Immunity
Article

The Transcription Factor GATA-3 Controls Cell Fate and Maintenance of Type 2 Innate Lymphoid Cells

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http://dx.doi.org/10.1016/j.immuni.2012.06.020

SUMMARY

Innate lymphoid cells (ILCs) reside at mucosal surfaces and control immunity to intestinal infections. Type 2 innate lymphoid cells (ILC2s) produce cytokines such as IL-5 and IL-13, are required for immune defense against helminth infections, and are involved in the pathogenesis of airway hyperreactivity. Here, we have investigated the role of the transcription factor GATA-3 for ILC2 differentiation and maintenance. We showed that ILC2s and their lineage-specified bone marrow precursors (ILC2Ps), as identified here, were characterized by continuous high expression of GATA-3. Analysis of mice with temporary deletion of GATA-3 in all ILCs showed that GATA-3 was required for the differentiation and maintenance of ILC2s but not for RORγt$^+$ ILCs. Thus, our data demonstrate that GATA-3 is essential for ILC2 fate decisions and reveal similarities between the transcriptional programs controlling ILC and T helper cell fates.

INTRODUCTION

Immunity to infections requires a highly coordinated response by the innate and adaptive immune systems. In order to deal efficiently with different types of pathogens, distinct effector programs are initiated. For example, intracellular infections lead to the induction of a T helper (Th) cell response characterized by the expression of IFN-γ (i.e., Th1 cells). Immunity against worm infections is, in part, coordinated by Th2 cells that release cytokines such as IL-4, IL-5, and IL-13. Such diverse Th cell responses are instructed by a distinct set of signals from the innate immune system. Fate decisions of Th cells are controlled by the induction of cell fate-determining transcription factors such as T-bet, GATA-binding protein 3 (GATA-3), or retinoic acid receptor-related orphan receptor (ROR)γt for Th1, Th2, or Th17 cells, respectively. Strikingly similar effector programs exist within populations of innate lymphocytes.

A group of cells, widely referred to as innate lymphoid cells (ILCs), has attracted particular attention recently (Spits and Di Santo, 2011). They consist of RORγt-expressing lymphoid tissue inducer (LTi) cells (also referred to as RORγt$^+$ ILCs) and type 2 ILCs (ILC2s). In contrast to IL-22- and IL-17-producing RORγt$^+$ ILCs, ILC2s can produce IL-5 and IL-13 and possibly also IL-4 (Saenz et al., 2010). Such an effector profile is reminiscent of Th2 cells and this ILC subset is variably referred to as natural helper cells (Moro et al., 2010), nuocytes (Neill et al., 2010), ILC2s (Spits and Di Santo, 2011), or type 2 innate helper (Ih2) cells (Price et al., 2010). We will refer to these cells as ILC2s. Similar to the biological role of Th2 cells, ILC2s are involved in the defense against worm infections (Liang et al., 2012; Moro et al., 2010; Neill et al., 2010) and in tissue repair after influenza virus infection (Monticelli et al., 2011). They also have a role in autoimmunity, specifically the initiation of airway hyperreactivity (Chang et al., 2011). The transcription factors determining ILC2 fate are currently not known and ILC2s are, therefore, defined by (1) various cell surface markers (i.e., IL-7Rα, Sca1, Kit, ICOS); (2) the expression of receptors for the cytokines IL-33 (ST1-T2) and IL-25 (IL-17RB); or (3) their production of the "type 2" cytokines IL-5 and IL-13. GATA-3 is a double zinc-finger transcription factor that is required for the effector fate decision of Th2 cells (Zheng and Flavell, 1997; Zhu et al., 2004). Various reports have shown that ILC2s contain *Gata-3* transcripts (Moro et al., 2010; Price et al., 2010; Yang et al., 2011) and GATA-3 was expressed by subsets of ILC2 after *Nippostrongylus* (*N.*) *brasiliensis* infection, suggesting that GATA-3 may be upregulated after infection (Liang et al., 2012). It remains a fundamental and unresolved issue as to whether GATA-3 is essential for lineage commitment and/or differentiation of ILC2s, comparable to the role of RORγt for the differentiation and function of RORγt$^+$ ILCs.

Although RORγt$^+$ ILCs and ILC2s have distinct functional profiles, they share developmental requirements, indicating developmental relationships. Both ILC subsets develop from the common lymphoid progenitor (CLP) in a process that requires Notch signaling (Possot et al., 2011; Wong et al., 2012). Development of the two ILC lineages and of NK cells but not of B or T cells requires the inhibitor of DNA binding 2 (Id2), suggesting that innate lymphocytes may have a common Id2-dependent progenitor (Moro et al., 2010; Yokota et al., 1999). Id2 is a helix-loop-helix (HLH) protein, which heterodimerizes with E proteins, that then can no longer initiate transcription of target genes because their DNA binding is impaired (Boos et al., 2007; Kee, 2009). Although the requirement of Id2 for the development of innate lymphocytes is well recognized, its expression by ILCs remains uncharacterized, as does the precise stage during differentiation at which induction occurs.

To assess the role of GATA-3 in the differentiation, maturation, and function of ILC2s, we utilized Id2 and GATA-3 reporter mice and also genetically modified mice, allowing for controlled temporary deletion of *Gata-3* in all innate lymphocyte subsets and in T cells (*Gata-3*$^{i\Delta ILC,T}$ mice). Although all innate lymphocytes (i.e., RORγt$^+$ ILCs, ILC2s, and NK cells) expressed high amounts of Id2, high GATA-3 expression was an exclusive and continuous attribute of all ILC2s. We also identified a GATA-3^{hi} population among lineage marker-negative (Lin$^-$) Sca1^{hi}Id2^{hi} cells in the bone marrow that we refer to as Lin$^-$Sca1^{hi}Id2^{hi} GATA-3^{hi} (LSIG) cells. Transfer studies and clonal in vitro cultures revealed that LSIG cells constitute a lineage-specified progenitor to mature ILC2s that we have termed the ILC2 progenitor, ILC2P. Genome-wide transcriptome profiling demonstrated that ILC2Ps are highly related to ILC2s but lacked expression of maturation markers such as the killer lectin-like receptor (KLR)G1 and of type 2 cytokine genes (i.e., IL-5 and IL-13). Induced deletion of GATA-3 in ILCs led to a selective loss of mature ILC2s, as well as of the ILC2Ps in the bone marrow, whereas development of the RORγt$^+$ ILC lineage was not affected. These data demonstrate that GATA-3 is required for the differentiation of ILC2Ps and the maintenance of mature ILC2s. Our data establish GATA-3 both as a transcription factor continuously expressed by all ILC2s and as being essential for ILC2 fate decisions.

RESULTS

ILC2s Are GATA-3-Expressing Innate Lymphocytes

To date, ILC2s are defined by a combination of cell surface markers (e.g., Sca1, CD25, and CD127) and their propensity to produce IL-5 and IL-13. However, these cell surface markers are nonspecific markers also expressed by other innate lymphocyte subsets (e.g., RORγt$^+$ ILCs) and lymphoid precursors (Buonocore et al., 2010; Sawa et al., 2010; Vonarbourg et al., 2010). We tested our hypothesis that GATA-3 is the lineage-defining transcription factor of ILC2s by examining GATA-3 expression by CD25$^+$Sca1^{hi} or CD127$^+$Sca1^{hi} cells among CD3$^-$CD19$^-$ cells, marker combinations widely used to "define" ILC2s (Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Saenz et al., 2010). CD25$^+$Sca1^{hi} cells found in the lamina propria of the small intestine were largely GATA-3 positive (79.6% ± 7.1%) (Figure 1A). The CD127$^+$Sca1^{hi} subset was less well defined but still showed GATA-3 expression in more than 60% of the population (64.8% ± 11.4%) (Figure 1A).

We next examined whether costaining with GATA-3 and Sca1 was sufficient to define ILC2s. Staining of leukocytes with antibodies specific for GATA-3 and Sca1 revealed a well-defined population of GATA-3^{hi}Sca1^{hi} cells that was prominently represented in the lamina propria of small intestine and colon and, to a lesser extent, the mesenteric lymph nodes (mLNs) and lungs (Figures 1B and S1A and S1B available online). GATA-3^{hi}Sca1^{hi} cells in the CD3$^-$CD19$^-$ subset coexpressed all of the markers normally associated with ILCs, such as CD25 (Figure 1B), CD127, CD90 (Thy1), and Kit (Figure 1C). GATA-3^{hi}Sca1^{hi} cells also displayed more specific ILC2 markers, for instance, the receptor for the cytokine IL-33 (T1-ST2) (Figure 1C). Importantly, GATA-3^{hi} ILCs did not express markers associated with either the myeloid lineage or IL-33R-expressing granulocytes (FcεRI) (Figure S1C). In addition, GATA-3^{hi} ILCs were negative for markers of RORγt$^+$ ILCs such as CD4, RORγt, or CCR6, and they did not express NK cell receptors (e.g., NKG2D or NK1.1) or CD122 expressed by all NK cells (Figure S1D). We noticed a population of Sca1$^{int\text{-}hi}$ innate (CD3$^-$CD19$^-$) lymphocytes in the small intestine that expressed intermediate levels of GATA-3 (GATA-3^{int}) (Figure S1E). Further characterization revealed that the majority of these were RORγt or NKp46-expressing cells of the NK and RORγt$^+$ ILC lineage, respectively (Figure S1E). Thus, the markers typically used to define ILC2s also define GATA-3^{hi} ILCs. The other innate lymphocyte lineages and myeloid cells did not contain a GATA-3^{hi} population.

ILC2s are functionally defined by their expression of IL-5 and IL-13. We investigated whether GATA-3^{hi} ILCs could also produce these cytokines. We used mice expressing green fluorescent protein (GFP) under the control of the *Gata-3* promoter (Grote et al., 2006). Direct comparison of GATA-3 reporter expression and intracellular staining with a GATA-3-specific antibody revealed that the fluorescent reporter faithfully marked all GATA-3$^+$ cells (Figure S1F). GATA-3^{hi} ILCs, but not GATA-3$^-$ or GATA-3^{int} innate lymphocytes, produced IL-5 and IL-13 after incubation with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Figures 1D and 1E). Thus, IL-5 and IL-13 expression by ILCs was strictly correlated with high GATA-3 expression. In contrast, GATA-3^{int} cells but not GATA-3^{hi} cells released IL-22 and IFN-γ, cytokines produced by RORγt$^+$ ILCs and NK cells, respectively (Figure 1D). These data further demonstrate that GATA-3^{int} cells also do not fulfill the functional criteria of ILC2s.

Further support for our hypothesis that ILC2s are GATA-3^{hi} ILCs arose from the analysis of their representation in various gene-deficient mouse strains. Consistent with previous findings regarding ILC2 development (Moro et al., 2010), GATA-3^{hi} ILCs were reduced in *Il7r*$^{-/-}$ and *Il7*$^{-/-}$ mice as well as in alymphoid *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (Figures 1F and 1G). In contrast, mice genetically lacking (1) recombination activating gene 2 (*Rag2*$^{-/-}$ mice lacking B and T cell development); (2) IL-15 (*Il15*$^{-/-}$ mice lacking the NK cell lineage); (3) RORγt (*Rorc(γt)*$^{-/-}$) or aryl hydrocarbon receptor (*Ahr*$^{-/-}$), both of which have reduced intestinal RORγt$^+$ ILCs; and (4) the thymic stromal lymphopoietin receptor (*Tslpr*$^{-/-}$) showed normal development and differentiation of GATA-3^{hi} ILCs (Figures 1F and 1G). Collectively, our data demonstrate that GATA-3-expressing ILCs are functionally, phenotypically, and developmentally identical to the ILC2 subset.

