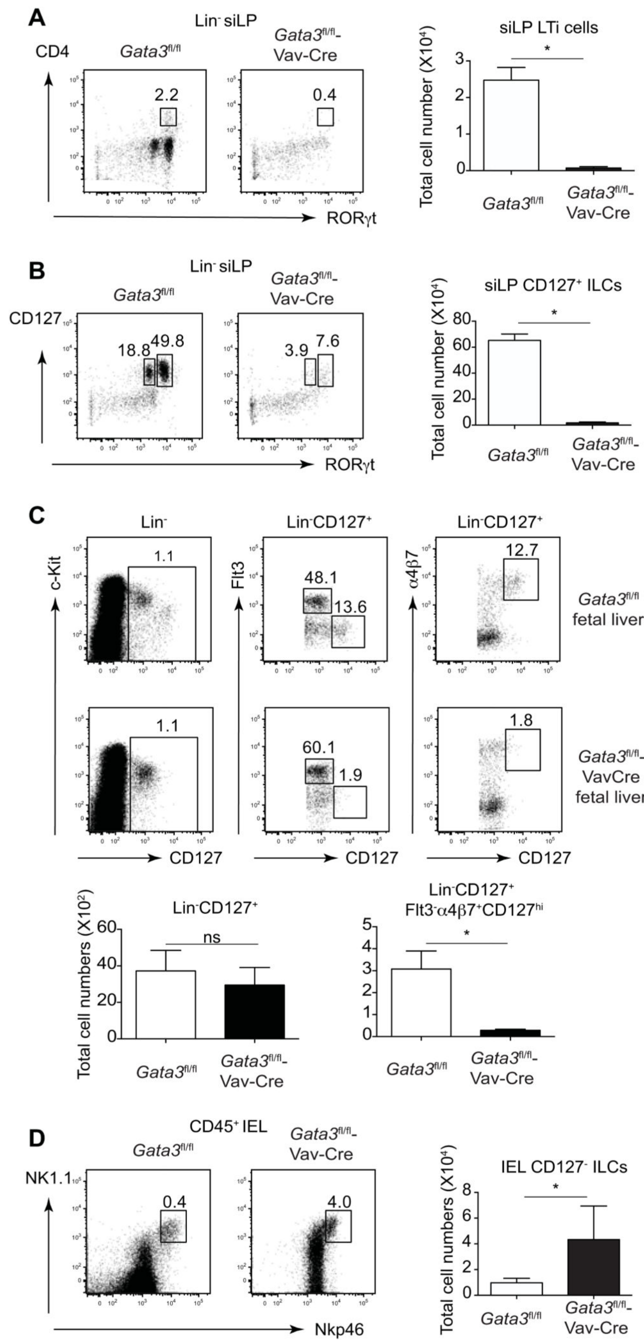

Figure 2. GATA3 is critical for the development of all IL-7Rα $^+$ ILCs but not IL-7Rα$^-$ ILCs

(A) Cells prepared fromsmall intestine lamina propria (siLP) of *Gata3*$^{fl/fl}$ or *Gata3*$^{fl/fl}$-Vav-Cre mice (3 mice per group) were stained with a cocktail of antibodies to various lineage markers, CD4 and RORγt. Plots gated on Lin$^-$ cells (left panel) and total cell numbers of LTi cells (right panel) were shown.

(B) Cells prepared from siLP of *Gata3*$^{fl/fl}$ or *Gata3*$^{fl/fl}$-Vav-Cre mice (3 mice per group) were stained with a cocktail of antibodies to various lineage markers, CD127 and RORγt.

Plots gated on Lin^- cells (left panel) and total cell numbers of IL-7Rα^+ ILCs (right panel) were shown.

(C) Fetal liver CLPs (Lin^-CD127^+) from *Gata3*$^{fl/fl}$ (n=6) or *Gata3*$^{fl/fl}$-Vav-Cre (n=2) embryos at embryonic day 15.5 (E15.5) were fractionated by the expression of Flt3 and $\alpha4\beta7$ expression. Plots were gated on Lin^- (upper left panels) or Lin^-CD127^+ cells (upper middle and right panels). Total cell numbers of CLPs (Lin^-CD127^+) cells and LTi progenitors ($Lin^-CD127^+Flt3^-\alpha4\beta7^+CD127^{hi}$) were calculated (lower panels). (D) Intraepithelial lymphocytes (IELs) prepared from *Gata3*$^{fl/fl}$ or *Gata3*$^{fl/fl}$-Vav-Cre mice (3 mice per group) were stained with a cocktail of antibodies to various lineage markers, CD45, NK1.1 and Nkp46. Plots gated on $CD45^+$ cells (left panel) and total cell numbers of IEL ILCs (right panel) were shown.

