Identification and distribution of developing innate lymphoid cells in the fetal mouse intestine

Jennifer K Bando¹, Hong-Erh Liang¹,² & Richard M Locksley¹,²

Fetal lymphoid tissue inducer (LTi) cells are required for lymph node and Peyer’s patch (PP) organogenesis, but where these specialized group 3 innate lymphoid cells (ILC3s) develop remains unclear. Here, we identify extrahepatic arginase-1+ Id2+ fetal ILC precursors that express a transitional developmental phenotype (ftILCPs) and differentiate into ILC1s, ILC2s and ILC3s in vitro. These cells populate the intestine by embryonic day (E) 13.5 and, before PP organogenesis (E14.5–15), are broadly dispersed in the proximal gut, correlating with regions where PPs first develop. At E16.5, after PP development begins, ftILCPs accumulate at PP anlagen in a lymphotoxin-α-dependent manner. Thus, ftILCPs reside in the intestine during PP development, where they aggregate at PP anlagen after stromal cell activation and become a localized source of ILC populations.

PPs are aggregated lymphoid follicles in the small intestine that sample intestinal luminal antigens and facilitate mucosal immune responses. In the mouse, the formation of the primordial PP, or PP anlage, is induced during fetal development by LTi cells, lineage (Lin−) c-Kit+ interleukin 7 receptor-α (IL-7Rα)+ cells that express the transcription factor RORγt and can express CD4 (refs. 1–2). These cells activate lymphotixin-β receptor (LTβR) signaling in stromal cells at the developing site with the LTβR ligand LTα1β1 (refs. 3–6). Lymphotoxin-activated stromal cells express chemokines that attract additional LTi cells to the developing PP, initiating a positive feedback loop that enhances stromal activation at the anlage5,7. This specific interaction between LTi and stromal cells is required for PP development, as these structures are absent in both LTα1β1-deficient Lta−/− mice and LTi cell–deficient Rorc(γt)−/− animals that specifically lack the RORγt isoform owing to disruption of exon 1γt (ref. 1).

LTi cells belong to a family of ILCs, which are dependent on common γ-chain (γc) cytokines but lack most lineage markers and do not require the recombination-activating genes Rag1 and Rag2 for development8. ILCs participate in a wide range of immune responses and have been divided into groups on the basis of the transcription factor and cytokine expression. ILC1s express the transcription factor T-bet and produce the cytokine IFN-γ; ILC2s highly express the transcription factor GATA-3 and produce the cytokines IL-5 and IL-13; and ILC3s, which include fetal LTi cells, require the transcription factor RORγt and produce the cytokines IL-22 and IL-17A. In contrast to other innate lymphoid populations, LTi cells are abundant in the fetal intestine and are the only ILCs described in the fetal mouse that function in organ development.

How these innate lymphoid subsets develop is a topic under active investigation. LTi cells and other ILC subsets require the E2A transcriptional inhibitor Id2, which indicates a shared developmental pathway for ILC lineages9–11. Indeed, a common precursor to multiple ILC subsets has been described in fetal liver and adult bone marrow, the major sites of hematopoiesis in fetuses after E10.5 and adults, respectively12. These Lin−Id2+αβγTcFlt3−CD25+ cells differentiate into NK1.1+IL-7Rα+T-bet+ ILC1s, GATA-3+ ILC2s and RORγt+ ILC3s but not T cells, B cells or conventional natural killer (NK) cells. A subset of Id2+ ILC progenitors also express the transcription factor PLZF and appear to have restricted lineage potential12,13.

Although ILC precursors have been described at sites of hematopoiesis, little is known about these cells in peripheral tissues. In the fetal mouse, there is evidence that precursor activity exists outside of the liver, as LTi cells have been derived in vitro from Lin−c-Kit+ IL-7Rα+αβγTcFlt3−RORγt-GFP− cells from the intestines of E14 Rorc(γt)GFP knock-in reporter mice14. Although these data suggest that undifferentiated ILC precursors may migrate to the fetal intestine and continue their development in tissue, the location and lineage potential of these extrahepatic ILC precursors remains unclear.

Arginase-1 (Arg1) is a urea cycle enzyme that is induced in macrophages during type 2 immune responses and wound repair15–18. We have reported that in the immune system, Arg1 is expressed not only by activated myeloid populations but also, constitutively, by ILC2s in adult mice19. Here we show that Arg1 expression additionally marks fetal ILC precursors that are in transitional developmental stages (ftILCPs) and their progeny. Arg1+ ftILCPs are capable of differentiating into functional ILC1, ILC2 and ILC3 populations and are present in the fetal intestine during PP organogenesis. These cells are dispersed in proximal portions of the fetal small intestine before PP development and accumulate at the developing PP in a lymphotixin-α (LTα)-dependent manner once intestinal lymphoid tissue organogenesis is initiated. These results indicate that fetal ILC precursors leave the liver and continue differentiating in intestinal tissues during active lymphoid tissue organogenesis.