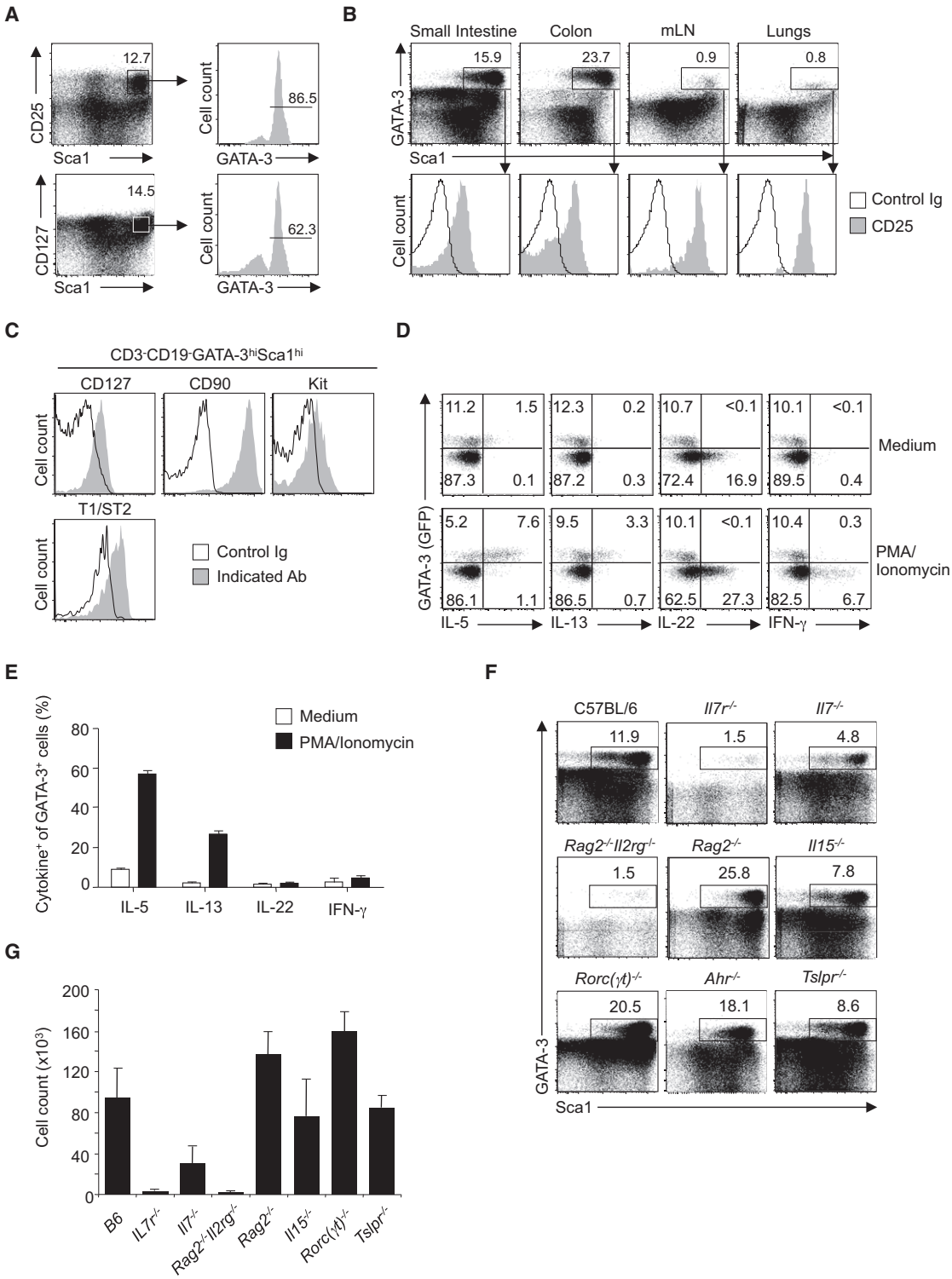

Figure 1. ILC2 Cells Are GATA-3-Expressing Innate Lymphoid Cells

(A) Expression of GATA-3 by CD25^{+}Sca1^{hi} (top) or CD127^{+}Sca1^{hi} (bottom) lamina propria lymphocytes from the small intestine. Dot plots were gated on CD45^{+}CD3^{-}CD19^{-} lymphocytes. Numbers represent percentages of cells in the indicated gates.

(B) Representative flow cytometry analyses of Sca1 and GATA-3 expression (top) by CD45^{+}CD3^{-}CD19^{-} lymphocytes from the indicated organs. Histograms (bottom) represent staining with CD25 antibody (gray) or control Ig (open) by electronically gated GATA-3^{hi}Sca1^{hi} cells. Numbers represent percentage of cells in quadrant.

(C) Lamina propria lymphocytes from the small intestine were costained with CD3, CD19, Sca1, and GATA-3 antibodies and with antibodies specific for the indicated markers (gray) or isotype control Ab (open). Histograms are electronically gated on Sca1^{hi}GATA-3^{hi} cells within the CD45^{+}CD3^{-}CD19^{-} population.

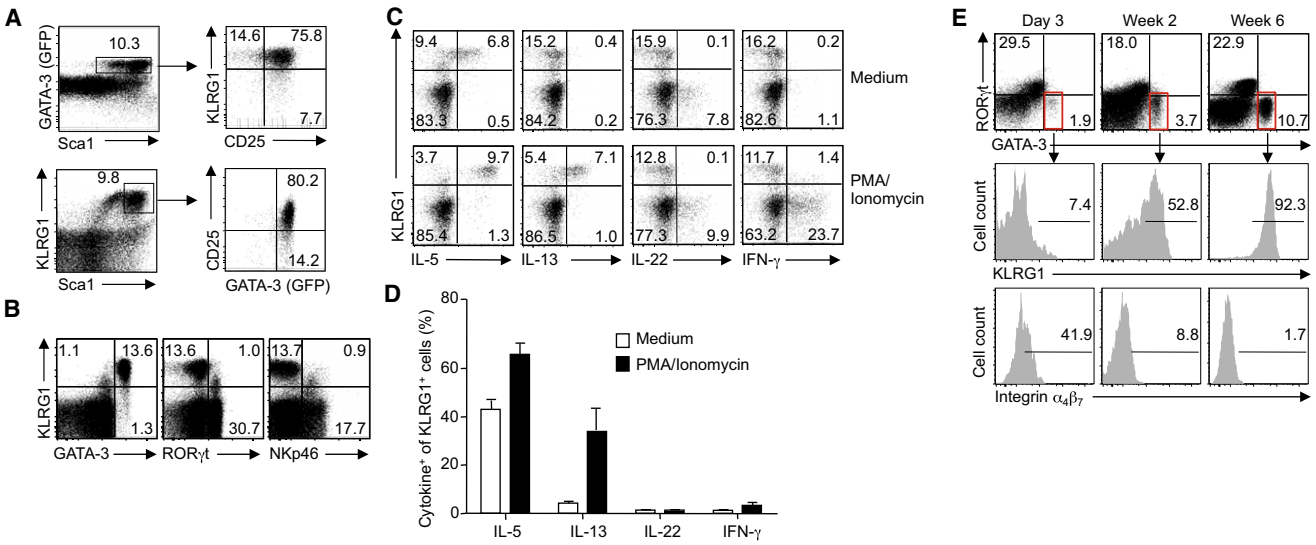

Figure 2. GATA-3^{hi} ILCs Express KLRG1

(A) Coexpression of KLRG1 and CD25 or CD25 and GATA-3 by electronically gated GATA-3^{hi}Sca1^{hi} (top) or KLRG1^{hi}Sca1^{hi} (bottom) CD45$^+$CD3$^-$CD19$^-$ lamina propria leukocytes. Numbers indicate percent of cells in the indicated regions.

(B) Flow cytometry analysis of KLRG1 coexpression with the indicated cell surface markers and transcription factors by CD45$^+$CD3$^-$CD19$^-$ lamina propria leukocytes. Numbers represent percentage of cells per quadrant.

(C and D) Intracellular cytokine staining of lamina propria lymphocytes after 4 hr stimulation with PMA and ionomycin.

(C) Dot plots are gated on CD45$^+$CD3$^-$CD19$^-$CD90$^+$ lymphocytes. Numbers represent percentage of cells per quadrant.

(D) Percentage (±SEM; n = 6) of cytokine-producing GATA-3^{hi} ILCs with (black bars) and without (white bars) stimulation.

(E) Lamina propria cells from mice at the indicated age were analyzed for GATA-3 and RORγt expression (top). Dot plots are electronically gated on CD45$^+$CD3$^-$CD19$^-$CD127$^+$ cells. Numbers represent percentage of cells per quadrant. Histograms represent expression of KLRG1 and integrin $\alpha_4\beta_7$ by electronically gated GATA-3^{hi}RORγt$^-$ cells.

All data shown are representative of at least three independent experiments.

KLRG1 Is a Marker of Mature ILC2s

Upon screening a large panel of cell surface markers to identify genes coexpressed by GATA-3^{hi} ILCs, we found that KLRG1 was expressed by virtually all ILC2s in adult mice (Figures 2A and 2B). This is consistent with previously published data showing elevated KLRG1 mRNA expression in ILC2s (Neill et al., 2010). Indeed, virtually all GATA-3^{hi}Sca1^{hi} cells were KLRG1 positive (Figure 2A, top) and all KLRG1^{hi}Sca1^{hi} cells were GATA-3^{hi} ILCs (Figure 2A, bottom). In further support of this view, IL-5 and IL-13 production was found only in KLRG1$^+$ cells. KLRG1$^-$ cells were producers of IL-22 and IFN-γ (Figures 2C and 2D). NKp46$^+$ NK cells of the small intestine were found to be KLRG1 negative (Figure 2B). This is consistent with our previous data (Sanos et al., 2009) and in contrast to splenic NK cells (Hanke et al., 1998). Thus, KLRG1 can be described as a reasonably selective marker of mature intestinal ILC2s.

We observed that ~10% of ILC2s were KLRG1 negative (Figures 2A and 2B). KLRG1 has been recognized as a marker of mature lymphocytes (Huntington et al., 2007; Voehringer et al., 2001), prompting us to analyze KLRG1 expression by ILC2s during ontogeny. Although RORγt$^+$ ILCs were substantially represented among CD3$^-$CD19$^-$ cells in the intestinal lamina propria of newborn mice, only a few GATA-3^{hi} ILCs were present (Figure 2E). However, their fraction steadily increased during the first 2 months after birth (Figure 2E). Intriguingly, GATA-3^{hi} ILCs of newborn mice expressed substantial levels of integrin $\alpha_4\beta_7$, an integrin required for lymphocyte homing to and retention in the intestine (Gorfu et al., 2009). Increasing age brought about a steady decline in integrin $\alpha_4\beta_7$ levels (Figure 2E). These findings suggest a maturation program of ILC2s characterized by the acquisition of KLRG1 and a loss of integrin $\alpha_4\beta_7$.

Lin$^-$Id2^{hi} Bone Marrow Lymphocytes Coexpress High Amounts of GATA-3 and Sca1

It has been reported that ILC2s are the progeny of bone marrow CLPs (Wong et al., 2012; Yang et al., 2011). To obtain further insights into the distinct stages of ILC2 differentiation, we analyzed GATA-3 and Id2 expression by hematopoietic precursor cells (lineage marker-negative cells) in the bone marrow. We chose to monitor Id2 in conjunction with GATA-3 for two

(D and E) Intracellular cytokine staining of lamina propria lymphocytes from *Gata-3*$^{Gfp/+}$ mice after 4 hr stimulation with PMA and ionomycin.

(D) Dot plots are gated on CD3$^-$CD19$^-$CD45$^+$CD90$^+$ lymphocytes. Numbers represent percentage of cells per quadrant.

(E) Percentage (±SEM; n = 5) of cytokine-producing GATA-3^{hi} ILCs with (black bars) and without (white bars) stimulation.

(F) Flow cytometry analysis of GATA-3 and Sca1 expression by CD45$^+$CD3$^-$CD19$^-$ cells from the intestinal lamina propria of the indicated mouse strains.