Numbers indicate the percentages in each box. Error bars represent Mean ±SD. “ns” indicates non-significant. “*” indicates $p<0.05$. Data are representative of three independent experiments (A and B) and two independent experiments (C and D). See also Figure S2.

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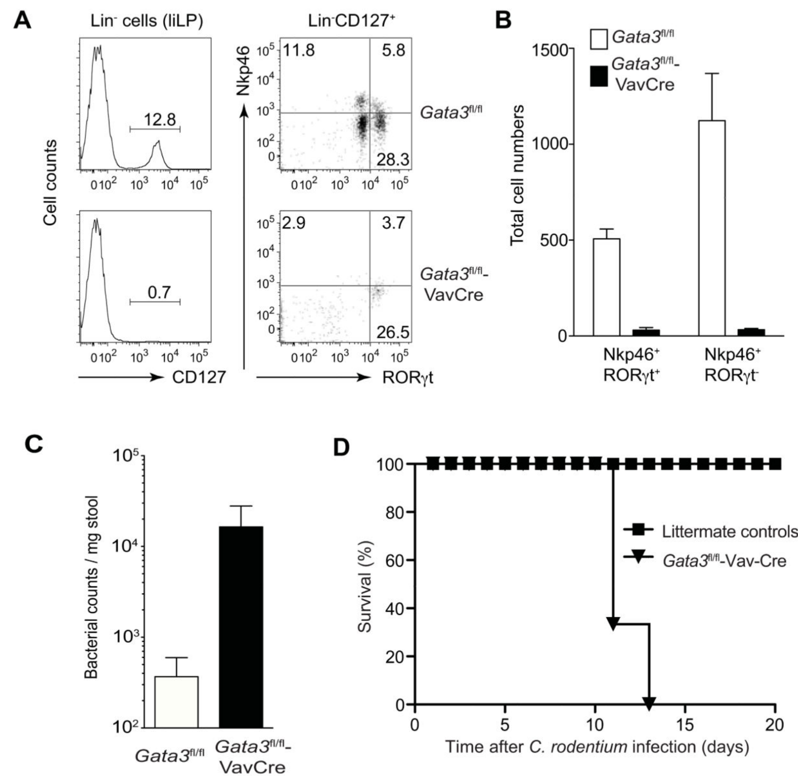

Figure 3. GATA3 deficiency results in susceptibility to *Citrobacter rondentium* infection

Gata3$^{fl/fl}$ or *Gata3*$^{fl/fl}$-Vav-Cre mice (3–4 mice per group) were orally infected with *Citrobacter rondentium*. Four days after infection (A–C), cells were harvested from the large intestine lamina propria (liLP) and stained with a cocktail of antibodies to various lineage markers and CD127, RORγt and Nkp46.

(A) Plots were gated on Lin$^-$ cells (left panel) or Lin$^-$ CD127$^+$cells (right panel).

(B) Absolute number of Nkp46$^+$ populations from liLP of each mouse was plotted.

(C) Feces were collected to assess bacterial loads. Numbers indicate the percentages in each quadrant or gate. Error bars represent Mean ± SD.

(D) Survival curves for the *Gata3*$^{fl/fl}$ or *Gata3*$^{fl/fl}$-Vav-Cre mice after infection. A–C represents one experiment and D represents another experiment.

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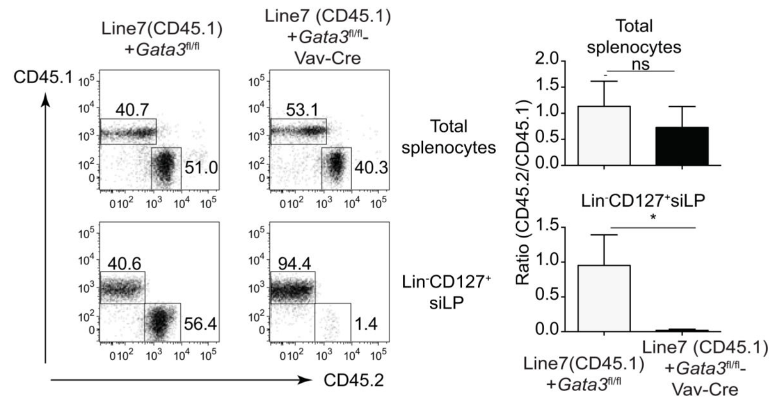

Figure 4. GATA3 effect on the development of IL-7Rα$^+$ ILCs is cell intrinsic

Bone marrow cells from *Gata3*$^{fl/fl}$ or *Gata3*$^{fl/fl}$-Vav-Cre were mixed with bone marrow cells from CD45.1 congenic (Line7) mice and co-transferred into irradiated *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice. Eight weeks after mixed bone marrow transplant, cells were harvested from spleen or siLP of chimeric mice (3 mice per group) and stained with CD45.1 and CD45.2 together with a cocktail of antibodies to various lineage markers and CD127. Plots were gated on total splenocytes (upper panel), or Lin$^-$ CD127$^+$ cells from siLP (lower panel). Ratio of CD45.2/CD45.1 was calculated and shown on the right. Numbers indicate the percentages in each box. Error bars represent Mean ± SD. “ns” indicates non-significant.“*” indicates $p<0.05$.

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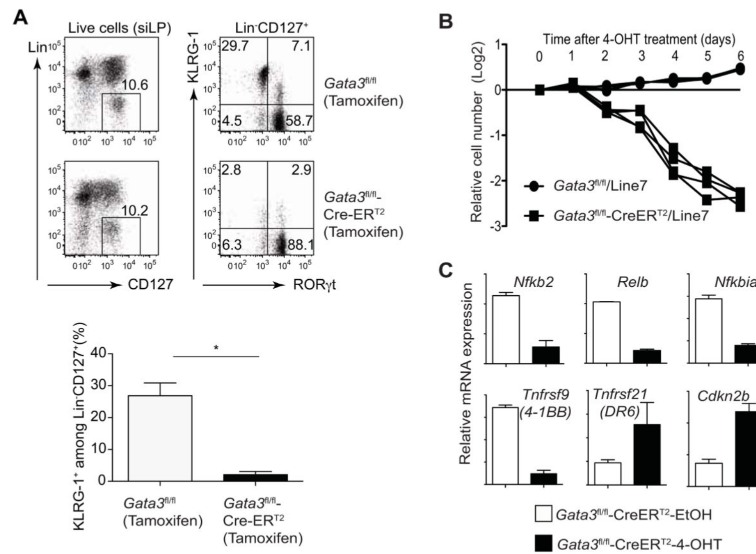

Figure 5. GATA3 is critical for the maintenance of ILC2 cell numbers both in vivo and in vitro

(A) *Gata3*$^{fl/fl}$ or *Gata3*$^{fl/fl}$-CreER^{T2} mice (2–3 mice per group) were injected 5 times with tamoxifen i.p., every other day. Three weeks after first injection, siLP cells were prepared and stained with a cocktail of antibodies to various lineage markers, CD127 (IL-7Rα), KLRG-1 and RORγt. Plots were gated on live cells (left panel) or Lin^{-}CD127^{+} cells (right panel). Numbers indicate the percentages in each quadrant.

(B) Lineage negative cells were purified from mesenteric lymph node of IL-25-treated *Gata3*$^{fl/fl}$-CreER^{T2}, *Gata3*$^{fl/fl}$ and CD45.1 congenic (Line7) mice (2–3 mice per group) by cell sorting. After cultured with IL-7, IL-25 and IL-33 for 5 days, they were mixed as indicated at a 1:1 ratio and then treated with 4-hydroxytamoxifen (4-OHT). The relative cell numbers were calculated based on the change of ratio over a period of 6 days. Four independently mixed cultures were tested in both groups. Data are representative of two independent experiments.

(C) Lineage negative cells were purified from mesenteric lymph node of IL-25-treated *Gata3*$^{fl/fl}$-CreER^{T2} mice (2–3 mice per group in duplicates) by cell sorting. After cultured with IL-7, IL-25 and IL-33 for 5 days, they were then treated with either 4-hydroxytamoxifen (4-OHT) or ethanol (EtOH) for 2 days. RNA-seq analysis was carried out. Relative mRNA expression of several genes was calculated based on the RNA-seq results with duplicates.

Error bars represent Mean ± SD.

See also Figure S3.