(G) Absolute cell numbers of GATA-3^{hi}Sca1^{hi} ILCs (±SD; n = 4) in the lamina propria of the small intestine isolated from the indicated mouse strains.

Data are representative of at least three independent experiments. See also Figure S1.

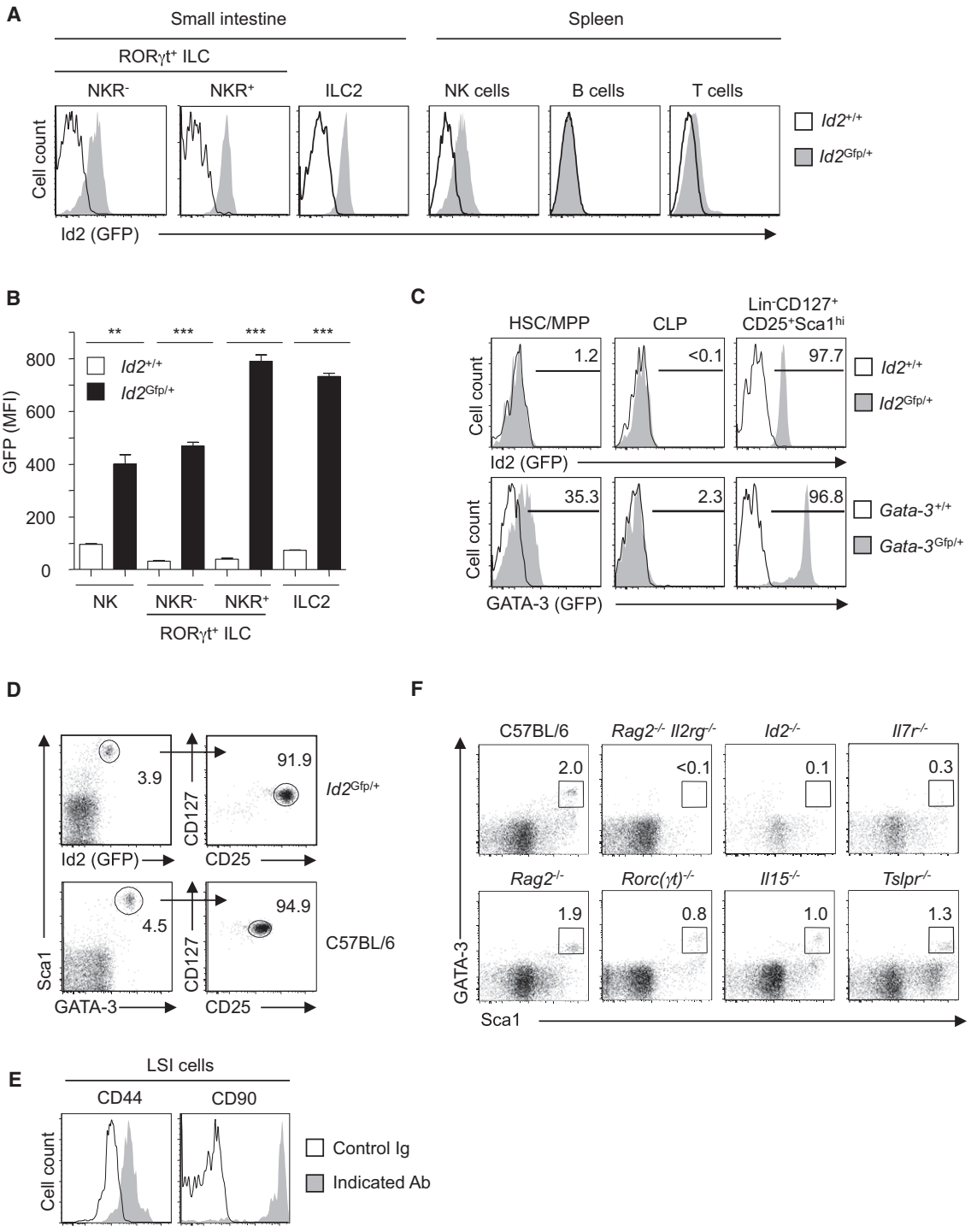

Figure 3. Identification of GATA-3^{hi} ILCs in the Bone Marrow

(A and B) Expression of Id2 (GFP) (gray) by the indicated cell subsets obtained from spleen or intestinal lamina propria of *Id2*$^{Gfp/+}$ mice. Open histograms represent analysis of the same population from control mice (A). (B) Mean fluorescence intensity (MFI ± SEM; n = 4) of Id2 (GFP) expression in comparison to control mice by the indicated cell subsets isolated from the intestinal lamina propria.

(C) Id2 (GFP) and GATA-3 (GFP) expression (gray) by the indicated bone marrow cell populations (HSC/MPP: Lin$^-$CD127$^-$Sca1$^+$Kit$^+$; CLP: Lin$^-$CD127$^+$CD135$^+$Sca1^{lo}Kit^{lo}). Open histograms represent analysis of the same population from control mice. Numbers indicate percentage of positive cells.

(D) Analyses of Sca1, Id2 (GFP), and GATA-3 expression by Lin$^-$ cells. The dot plots to the right show CD127 and CD25 expression by electronically gated Sca1^{hi}Id2^{hi} (top) or Sca1^{hi}GATA-3^{hi} (bottom) cells. Numbers represent percentage of cells in the indicated gates.

(E) Flow cytometry analysis of CD44 and CD90 expression (gray) by bone marrow LSI cells. Open histograms show staining with an isotype-matched control antibody.

reasons: (1) a precursor to the lineage of ILC2 may not yet express GATA-3 and (2) Id2 is required for the development of all innate lymphocytes (Moro et al., 2010; Yokota et al., 1999). Although the role of Id2 in innate lymphocyte development is well established, its expression pattern lacks definition. We have studied Id2 expression in *Id2*$^{Gfp/+}$ mice, a genetically modified mouse strain that expresses a fluorescent reporter (GFP) under the control of the *Id2* promoter to allow faithful tracking of Id2-expressing cells (Rawlins et al., 2009). Utilizing these mice, we discovered that all mature innate lymphocyte lineages (NK cells, ILC2s, and NKR$^+$ and NKR$^-$ RORγt$^+$ ILCs) were Id2^{hi} (Figures 3A and 3B). Thymic CD4$^-$CD8$^-$ (double-negative, DN) T cells and CD4$^+$CD8$^+$ (i.e., DP) T cells were negative for Id2, whereas CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$ (i.e., SP) T cells expressed low amounts of Id2 (Figure S2A), which was maintained in peripheral T cells (Figure 3A). As previously reported (Jackson et al., 2011), B cells (Figure 3A) and most mononuclear phagocytes (Figure S2B) did not express appreciable amounts of Id2. CD11c$^+$ DCs were Id2^{lo} (Figure S2B), a finding consistent with a role of Id2 for the development of DC subsets (Hacker et al., 2003; Jackson et al., 2011). Thus, under steady-state conditions, high Id2 expression is a specific characteristic of all innate lymphocytes.

Among Lin-negative hematopoietic precursors in the bone marrow, Id2 is not expressed by hematopoietic stem cells (HSCs) and multipotent progenitors (MPPs) or common lymphoid progenitors (CLPs) (Figure 3C). We detected a substantial population of Lin-negative cells that uniformly coexpressed Id2 and Sca1 (Figure 3D). These Lin$^-$Sca1^{hi}Id2^{hi} (LSI) cells also homogenously expressed CD127 and CD25 (Figure 3D) together with CD44 and CD90 (Figure 3E), markers that are characteristic of ILCs. Conversely, Lin$^-$Sca1^{hi}CD127$^+$CD25$^+$ cells were uniformly Id2 positive (Figure 3C). These cells also, unexpectedly, showed a uniformly high expression of GATA-3 (Figures 3C and 3D). The population of LSI cells was virtually identical to Lin$^-$Sca1^{hi}GATA-3^{hi} cells (Figures 3C and 3D) and we will, therefore, refer to these as Lin$^-$Sca1^{hi}Id2^{hi}GATA-3^{hi} (LSIG) cells. LSIG cells were developmentally dependent on *Il2rg, Id2,* and *Il7r* but were independent of *Rag2*, *Rorc(*γ*t), Il15*, and *Tslpr* gene expression (Figure 3F), a developmental program reminiscent of intestinal ILC2s (Figure 1F).

Genome-wide Expression Profiling of LSIG Cells Reveals Close Relationship to ILC2s

LSIG cells may be multipotent progenitors to various innate lymphocyte lineages or may constitute lineage-specified progenitors to ILC2s. We analyzed expression of cell surface markers and the functional profile of LSIG cells. LSIG cells did not express markers associated with the NK cell lineage (Figure S3A), the RORγt$^+$ ILC fate (Figures S3B and S3C), the myeloid lineage (Figure S3D), or multipotent hematopoietic progenitors (Figure S3E). In addition to Id2 and GATA-3, these cells expressed a number of markers also found on mature intestinal ILC2s, including receptors for the cytokines IL-33 (T1-ST2) and IL-25 (IL-17RB) (Figures 4A and 4B). Other innate lymphocyte populations do not express these receptors, which provides support for the lineage-specified progenitor model. However, there were also conspicuous differences compared to mature ILC2s. Bone marrow LSIG cells lacked expression of KLRG1 and Kit (Figures 4A and 4B). Similar to the immature KLRG1$^-$ GATA-3^{hi} ILCs found in newborn mice (Figure 2E), a subset of LSIG cells expressed integrin $\alpha_4\beta_7$ (Figure 4A), which was absent from mature KLRG1$^+$ ILC2s (Figure 2E). Direct comparison of the functional profile of LSIG cells to that of mature ILC2s via qRT-PCR revealed that *Il4*, *Il5*, and *Il13* but not *Il22*, *Il17a*, or *Ifng* were highly expressed in mature ILC2s (Figure 4C, black bars), whereas expression was low in LSIG cells (Figure 4C, open bars). To confirm these findings, we directly compared IL-5 production by LSIG cells and mature intestinal ILC2s in vitro. Although intestinal ILC2s were potent producers of IL-5, bone marrow LSIG cells produced only very low amounts (Figure 4D).

We performed genome-wide transcriptome analysis of highly purified LSIG cells, intestinal ILC2s, and NKR$^+$ or NKR$^-$ RORγt$^+$ ILCs to better assess the similarities and discrepancies of gene expression among these different ILC populations. The hierarchical clustering analysis of the four ILC subsets generated two distinct cell clusters: the first cluster grouped together NKR$^-$ and NKR$^+$ RORγt$^+$ ILCs, as already revealed by previous data (Reynders et al., 2011), and the second cluster combined LSIG cells and mature ILC2s (Figure 4E). These data demonstrate a profound relationship between the transcriptional programs of the GATA-3^{hi} ILC subsets in the intestine and the bone marrow, whereas the profiles of the RORγt$^+$ ILC subsets were quite distant. We clustered and annotated selected genes that showed the most robust and consistent differences in expression (at least 2-fold) among the various ILC subsets (Figure 4F). A group of genes was found to be common to LSIG cells and mature ILC2s, but not the RORγt$^+$ ILC subsets (cluster 8, ILC2-LSIG core cluster). Among them were the genes encoding GATA-3, CD27, and Sca1 (Ly6A) and receptors of IL-33, IL-25, and IL-9 (Figure 4G, top). Importantly, we could identify clusters of genes differentially expressed by LSIG cells and mature ILC2s (Figure 4G, bottom). Of note, the ILC2 signature cytokine genes IL-5 and IL-13, as well as the gene for KLRG1, were strongly represented in intestinal ILC2s but not in bone marrow LSIG cells (Figure 4G), a finding that concurred with the results described above (Figures 4A–4D).

LSIG Cells Are Lineage-Specified Progenitors to ILC2s

Collectively, our findings from functional and genome-wide transcriptional analyses demonstrate that LSIG cells and intestinal ILC2s are highly related, albeit with differential expression of gene clusters between the two ILC subsets. Thus, we propose that LSIG cells are the lineage-specified progenitors of ILC2s. We tested this model by examining the developmental potential of LSIG cells in vitro and in vivo. We cultured highly purified LSIG cells on mitotically inactivated OP9 feeder cells or Notch ligand

(F) The indicated mouse strains were analyzed for the presence of bone marrow Lin$^-$Sca1^{hi}GATA-3^{hi} cells. Numbers represent percentage of cells in the indicated gates.

Data are representative of at least three independent experiments. **p $\leq$ 0.01, ***p $\leq$ 0.001. LSI: Lin$^-$Sca1^{hi}Id2^{hi} bone marrow cells. See also Figure S2.

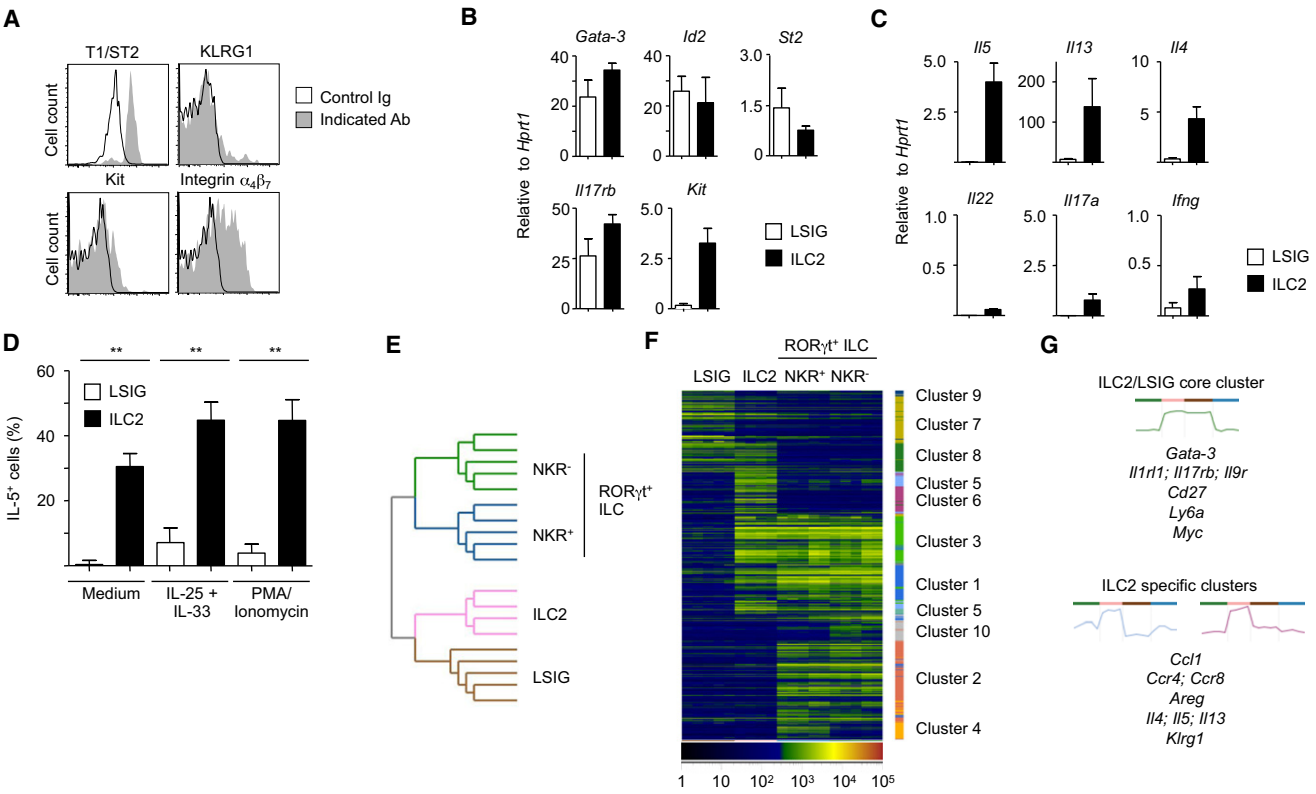

Figure 4. Transcriptome Analysis Reveals Close Relationship between LSIG Cells and ILC2s

(A) Flow cytometry analysis of the indicated cell surface markers (gray) by bone marrow LSIG cells. Open histograms depict staining with isotype-matched control antibodies.

(B and C) Quantitative RT-PCR analysis (±SEM; n = 3) of expression of the indicated genes by LSIG cells (white bars) or ILC2s (black bars).

(D) Percentage (±SEM; n = 4) of IL-5-producing bone marrow LSIG cells (white bars) compared to ILC2s from the small intestine (black bars).

(E) Hierarchical clustering of normalized microarray data of replicate RNA samples from the indicated cell subsets. Dendrogram was obtained by analyzing 911 out of 28,441 probe sets.

(F) Heat map representation of genes clustering with at least 2-fold differences in expression pattern across different cell subsets as assessed by *k-means*. Columns represent the indicated cell subsets in four or five biological replicates. Each row represents one examined gene. Hierarchical clustering revealed ten different clusters as indicated. The color code at the bottom defines the expression intensity of each individual gene in all examined cell subsets.

(G) Functional classification of the gene profiles shared by LSIG and ILC2s (i.e., ILC2/LSIG core cluster) and specific for ILC2s as assessed by *k-means*. Representative genes within these clusters are indicated. Signature gene expression profiles of the respective clusters used in the functional annotation are indicated above.

Data are representative of three independent experiments. $^{**}p \leq 0.01$. LSIG: $Lin^{-}Sca1^{hi}Id2^{hi}GATA\text{-}3^{hi}$ bone marrow cells. See also Figure S3.

delta-like 1 (Dll1)-expressing OP9 cells, which is required to generate T cells from uncommitted precursor cells (Schmitt and Zúñiga-Pflücker, 2002). When cultured on OP9 or OP9-Dll1 cells in the presence of IL-7 or IL-7 plus IL-33, LSIG cells did not show any appreciable T or B cell potential (Figure S4A) but, instead, remained uniformly Id2- and GATA-3-expressing cells (Figures 5A and 5B). We were also unable to find any evidence of innate multilineage potential in LSIG cells. When cultured in the presence of cytokines permissive for NK cell differentiation, LSIG cells showed no signs of NK cell differentiation such as upregulation of NKR (Figure 5A). In addition, LSIG cells cultured in the presence of cytokines promoting differentiation of RORγt^{+} ILCs (IL-1β, IL-23, IL-2, and IL-7) did not upregulate expression of IL-17A, IL-22, IL-23R, or RORγt, indicative of the RORγt^{+} ILC fate (Figures 5C and S4B). Interestingly, bone marrow LSIG cells, which are KLRG1 negative, upregulated KLRG1 expression when cultured in medium containing IL-7 and IL-2, in particular if IL-33 (Figures 5B and 5D) or IL-25 (Figure 5D) were present. In contrast, culture of LSIG cells in the presence of thymic stromal lymphopoietin (TSLP) did not lead to the upregulation of KLRG1 (Figure 5D). Clonal differentiation assays confirmed that LSIG cells remained stably GATA-3- and Id2-expressing ILCs (Figure S4C). In addition, a fraction of clones upregulated KLRG1 (Figure S4D).

To further test our theory that LSIG cells differentiate into ILC2s, we assessed cytokine production after in vitro differentiation. Culture of LSIG cells with IL-7 and SCF, conditions in which they do not substantially expand (Figure S4E), led to low-level production of IL-5 and IL-13 (Figures 5E and 5F and data not shown), similar to that observed in in vivo nondifferentiated bone marrow LSIG cells (Figures 4C and 4D). TSLP did not show any significant effect on the maturation (Figure 5D) or cytokine production (Figure 5F) of LSIG cells in vitro. In contrast, LSIG cells differentiating in the presence of IL-33 or IL-25 became

potent producers of IL-5 and IL-13, indicative of differentiation into ILC2s (Figures 5E and 5F). However, cytokines such as IFN-γ or IL-22, which are associated with different innate lymphocyte fates, could not be detected (Figures 5C and 5E). Collectively, these data demonstrate that LSIG cells exclusively differentiate into the IL-5- and IL-13-producing KLRG1$^+$ ILC2 subset.

To provide additional support for our hypothesis that LSIG cells are the lineage-specified precursors of ILC2s, we transferred highly purified LSIG cells (H-2^b) into alymphoid *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (H-2^d) and tracked their differentiation potential. After transfer, donor-derived cells were found in organs in which ILC2s reside (e.g., intestine) (Figures 5G, S4F, and S4G) but not in lungs, liver, or spleen (Figures S4F and S4G). We tested whether injection of inflammatory cytokines such as IL-25 and IL-33 led to recruitment of LSIG-derived ILC2s to extraintestinal sites. Interestingly, 4 days after cytokine injection, a small but reproducible population of LSIG-derived cells was found in lungs, liver, and spleen (Figure S4G). Whereas KLRG1$^-$ LSIG cells had no T cell, B cell, NK cell, or RORγt$^+$ ILC potential, donor-derived cells maintained expression of Id2 and GATA-3. Intriguingly, virtually all precursor cells differentiated into KLRG1$^+$ ILC2s (Figure 5G), collectively demonstrating that KLRG1$^-$ LSIG cells are precursors to mature intestinal KLRG1$^+$ ILC2s. To obtain further evidence that KLRG1$^-$ LSIG cells have precursor potential and do not simply constitute mature ILC2s lacking KLRG1 expression (Brickshawana et al., 2011), we directly compared the reconstitution efficiency of bone marrow-derived KLRG1$^-$ LSIG cells to that of mature small intestinal KLRG1$^+$ ILC2s upon transfer into alymphoid mice. The population of mature KLRG1$^+$ ILC2s recovered from alymphoid mice was approximately the same size as that injected. In contrast, the KLRG1$^-$ LSIG population generated ~20- to 30-fold more progeny (Figures 5H and 5I), indicating that they had proliferated after transfer to produce mature KLRG1$^+$ ILC2s. From now on, we will refer to the bone marrow KLRG1$^-$ LSIG population as lineage-specified precursors to ILC2s (i.e., ILC2Ps).

It was recently reported that mice genetically lacking *Rora* (encoding RORα) have reduced numbers of ILC2s after injections of IL-25 and that in vitro generation of ILC2s from *Rora*-deficient CLP was impaired (Wong et al., 2012). However, it remained unknown at which stage RORα is upregulated and whether RORα expression or the effects of its deletion are specific to ILC2s. We analyzed *Rora* expression by hematopoietic progenitors, ILC2Ps, and mature ILC subsets by using qRT-PCR. Although HSCs-MPPs and CLPs did not express detectable levels of *Rora*, all mature innate lymphocyte subsets showed robust expression of the *Rora* gene. ILC2Ps reproducibly contained lower but considerable numbers of *Rora* transcripts, indicating that upregulation of RORα occurs in two waves, during lineage specification of ILCs and at the transition from ILC2Ps to mature ILC2s (Figure 5J).

ILC2Ps Develop into Functional ILC2s Conferring Immunity to Helminth Infection

It has been recently demonstrated that ILC2s play an important role in the clearance and prevention of infections with the helminth parasite *N. brasiliensis* (Liang et al., 2012; Neill et al., 2010). Alymphoid *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ cannot control *N. brasiliensis* infection (Figure 6A; Moro et al., 2010; Price et al., 2010). However, adoptive transfer of ILC2Ps prior to worm challenge led to a control of infection comparable to that in lymphoreplete mice (Figure 6A). Donor-derived ILC2s were found in the intestine and only very few cells homed to lungs and spleen prior to infection (Figure 6B). Interestingly, infection of mice with *N. brasiliensis* led to a significant increase of ILC2P-derived ILC2s in small intestine but also in lungs and spleen. Although eosinophils were not recruited to the lungs of alymphoid mice, probably because of the lack of an innate source of IL-5 and/or IL-13 (Liang et al., 2012), mice receiving ILC2Ps prior to worm challenge showed a profound accumulation of eosinophils in the lungs at day 5 postinfection (Figures 6C and 6D). In the intestine, IL-13 expression has been shown to lead to increased differentiation of mucin-producing goblet cells and to increased angiogenin expression by Paneth cells supporting a milieu conducive for worm expulsion (Steenwinckel et al., 2009). Although *N. brasiliensis*-infected *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice showed low-level expression of angiogenin 4 or mucin 2, transfer of ILC2Ps led to a substantial increase in the expression of these genes (Figures 6E and 6F). Only few mucus-producing (i.e., Alcian blue-positive) cells were found in the colon of *N. brasiliensis*-infected *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice. Transfer of ILC2Ps into alymphoid mice and consecutive infection led to goblet cell hyperplasia surpassing that of control mice (Figure 6G). Collectively our data demonstrate that ILC2Ps can differentiate into functional ILC2s, conferring protection against murine helminth infection by inducing epithelial expression of the genes involved in the defense against worms.