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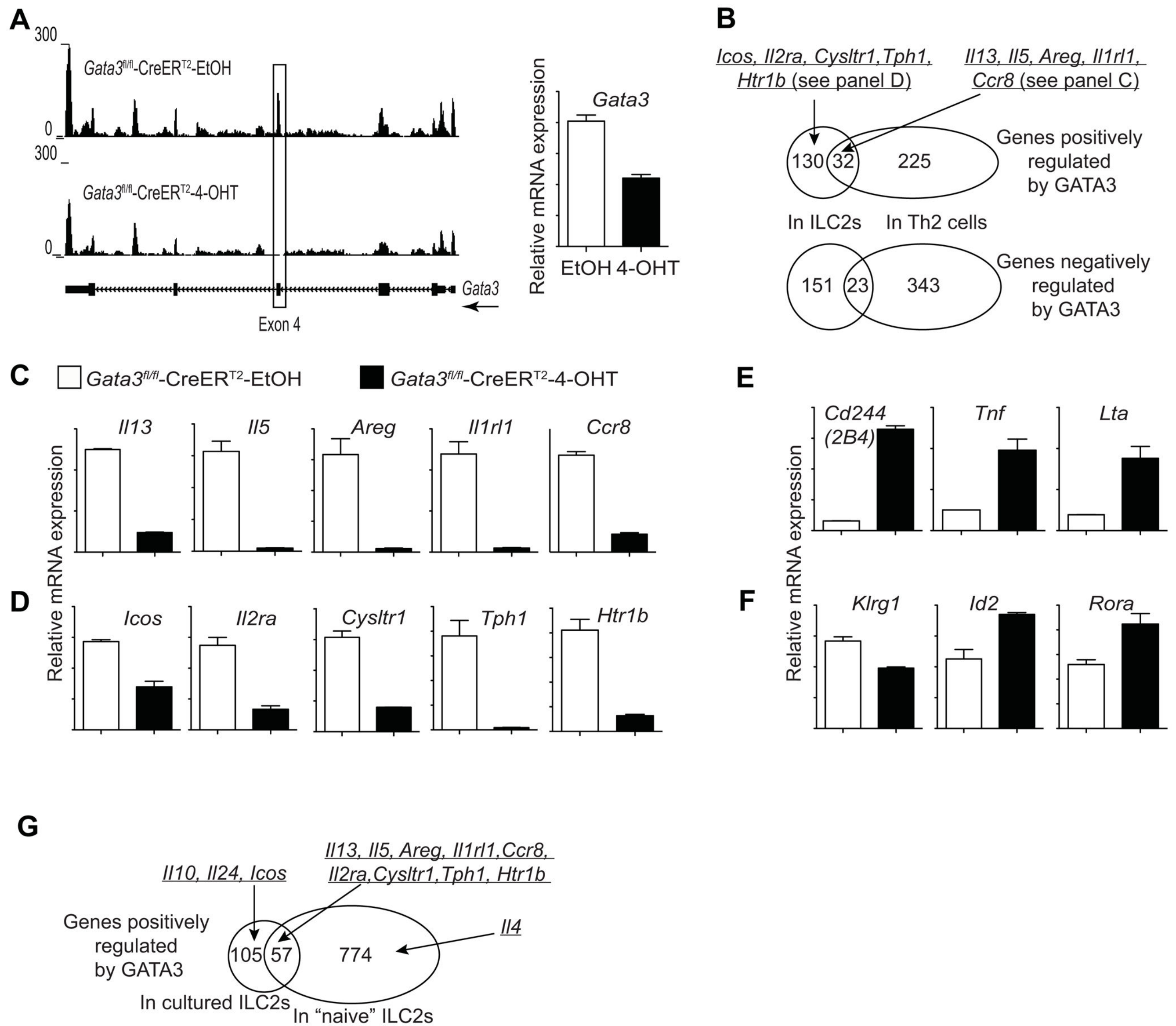

Figure 6. GATA3 positively and negatively regulates the expression of many critical genes in ILC2s

(A–F) Lineage negative cells were purified from mesenteric lymph node of IL-25-treated *Gata3*$^{fl/fl}$-CreER^{T2} mice (2–3 mice per group in duplicates) by cell sorting. After cultured with IL-7, IL-25 and IL-33 for 5 days, they were then treated with either 4-hydroxytamoxifen (4-OHT) or ethanol (EtOH) for 2 days.

(A) UCSC genome browser view of the RNA-seq data at the *Gata3* locus (A, left panel). Boxed area indicates the deletion of exon 4 by Cre-ER^{T2}. Relative *Gata3* mRNA expression was calculated based on the RPKM values obtained from RNA-seq results with duplicates (A, right panel).

(B) Venn diagrams showing overlap of total genes that are positively (upper) or negatively (lower) regulated by GATA3 in ILC2s and Th2 cells.