CCR9 Signals Are Required for the Migration of ILC2Ps to the Intestine

We investigated the molecular signals involved in coordinating the migration of ILC2s to the intestine. Our phenotypical characterization of ILC2Ps and mature ILC2s revealed that both populations expressed high levels of the chemokine receptor CCR9 (Figure 6H), a chemokine receptor with essential function in the homing of lymphocytes from bone marrow to the lamina propria (Zabel et al., 1999). *Ccr9* deficiency led to a significant decrease of ILC2s in the intestinal lamina propria. However, *Ccr9*$^{-/-}$ ILC2s were not significantly diverted to extraintestinal organs, although we consistently observed a larger fraction of ILC2s in bone marrow, lungs, and mesenteric lymph nodes (Figures 6I and 6J). Collectively, our findings illustrate that migration of ILC2Ps to the intestine is in part dependent on CCR9-mediated signals.

GATA-3 Is Required for ILC2 Cell Fate Decisions and Maintenance of Mature ILC2s

Our data demonstrate that GATA-3 is a transcription factor stably and continuously expressed by ILC2s and by ILC2Ps, their lineage-specified bone marrow precursors. However, it remains unknown whether GATA-3 is required for their development and/or differentiation. We developed a genetic model in which the *Gata-3* gene can be inducibly ablated in all ILCs (Figure S5A). We crossed *Id2*$^{CreERt2/+}$ mice (Rawlins et al., 2009) to mice carrying a conditional *Gata-3* allele (*Gata-3*$^{f/f}$) (Grote et al., 2008). Tamoxifen application induces nuclear translocation of Cre allowing for the inducible inactivation of *Gata-3*. To faithfully monitor Cre activity, we further crossed these mice to a strain

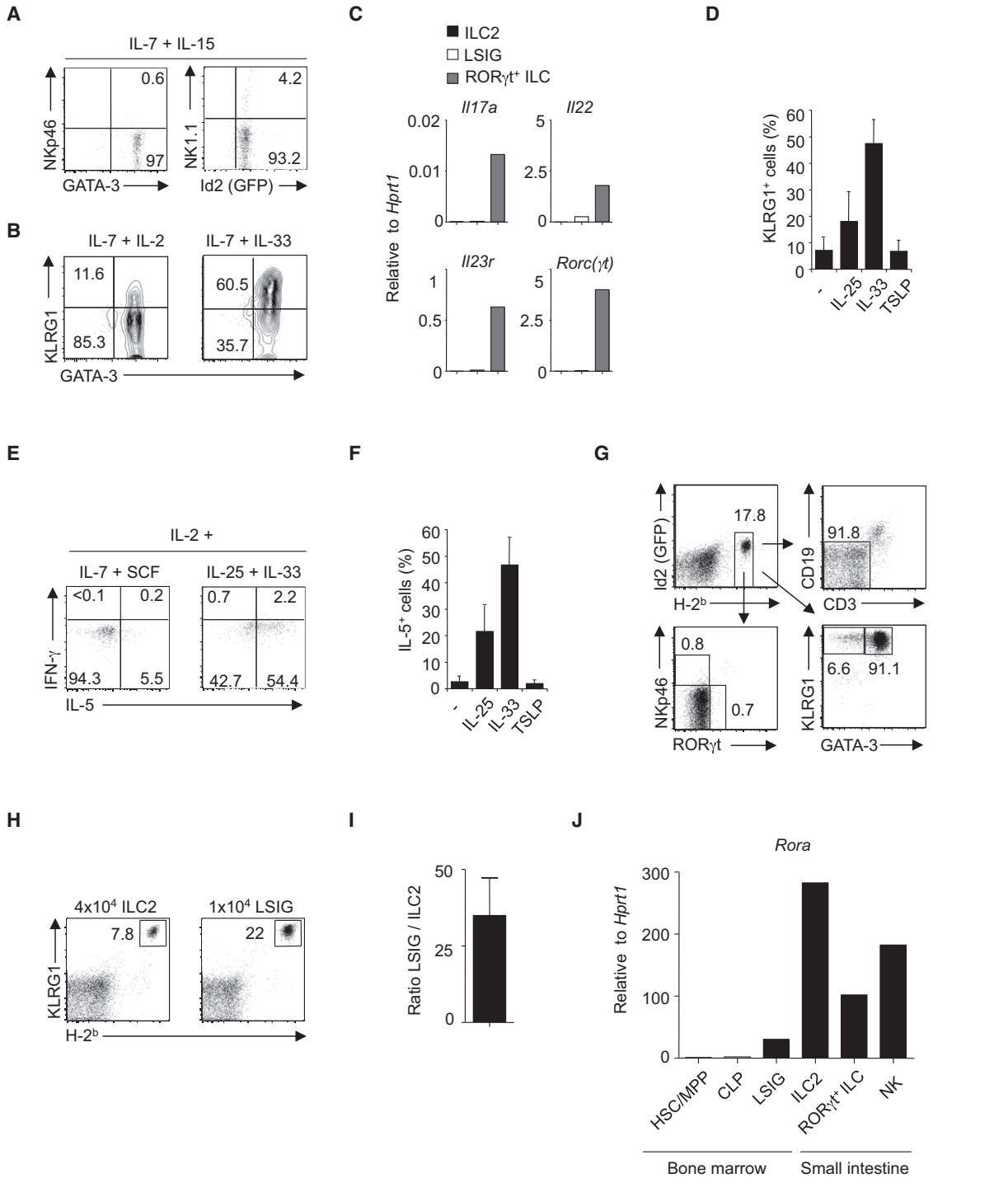

Figure 5. LSIG Cells Are Lineage-Specified Progenitors of ILC2s

(A) Highly purified LSIG cells from *Gata-3*$^{Gfp/+}$ mice or *Id2*$^{Gfp/+}$ mice cells were cultured for 6 days on OP9 feeder cells in medium containing IL-7 and IL-15 and tested for the expression of NKp46 and GATA-3 (left) or NK1.1 and Id2 (right). Numbers represent percentage of cells in quadrant.

(B) Highly purified LSIG cells were cultured on OP9 feeder cells in the presence of the indicated cytokines. KLRG1 and GATA-3 expression were determined after 7 days of culture. Numbers represent percentage of cells in quadrant.

(C) Quantitative RT-PCR analysis of expression of the indicated genes by ILC2s (black bars), by LSIG cells cultured for 7 days with cytokines favoring differentiation of RORγt$^+$ ILCs (IL-2, IL-7, IL-23, IL-1β) (white bars), or by RORγt$^+$ ILCs (gray bars).

(D) Percentage (±SD; n = 3) of KLRG1$^+$ cells after 7 day culture of purified LSIG cells on OP9 feeder cells in the presence of the indicated cytokines.

(E) Highly purified LSIG cells were cultured on OP9 feeder cells in the presence of the indicated cytokines. After 11 days of culture, IL-5 and IFN-γ production was determined by intracellular cytokine staining. Numbers represent percentage of cells per quadrant.

carrying a fluorescent reporter (yellow fluorescent protein, YFP), which comes under the transcriptional control of the ubiquitous *Rosa26* promoter once a transcriptional roadblock is excised by Cre-mediated recombination (Figure S5A; Srinivas et al., 2001). Id2 is highly expressed in ILC2Ps, innate lymphocytes, and during T cell differentiation in the thymus (Figures 3A, 3C, and S2A), so this approach produced mice in which the *Gata-3* gene can be inducibly deleted in all innate lymphocyte lineages and T cells (*Gata-3*$^{i\Delta ILC,T}$). Cre activity correlated with Id2 expression so that Id2^{hi} cells (e.g., ILC2s, ILC2Ps, RORγt^{+} ILCs) had higher Cre activity than Id2^{lo} cells (e.g., T cells) (Figure S5B). Development of GATA-3^{int}RORγt^{+} ILCs (Figure S1E) was unaffected by the deletion of GATA-3 (Figure 7A), which allowed us to investigate the efficiency of GATA-3 inactivation in the YFP^{+} (Cre-on) subset of RORγt^{+} ILCs. Upon induction of Cre expression by tamoxifen, GATA-3 expression was abolished in YFP^{+} RORγt^{+} ILCs (Figure 7B). Thus, *Gata-3*$^{i\Delta ILC,T}$ mice allow for efficient inducible deletion of *Gata-3* in innate lymphocytes.

We next treated *Gata-3*$^{i\Delta ILC,T}$ mice for 3 weeks with a tamoxifen-containing diet and analyzed ILC2s in the intestinal lamina propria. Although the pool of RORγt^{+} ILCs was unaffected by *Gata-3* deficiency (Figures 7A and 7B), KLRG1^{+}GATA-3^{hi} cells or RORγt^{-}KLRG1^{+} cells (both representing ILC2s) were virtually absent in the GATA-3-deficient (i.e., YFP^{+}) subset (Figures 7A and 7C). This was also reflected in our analysis of the proportion of KLRG1^{+} cells within all innate lymphocytes with *Gata-3* deletion (YFP^{+}) (Figure 7D). In contrast, ILC2s were normally represented in the YFP-negative (Cre-off) subset spared from Cre-mediated inactivation of *Gata-3* (Figures 7A and 7C). Whereas ILC2s are entirely lost in mice with conditional deletion of *Gata-3*, the total pool of ILCs with *Gata-3* deletion (YFP^{+}) was only mildly reduced (Figure 7E), which reflects the normal maintenance of RORγt^{+} ILCs and of additional ILC subsets in the absence of GATA-3. It is unlikely that KLRG1, CD25, and Sca1 are under the direct transcriptional control of GATA-3, meaning that a deficiency in GATA-3 is unlikely to have the knock-on effect of obscuring detection of these markers. In support of this, ILC2Ps do not express KLRG1 despite high GATA-3 expression (Figures 3C, 3D, and Figures 4A). Nevertheless, we interrogated cytokine production by CD3^{-}CD19^{-} cells in order to visualize residual innate type 2 cytokine producers in the absence of GATA-3. Whereas the GATA-3-proficient (YFP^{-}) fraction of innate lymphocytes produced IL-5 and IL-13 after stimulation comparable to control mice, no cytokine producers were detected in the GATA-3-deficient (YFP^{+}) population (Figure 7F).

The entire intestinal pool of ILC2s was lost within few weeks of *Gata-3* inactivation (Figures 7A, 7C, and 7D). Maintenance of the pool size of intestinal lymphocytes is the aggregate of two parameters; cell half-life and the rate of replacement by newly arriving progenitors. Therefore, our data have two important implications. First, that mature ILC2s cannot be maintained when *Gata-3* is ablated and, second, that bone marrow-derived ILC2Ps do not efficiently replenish the peripheral pool of KLRG1^{+} ILC2s when GATA-3 is inactivated. Because we could detect ILC2s more than 4 months after transfer (Figures 5G and 5I), our data do not favor the view that the absence of ILC2s 3–4 weeks after *Gata-3* inactivation reflects the natural half-life of this subset. To more directly address this issue, we cultured highly purified YFP^{-} (i.e., Cre-off) KLRG1^{+} ILC2s from the lamina propria of *Gata-3*$^{f/f}$ mice and of control mice in the presence of tamoxifen for 7 days. Roughly 20% of ILC2s from *Gata-3*$^{+/+}$ controls switched on Cre expression (i.e., became YFP positive) during the 7 days of culture (Figure 7G). Interestingly, when Cre activity was induced in ILC2s from *Gata-3*$^{f/f}$ mice, only very few if any YFP^{+} cells could be found whereas the YFP^{-} (*Gata-3*-proficient) ILC2s were normally maintained (Figure 7G). These data establish that GATA-3 is required for the maintenance of mature ILC2s.

Given the failure of ILC2Ps to repopulate the peripheral pool of KLRG1^{+} ILC2s when *Gata-3* is inactivated, we investigated the role of GATA-3 in the development or maintenance of bone marrow ILC2Ps. After inducible deletion of *Gata-3*, LSI cells (i.e., ILC2Ps) were 6- to 10-fold reduced (Figure 7H). The remaining cells phenotypically resembled ILC2Ps in that they expressed high levels of Sca1 and CD25 (Figure 7I). It was remarkable to find such a population of Lin^{-}CD25^{+}Sca1^{hi} bone marrow cells within the YFP^{+} (Cre-on) population (Figure 7I, bottom), and we suspected that they represented either precursor cells that could not differentiate in the absence of GATA-3 or remaining ILC2Ps. Culture of YFP^{+} (i.e., Cre-on) Lin^{-}CD25^{+}Sca1^{hi} cells from GATA-3-proficient control mice led to the generation of KLRG1^{+} ILC2s (Figure 7J) as observed before (Figure 5B). In contrast, culture of equal numbers of the same subset isolated from *Gata-3*-deficient mice did not generate any progeny (Figure 7J). Collectively, our data provide evidence for a dominant role for GATA-3 at two central checkpoints of the ILC2 lineage. First, GATA-3 is required for the maintenance of mature ILC2s and of ILC2Ps. Second, GATA-3 is an important factor in the maturation of ILC2Ps. Thus, similar to the role of RORγt in RORγt^{+} ILCs, GATA-3 is an essential determinant of ILC2 fate.

DISCUSSION

By using genetically modified mice to allow faithful tracking of GATA-3-expressing cells and intracellular staining for this transcription factor, we demonstrated that ILC2s are, by any definition (phenotypic, functional, and developmental), GATA-3^{hi}

(F) Percentage (±SD; n = 3) of IL5^{+} cells after 7 day culture of purified LSIG cells on OP9 feeder cells in the presence of the indicated cytokines.
(G) Highly purified bone marrow LSIG cells from *Id2*$^{Gfp/+}$ mice (H-2^{b}) were adoptively transferred into irradiated *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ hosts (H-2^{d}). Six weeks after transfer, donor-derived cells from the intestinal lamina propria were analyzed for the expression of the indicated markers. Numbers represent percentage of cells in the indicated gates.
(H and I) The indicated numbers of highly purified bone marrow LSIG cells (H-2^{b}) or intestinal ILC2s (H-2^{b}) were adoptively transferred into irradiated *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ hosts (H-2^{d}). Four months after transfer, the fraction of donor-derived KLRG1^{+} ILC2s among lamina propria leukocytes was determined. As a measure of repopulation efficiency, the ratio (±SEM, n = 3) of LSIG cell-derived to ILC2-derived KLRG1^{+} cells was determined (H).
(J) Quantitative RT-PCR analysis of *Rora* expression by the indicated cell subsets from bone marrow or the small intestine.
All experiments are representative of at least two independent experiments. LSIG: Lin^{-}Sca1^{hi}Id2^{hi}GATA-3^{hi} bone marrow cells. See also Figure S4.

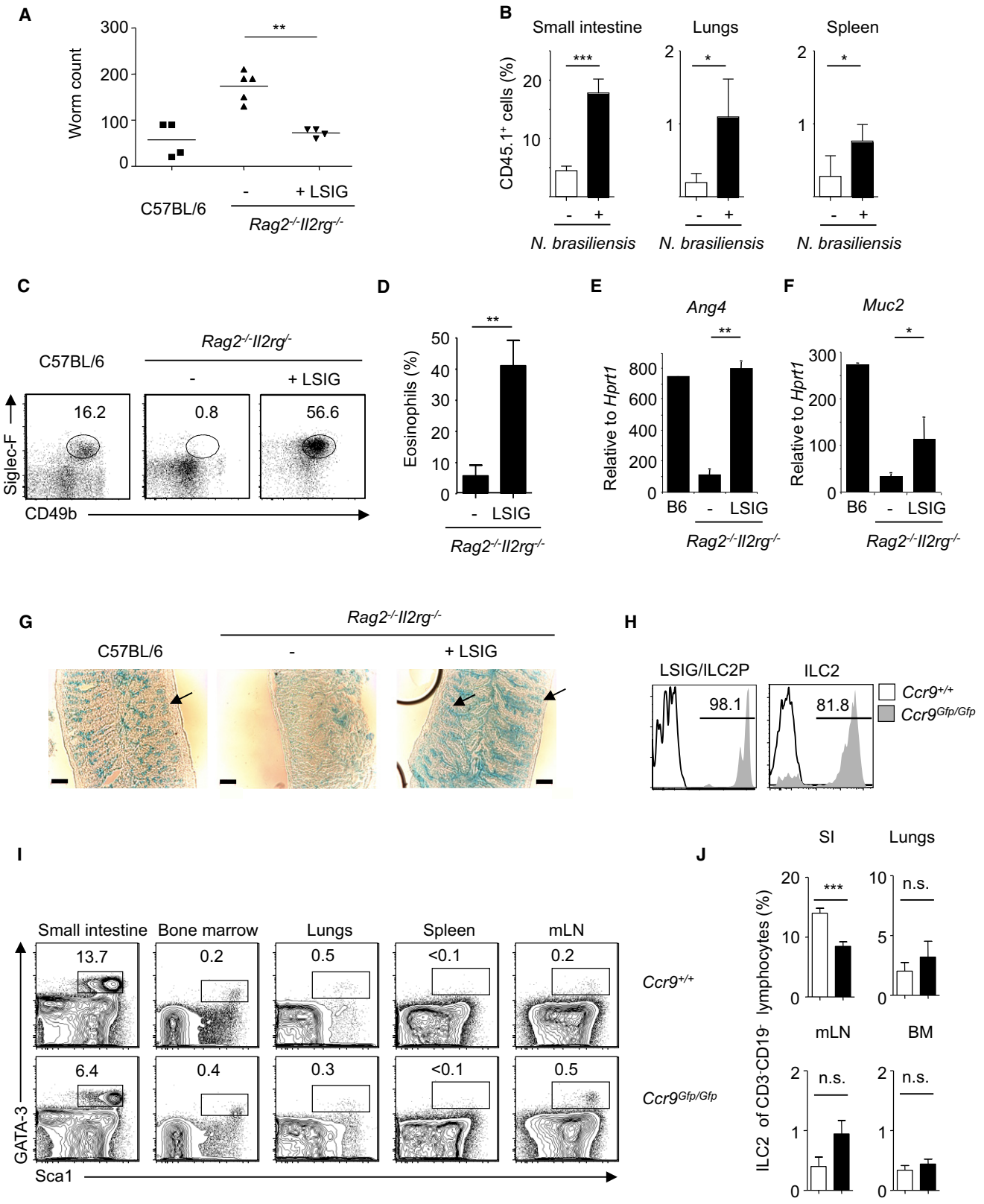

Figure 6. Transfer of LSIG Cells Confers Immunity to *N. brasiliensis* Infection

(A–G) Highly purified LSIG cells were transferred into groups of *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ hosts. Four weeks after transfer, the indicated groups of mice (n = 4–5) were infected with *N. brasiliensis* and analyzed on day 5 of infection.

ILCs. Given that other innate lymphocyte lineages (i.e., RORγt$^+$ ILCs and NK cells) expressed only low amounts of GATA-3, high GATA-3 expression can be said to be a specific characteristic of ILC2s. The presence of *Gata-3* transcripts in ILC2s has previously been reported (Moro et al., 2010; Price et al., 2010; Yang et al., 2011). One recent report drew an interesting parallel to Th2 cells by showing that a subset of lung-resident ILC2s (i.e., CD4$^-$CD8$^-$CD19$^-$ICOS$^+$ cells) isolated from *N. brasiliensis*-infected mice expressed GATA-3 (Liang et al., 2012). Our data now demonstrate that all ILC2s constitutively express high amounts of GATA-3.

We observed only a very small fraction of ILC2s in the intestine of newborn mice. However, their proportion steadily increased during the first 2 months after birth, indicating that ILC2s populate the intestine in substantial numbers only postnatally. Phenotypical profiling showed that >90% of ILC2s of adult mice expressed the lectin-like receptor KLRG1, which was absent from GATA-3^{hi} ILCs of newborn mice. Thus, we propose that KLRG1 acts as a marker of mature ILC2s. KLRG1 is expressed by a subset of mature splenic NK cells and by subsets of activated CD8$^+$ T cells and its ligation can inhibit effector functions in these lymphocyte subsets (Blaser et al., 1998; Hanke et al., 1998; Huntington et al., 2007; Sarkar et al., 2008). KLRG1 is an inhibitory immunoreceptor that binds to classical cadherins (e.g., E-cadherin) (Gründemann et al., 2006; Ito et al., 2006), highly expressed by intestinal epithelial cells. It is tempting to speculate that the interaction of KLRG1 with E-cadherins may dampen the function of ILC2s.

Previous data have indicated that ILC2s develop from CLPs and are part of the lymphoid lineage (Wong et al., 2012; Yang et al., 2011). However, the distinct stages of ILC2 development or differentiation have not yet been recognized. Id2 is a transcription factor required for the development of all innate lymphocyte lineages (Moro et al., 2010; Yokota et al., 1999). We identified a distinct population of Lin-negative Id2^{hi} cells in the bone marrow that coexpressed Sca1, CD25, CD127, and receptors for IL-33 and IL-25. Phenotypic and genome-wide transcriptome analysis of these Lin$^-$Sca1^{hi}Id2^{hi} (LSI) cells revealed that they were very closely related to ILC2s and only distantly related to other innate lymphocyte lineages such as RORγt$^+$ ILCs. LSIG cells are lineage-specified precursors to mature KLRG1$^+$ ILC2s that we propose to refer to as ILC2Ps. Previous reports had already indicated the presence of ILC2-like cells in the bone marrow but failed to probe the developmental potential of these cells (Brickshawana et al., 2011; Price et al., 2010). Importantly, CD25$^+$Lin$^-$Sca1$^+$Kit$^-$ (CD25$^+$LSK$^-$) cells that are virtually identical to the LSIG population were recognized more than a decade ago and considered perplexing because, after adoptive transfer, and despite expression of "stem cell antigen," they did not generate any appreciable progeny in secondary lymphoid organs (Kumar et al., 2008; Randall and Weissman, 1998). Indeed, LSIG cells failed to home to secondary lymphoid organs but were precursors to mature intestinal ILC2s.

GATA-3 was required for the maintenance of mature ILC2s and bone marrow-resident ILC2Ps. It remains formally possible that ILC2Ps cannot migrate to the intestinal mucosa in the absence of GATA-3. This is another striking parallel to the role of GATA-3 in Th2 cells, as they also require continuous GATA-3 expression for their maintenance (Pai et al., 2003; Zhu et al., 2004). Interestingly, a subset of GATA-3-negative LSI cells were found in the bone marrow of GATA-3-deficient mice. These cells underwent in vitro differentiation into ILC2s only in the presence of GATA-3, demonstrating that GATA-3 controls lineage specification in this innate lymphocyte lineage. A recent report indicated that RORα was required for efficient ILC2 development but the precise stage of differentiation at which it acted was not examined (Wong et al., 2012). We have now shown that, in contrast to GATA-3, RORα expression is not specific to the ILC2 lineage but it is expressed at high levels by all innate lymphocytes. RORα expression is probably upregulated in two waves, during ILC lineage specification and at the transition of ILC2P to ILC2. Altogether, our data demonstrate that, similar to the role of RORγt for RORγt$^+$ ILCs, GATA-3 is required for the ILC2 fate.