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(C–F) Representative genes regulated by GATA3 in different categories were shown. Relative mRNA expression was calculated based on the RPKM values obtained from RNA-seq results with duplicates.
(C) Examples represent 32 genes that were positively regulated by GATA3 in both ILC2s and Th2 cells.
(D) Examples represent 130 genes that were positively regulated by GATA3 in ILC2s but not in Th2 cells.
(E) Examples represent 151 genes that were negatively regulated by GATA3 in ILC2s but not in Th2 cells.
(F) Expression of *Klrg1*, *Id2* and *Rora* was not affected by *Gata3* inactivation in ILC2s.
(G) *Gata3*$^{fl/fl}$ or *Gata3*$^{fl/fl}$-CreER^{T2} mice (4 mice per group) were treated with tamoxifen in vivo for 2–3 days and then ILC2s were sorted based on KLRG1 expression. RNA-seq analyses were performed with *Gata3*-sufficient and *Gata3*-deficient cells. The Venn diagram showing overlap of total genes that are positively regulated by GATA3 in cultured ILC2s and ILC2s at the steady state.
Error bars represent Mean ± SD.
See also Figure S4 and Table S1.

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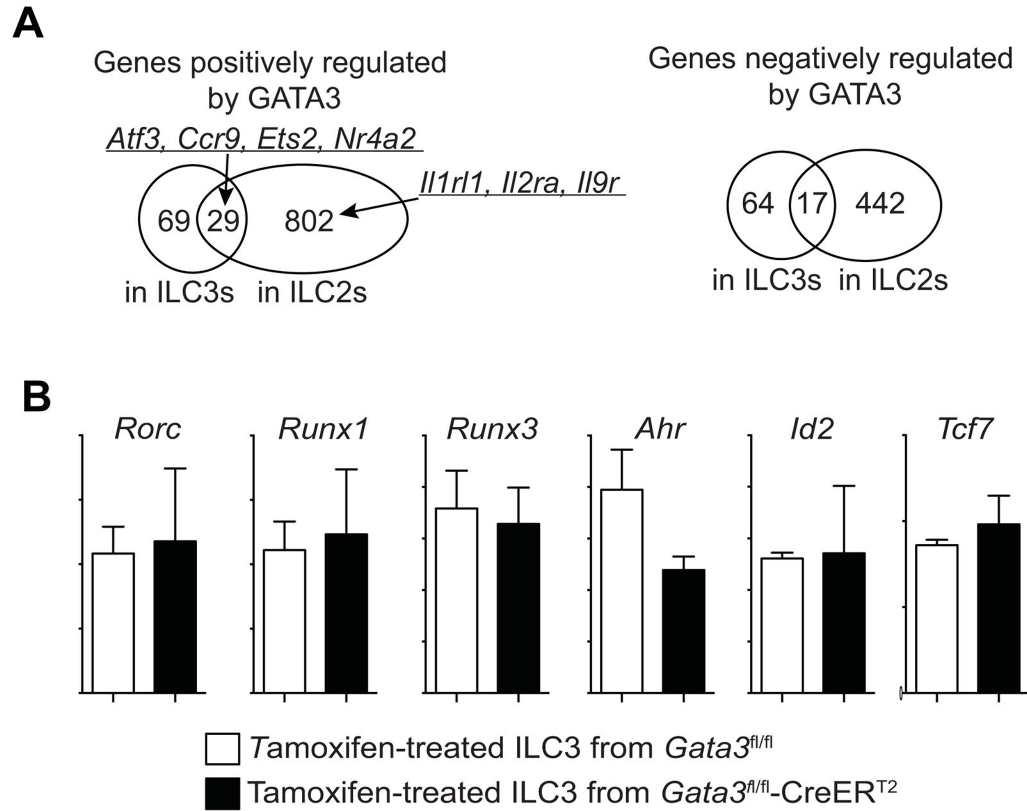

Figure 7. GATA3 does not regulate known key transcription factors in ILC3s

(A) *Gata3*$^{fl/fl}$ or *Gata3*$^{fl/fl}$-CreER^{T2} mice (4 mice per group) were treated with tamoxifen in vivo for 2–3 days and then KLRG1 negative ILCs (mostly ILC3s) were sorted. RNA-seq analyses were performed with *Gata3*-sufficent and *Gata3*-deficient ILC3s. RNA-seq analyses were also performed with *Gata3*-sufficent and *Gata3*-deficient ILC2s as described in Fig. 6G. The Venn diagram showing overlap of total genes that are positively or negatively regulated by GATA3 in ILC2s and ILC3s.

(B) Expression of *Rorc*, *Runx1, Runx3, Id2, Ahr* and *Tcf7* was not affected by *Gata3* inactivation in ILC3s. Error bars represent Mean ± SD.