It is an emerging theme that the distinct ILC subsets use transcriptional programs previously identified to be in control of various Th cell effector fates. For example, RORγt is required for both the Th17 cell program and the development and function of RORγt$^+$ ILCs. We have provided conclusive evidence that another ILC subset resembling Th2 cells requires GATA-3 for lineage specification and maintenance. These findings may indicate that these transcriptional programs are conserved in both the innate and adaptive arms of the immune system. However, there are also obvious dissimilarities. Under steady-state conditions, CD4$^+$ T cells are naive cells that do not express GATA-3 or RORγt before encountering their cognate antigen. It is only upon TCR ligation that the small pool of antigen-specific T cells expands and effector-fate determining transcription factors are upregulated. In contrast, the ILC lineages require RORγt or GATA-3 at the lineage specification stage in primary lymphoid organs (fetal liver or bone marrow), unlike

(A) Total intestinal helminth counts.
(B) Percentage (±SD; n = 3) of donor-derived (CD45.1$^+$) ILC2s in the indicated organs at the day of infection (white bars) and at day 5 after *N. brasiliensis* infection.
(C) Representative dot plot of eosinophil (CD49b^{lo}Siglec-F$^+$) accumulation in the lungs of the indicated mouse strains. Numbers in graph represent percentage of cells in the indicated gates.
(D) Percentage (±SEM; n = 4) of CD49b^{lo}Siglec-F$^+$ cells.
(E and F) Quantitative RT-PCR analysis (±SD; n = 4) of *Angiogenin4* (E) and *Mucin2* (F) expression by intestinal epithelial cells isolated from the indicated treatment groups.
(G) Alcian blue staining of cryosections of the small intestine. Arrowheads indicate goblet cells with high amounts of mucus production. Scale bars represent 100 μm. Original magnification, ×20.
(H) CCR9 (GFP) expression by intestinal ILC2s and bone marrow ILC2Ps. Histograms are electronically gated on LSIG cells or ILC2s. Open histograms represent analysis of the same population from control mice.
(I and J) Representation of GATA-3^{hi} ILCs (i.e., ILC2s or ILC2Ps) in C57BL/6 (I, top) and *Ccr9*-deficient (I, bottom) mice. Numbers represent percentage of cells in the indicated gates. (J) Percentage (±SEM; n = 3) of GATA-3^{hi} ILCs in the indicated organs of control (white bars) or *Ccr9*-deficient (black bars) mice.
All data are representative of three or more independent experiments. *$p \leq 0.05$, **$p \leq 0.01$. LSIG: Lin$^-$Sca1^{hi}Id2^{hi}GATA-3^{hi} bone marrow cells.

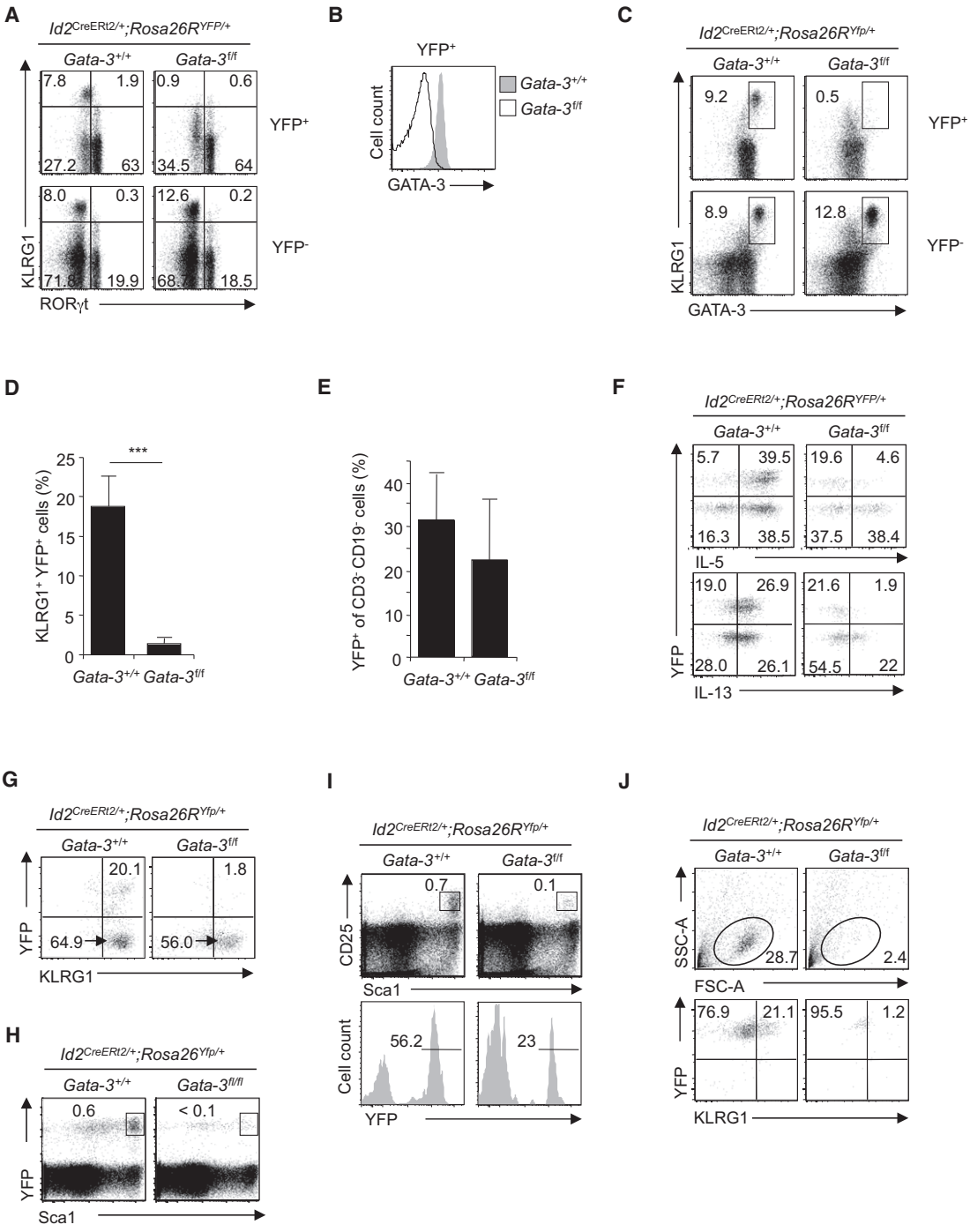

Figure 7. GATA-3 Controls Cell Fate and Maintenance of ILC2s

(A) Analyses of RORγt and KLRG1 expression by YFP$^+$ (Cre-on) and YFP$^-$ (Cre-off) fractions of CD45$^+$CD3$^-$CD19$^-$ lamina propria cells from the indicated mouse strains. Numbers represent percent cells in quadrant.

(B) GATA-3 expression by intestinal YFP$^+$ (Cre-on) RORγt$^+$ ILCs of *Gata-3*$^{+/+}$ (gray) or *Gata-3*$^{f/f}$ (open) animals.

(C) Expression of GATA-3 and KLRG1 by YFP$^+$ (Cre-on) and YFP$^-$ (Cre-off) fractions of CD45$^+$CD3$^-$CD19$^-$ intestinal lamina propria cells from the indicated mouse strains. Numbers represent percent cells in gate.

(D and E) Percentage (±SD; n = 4) of KLRG1$^+$YFP$^+$ cells (D) and of YFP$^+$ cells (E) among CD45$^+$CD3$^-$CD19$^-$ lamina propria lymphocytes from the indicated mice.

(F) Lamina propria lymphocytes from the indicated mouse strains were stimulated for 4 hr with PMA and ionomycin. IL-5 and IL-13 production was analyzed by intracellular cytokine staining and flow cytometry analysis of electronically gated CD45$^+$CD3$^-$CD19$^-$CD90$^+$ lamina propria lymphocytes. Numbers indicate percent cells in quadrant.

T cells, which can, in general, develop in the absence of these transcription factors. Such differences may well reflect the divergent design principles of the innate and adaptive immune systems.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice were purchased from Janvier. Information about the genetically modified mouse strains used in this study can be found in the Supplemental Experimental Procedures. Mice were kept under specific-pathogen-free conditions and experiments were performed in accordance with the guidelines of the animal care and use committees and the Regierungspräsidium Freiburg and Erlangen.

Microarray Analysis

RNA for microarray was isolated with the RNeasy Micro Kit (QIAGEN) according to the manufacturer's protocol. RNA integrity was determined before amplification with the Ovation Pico WTA V2 kit (Nugen). Amplified cDNA samples were further fragmented and labeled with the Affymetrix WT labeling kit. Labeled fragments were hybridized to GeneChip ST1.0 before analysis with the Affymetrix GeneChip Scanner 3000 7G.

Cell Sorting and Adoptive Transfer Experiments

The indicated cell populations were isolated from the respective organs and were highly purified (>98%) with a MoFlo Astrios cell sorter. Cells were transferred i.v. into nonirradiated *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice on a BALB/c background or were used for in vitro experiments.

N. brasiliensis Infection

C57BL/6 *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice were reconstituted with 1 × 10^4 LSIG cells. Four weeks later, reconstituted mice were injected s.c. with 500 purified *N. brasiliensis* stage 3 larvae (see also Supplemental Experimental Procedures). Injection of IL-25 and IL-33 (250 ng/ml each; BioLegends) was performed at day 0 and day 2 after infection and mice were sacrificed for analysis at day 5 postinfection. During infection, mice were provided drinking water supplemented with antibiotics (2 g/l neomycin sulfate, 100 mg/l polymixin B sulfate; Sigma-Aldrich).

Histology

Ileal tissues were fixed in 4% paraformaldehyde and embedded in OCT compound (Tissue-Tek) before freezing at −80°C. Histological sections with a thickness of 7 μm were stained with Alcian blue working solution for 15 min, followed by extensive washing, and were mounted with Aquatex (Merck).

In Vitro Differentiation

The indicated, highly purified cell populations were seeded onto mitomycin C-treated, mitotically inactivated OP9- or OP9Dll-1 feeder cells. Cytokines were added to the culture at a concentration of 20 ng/ml if not indicated otherwise. Clonal differentiation assays are described in detail in the Supplemental Experimental Procedures.

Statistical Analysis

Significance level of the data sets was determined by performing a two-tailed Student's t test. If equal variances between the groups could not be assumed, Welch's correction of the t test was applied. Tests for statistical significance were performed with Graph Pad Prism v4 software (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n.s., not significant).

ACCESSION NUMBERS

The gene array data have been deposited in ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under the accession number E-MEXP-3743.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.06.020.

ACKNOWLEDGMENTS

We thank G. Häcker for support; the members of the A.D. laboratory for valuable discussions; C.A. Connor and H. Pircher for comments on the manuscript; and N. Göppert for technical assistance. We are grateful to B. Hogan for providing *Id2*$^{CreERt2/+}$ and *Id2*$^{Gfp/+}$ mice and to J. Wersing for cell sorting. The work was supported by the Deutsche Forschungsgemeinschaft (SGBM, SFB620/A14 and GRK1104 to A.D., T.H., and C.S.N.K.), the Bundesministerium für Bildung und Forschung Centrum für Chronische Immundefizienz (to A.D.), an EU grant (PAS_241506 to D.V.), and Boehringer Ingelheim (to M.B.).

Received: March 28, 2012
Accepted: June 30, 2012
Published online: October 11, 2012

REFERENCES

Blaser, C., Kaufmann, M., and Pircher, H. (1998). Virus-activated CD8 T cells and lymphokine-activated NK cells express the mast cell function-associated antigen, an inhibitory C-type lectin. J. Immunol. *161*, 6451–6454.

Boos, M.D., Yokota, Y., Eberl, G., and Kee, B.L. (2007). Mature natural killer cell and lymphoid tissue-inducing cell development requires Id2-mediated suppression of E protein activity. J. Exp. Med. *204*, 1119–1130.

Brickshawana, A., Shapiro, V.S., Kita, H., and Pease, L.R. (2011). Lineage$^{(-)}$ Sca1$^+$c-Kit$^{(-)}$CD25$^+$ cells are IL-33-responsive type 2 innate cells in the mouse bone marrow. J. Immunol. *187*, 5795–5804.

Buonocore, S., Ahern, P.P., Uhlig, H.H., Ivanov, I.I., Littman, D.R., Maloy, K.J., and Powrie, F. (2010). Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. Nature *464*, 1371–1375.

Chang, Y.J., Kim, H.Y., Albacker, L.A., Baumgarth, N., McKenzie, A.N., Smith, D.E., Dekruyff, R.H., and Umetsu, D.T. (2011). Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. Nat. Immunol. *12*, 631–638.

Gorfu, G., Rivera-Nieves, J., and Ley, K. (2009). Role of beta7 integrins in intestinal lymphocyte homing and retention. Curr. Mol. Med. *9*, 836–850.

Grote, D., Souabni, A., Busslinger, M., and Bouchard, M. (2006). Pax 2/8-regulated Gata 3 expression is necessary for morphogenesis and guidance of the nephric duct in the developing kidney. Development *133*, 53–61.

Grote, D., Boualia, S.K., Souabni, A., Merkel, C., Chi, X., Costantini, F., Carroll, T., and Bouchard, M. (2008). Gata3 acts downstream of beta-catenin signaling to prevent ectopic metanephric kidney induction. PLoS Genet. *4*, e1000316.

(G) Highly purified YFP$^-$ ILC2s were cultured on mitotically inactivated OP9 feeder cells in the presence of IL-2, IL-7, and 4-OH tamoxifen. After 7 days, cells were analyzed for expression of YFP and KLRG1. Numbers represent percentage of cells in the indicated gates.
(H) Expression of Sca1 and YFP among Lin$^-$ cells of the bone marrow from the indicated mouse strains.
(I) Expression of CD25 and Sca1 by Lin$^-$ bone marrow cells. Numbers indicate percentage of cells in quadrant. The lower panel shows the expression of YFP (Cre-on) in electronically gated Lin$^-$CD25$^+$Sca1^{hi} bone marrow cells of the indicated mouse strains. Numbers indicate percentage of YFP-positive cells.
(J) Highly purified YFP$^+$ ILC2Ps were cultured on mitotically inactivated OP9 feeder cells in the presence of IL-2, IL-7, and 4-OH tamoxifen. After 7 days, the cells were analyzed for expression of YFP and KLRG1. Numbers represent percentage of cells in quadrants.
All data are representative of at least two independent experiments. ***$p \leq 0.001$; n.s., not statistically significant. See also Figure S5.

Gründemann, C., Bauer, M., Schweier, O., von Oppen, N., Lässing, U., Saudan, P., Becker, K.F., Karp, K., Hanke, T., Bachmann, M.F., and Pircher, H. (2006). Cutting edge: identification of E-cadherin as a ligand for the murine killer cell lectin-like receptor G1. J. Immunol. *176*, 1311–1315.

Hacker, C., Kirsch, R.D., Ju, X.S., Hieronymus, T., Gust, T.C., Kuhl, C., Jorgas, T., Kurz, S.M., Rose-John, S., Yokota, Y., and Zenke, M. (2003). Transcriptional profiling identifies Id2 function in dendritic cell development. Nat. Immunol. *4*, 380–386.

Hanke, T., Corral, L., Vance, R.E., and Raulet, D.H. (1998). 2F1 antigen, the mouse homolog of the rat "mast cell function-associated antigen", is a lectin-like type II transmembrane receptor expressed by natural killer cells. Eur. J. Immunol. *28*, 4409–4417.

Huntington, N.D., Tabarias, H., Fairfax, K., Brady, J., Hayakawa, Y., Degli-Esposti, M.A., Smyth, M.J., Tarlinton, D.M., and Nutt, S.L. (2007). NK cell maturation and peripheral homeostasis is associated with KLRG1 up-regulation. J. Immunol. *178*, 4764–4770.

Ito, M., Maruyama, T., Saito, N., Koganei, S., Yamamoto, K., and Matsumoto, N. (2006). Killer cell lectin-like receptor G1 binds three members of the classical cadherin family to inhibit NK cell cytotoxicity. J. Exp. Med. *203*, 289–295.

Jackson, J.T., Hu, Y., Liu, R., Masson, F., D'Amico, A., Carotta, S., Xin, A., Camilleri, M.J., Mount, A.M., Kallies, A., et al. (2011). Id2 expression delineates differential checkpoints in the genetic program of CD8α^+ and CD103$^+$ dendritic cell lineages. EMBO J. *30*, 2690–2704.

Kee, B.L. (2009). E and ID proteins branch out. Nat. Rev. Immunol. *9*, 175–184.

Kumar, R., Fossati, V., Israel, M., and Snoeck, H.W. (2008). Lin$^-$Sca1$^+$kit$^-$ bone marrow cells contain early lymphoid-committed precursors that are distinct from common lymphoid progenitors. J. Immunol. *181*, 7507–7513.

Liang, H.E., Reinhardt, R.L., Bando, J.K., Sullivan, B.M., Ho, I.C., and Locksley, R.M. (2012). Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. Nat. Immunol. *13*, 58–66.

Monticelli, L.A., Sonnenberg, G.F., Abt, M.C., Alenghat, T., Ziegler, C.G., Doering, T.A., Angelosanto, J.M., Laidlaw, B.J., Yang, C.Y., Sathaliyawala, T., et al. (2011). Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. Nat. Immunol. *12*, 1045–1054.

Moro, K., Yamada, T., Tanabe, M., Takeuchi, T., Ikawa, T., Kawamoto, H., Furusawa, J., Ohtani, M., Fujii, H., and Koyasu, S. (2010). Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit($^+$)Sca-1($^+$) lymphoid cells. Nature *463*, 540–544.

Neill, D.R., Wong, S.H., Bellosi, A., Flynn, R.J., Daly, M., Langford, T.K., Bucks, C., Kane, C.M., Fallon, P.G., Pannell, R., et al. (2010). Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. Nature *464*, 1367–1370.

Pai, S.Y., Truitt, M.L., Ting, C.N., Leiden, J.M., Glimcher, L.H., and Ho, I.C. (2003). Critical roles for transcription factor GATA-3 in thymocyte development. Immunity *19*, 863–875.

Possot, C., Schmutz, S., Chea, S., Boucontet, L., Louise, A., Cumano, A., and Golub, R. (2011). Notch signaling is necessary for adult, but not fetal, development of RORγt($^+$) innate lymphoid cells. Nat. Immunol. *12*, 949–958.

Price, A.E., Liang, H.E., Sullivan, B.M., Reinhardt, R.L., Eisley, C.J., Erle, D.J., and Locksley, R.M. (2010). Systemically dispersed innate IL-13-expressing cells in type 2 immunity. Proc. Natl. Acad. Sci. USA *107*, 11489–11494.

Randall, T.D., and Weissman, I.L. (1998). Characterization of a population of cells in the bone marrow that phenotypically mimics hematopoietic stem cells: resting stem cells or mystery population? Stem Cells *16*, 38–48.

Rawlins, E.L., Clark, C.P., Xue, Y., and Hogan, B.L. (2009). The Id2$^+$ distal tip lung epithelium contains individual multipotent embryonic progenitor cells. Development *136*, 3741–3745.

Reynders, A., Yessaad, N., Vu Manh, T.P., Dalod, M., Fenis, A., Aubry, C., Nikitas, G., Escalière, B., Renauld, J.C., Dussurget, O., et al. (2011). Identity, regulation and in vivo function of gut NKp46$^+$RORγt$^+$ and NKp46$^+$RORγt$^-$ lymphoid cells. EMBO J. *30*, 2934–2947.

Saenz, S.A., Siracusa, M.C., Perrigoue, J.G., Spencer, S.P., Urban, J.F., Jr., Tocker, J.E., Budelsky, A.L., Kleinschek, M.A., Kastelein, R.A., Kambayashi, T., et al. (2010). IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. Nature *464*, 1362–1366.

Sanos, S.L., Bui, V.L., Mortha, A., Oberle, K., Heners, C., Johner, C., and Diefenbach, A. (2009). RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. Nat. Immunol. *10*, 83–91.

Sarkar, S., Kalia, V., Haining, W.N., Konieczny, B.T., Subramaniam, S., and Ahmed, R. (2008). Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. J. Exp. Med. *205*, 625–640.

Sawa, S., Cherrier, M., Lochner, M., Satoh-Takayama, N., Fehling, H.J., Langa, F., Di Santo, J.P., and Eberl, G. (2010). Lineage relationship analysis of RORgammat+ innate lymphoid cells. Science *330*, 665–669.

Schmitt, T.M., and Zúñiga-Pflücker, J.C. (2002). Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. Immunity *17*, 749–756.

Spits, H., and Di Santo, J.P. (2011). The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. Nat. Immunol. *12*, 21–27.

Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M., and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev. Biol. *1*, 4.

Steenwinckel, V., Louahed, J., Lemaire, M.M., Sommereyns, C., Warnier, G., McKenzie, A., Brombacher, F., Van Snick, J., and Renauld, J.C. (2009). IL-9 promotes IL-13-dependent paneth cell hyperplasia and up-regulation of innate immunity mediators in intestinal mucosa. J. Immunol. *182*, 4737–4743.

Voehringer, D., Blaser, C., Brawand, P., Raulet, D.H., Hanke, T., and Pircher, H. (2001). Viral infections induce abundant numbers of senescent CD8 T cells. J. Immunol. *167*, 4838–4843.

Vonarbourg, C., Mortha, A., Bui, V.L., Hernandez, P.P., Kiss, E.A., Hoyler, T., Flach, M., Bengsch, B., Thimme, R., Hölscher, C., et al. (2010). Regulated expression of nuclear receptor RORγt confers distinct functional fates to NK cell receptor-expressing RORγt($^+$) innate lymphocytes. Immunity *33*, 736–751.

Wong, S.H., Walker, J.A., Jolin, H.E., Drynan, L.F., Hams, E., Camelo, A., Barlow, J.L., Neill, D.R., Panova, V., Koch, U., et al. (2012). Transcription factor RORα is critical for nuocyte development. Nat. Immunol. *13*, 229–236.

Yang, Q., Saenz, S.A., Zlotoff, D.A., Artis, D., and Bhandoola, A. (2011). Cutting edge: Natural helper cells derive from lymphoid progenitors. J. Immunol. *187*, 5505–5509.

Yokota, Y., Mansouri, A., Mori, S., Sugawara, S., Adachi, S., Nishikawa, S., and Gruss, P. (1999). Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. Nature *397*, 702–706.

Zabel, B.A., Agace, W.W., Campbell, J.J., Heath, H.M., Parent, D., Roberts, A.I., Ebert, E.C., Kassam, N., Qin, S., Zovko, M., et al. (1999). Human G protein-coupled receptor GPR-9-6/CC chemokine receptor 9 is selectively expressed on intestinal homing T lymphocytes, mucosal lymphocytes, and thymocytes and is required for thymus-expressed chemokine-mediated chemotaxis. J. Exp. Med. *190*, 1241–1256.

Zheng, W., and Flavell, R.A. (1997). The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell *89*, 587–596.

Zhu, J., Min, B., Hu-Li, J., Watson, C.J., Grinberg, A., Wang, Q., Killeen, N., Urban, J.F., Jr., Guo, L., and Paul, W.E. (2004). Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. Nat. Immunol. *5*, 1157–1165.