RESULTS

Adult LTi-like cells express the enzyme Arg1

We have reported that ILC2s constitutively express Arg1 and that ILC2s are >95% of all Arg1-expressing hematopoietic cells in the naive
mouse lung. We next investigated Arg1 expression in the enteric system to determine whether additional cell populations express the enzyme. Using Arg1<sup>YFP</sup> reporter mice, in which a construct encoding an internal ribosomal entry site and YFP (IRES-YFP) is inserted after exon 8 of the Arg1 gene without disrupting enzyme expression<sup>20</sup>, we determined that YFP<sup>+</sup> cells made up less than 1% of hematopoietic cells isolated from the small intestine (lamina propria and intraepithelial cells combined) (Fig. 1a). These cells were identified as ILCs on the basis of their expression of the glycoprotein Thy-1 and IL-7Rα and their lack of common myeloid and lymphoid lineage surface markers CD11b, CD11c, CD3, B220, NK1.1 and Nkp46 (Fig. 1b). In wild-type and Rag<sup>2<sup>−/−</sup></sup> mice, Arg1-expressing cells were present in cryptopatches (Fig. 1c,d), tertiary lymphoid structures that contain LTI-like cells and CD11c<sup>+</sup> dendritic cells<sup>21,22</sup>. To test whether YFP<sup>+</sup>

![Image]

**Figure 1.** Adult LTI-like cells express Arg1. (a) YFP<sup>+</sup> cells in the adult wild-type (WT) and Arg1<sup>YFP</sup> small intestine. Plots show live CD45<sup>+</sup> cells, and the numbers in the graphs indicate the percentage of these cells that express YFP. *Anemonia majano* cyan (Am cyan) is an open channel used here to detect autofluorescence. (b) Surface markers expressed by YFP<sup>+</sup> cells in the adult small intestine. Shaded histograms indicate isotype controls. (c) Histological sections of WT and Arg1<sup>YFP</sup> cryptopatches. Sections are counterstained with DAPI. (d) Anti-RORγ<sup>t</sup> staining in an Arg1<sup>YFP</sup>Rag2<sup>−/−</sup> cryptopatch. (e) Surface markers expressed by YFP<sup>+</sup> cells isolated from dissected cryptopatches. Numbers in quadrants indicate frequencies out of total YFP<sup>+</sup> cells. Data are representative of three independent experiments, with two-three mice per group (a-e).

![Image]

**Figure 2.** Innate lymphoid cells express Arg1 in the fetal gut. (a) YFP expression in E15.5 wild-type (WT) and Arg1<sup>YFP</sup> intestines. (b) Surface markers expressed by YFP<sup>+</sup> cells isolated from E15.5 Arg1<sup>YFP</sup> intestines. Shaded histograms indicate isotype controls. (c) RORγ<sup>ffm</sup>, NK1.1 and ST2 expression in distinct Arg1-YFP<sup>+</sup> populations in E15.5 intestines. Arg1-YFP<sup>RNT</sup> cells are also present. (d) Expression of T-bet, GATA-3 and RORγ<sup>t</sup> in Arg1-YFP<sup>+</sup>ST2<sup>+</sup> and Arg1-YFP<sup>+</sup>NK1.1<sup>+</sup> cells. Plots were gated on CD45<sup>+</sup>Arg1-YFP<sup>+</sup> cells. (e) Arg1-YFP<sup>+</sup>RORγ<sup>t</sup>-GFP<sup>+</sup> double-reporter intestines. (f) Arg1-YFP<sup>+</sup>RORα<sup>−/+</sup>-GFP<sup>−/−</sup> Ar1<sup>−/−</sup> ST2<sup>−/−</sup> mice isolated from E15–E15.5 Rorc<sup>−/−</sup> and Rorc<sup>−/−</sup> intestines (n = 4 mice per group). NS, not significant (P > 0.05, unpaired Student’s t-test). (g) RFP expression in intestinal Arg1-YFP<sup>+</sup>RORγ<sup>+</sup> Gfp<sup>+</sup> LTI cells from E15.5 and E16.5 Arg1-YFP<sup>+</sup> Rorc<sup>−/−</sup>rorc<sup>−/−</sup> Rosa26<sup>loxSTOP-RFP</sup> triple-reporter mice. (h) Left, GFP expression in Arg1-YFP<sup>+</sup>RNT<sup>−</sup> cells (identified by fate mapping) isolated from an E15.5 Arg1<sup>YFP</sup>Rorc<sup>−/−</sup>rorc<sup>−/−</sup> Rosa26<sup>loxSTOP-RFP</sup> intestine. Right, GFP expression in all Arg1<sup>−/−</sup> cells in an Arg1<sup>YFP</sup>Rorc<sup>−/−</sup>rorc<sup>−/−</sup> littermate as a positive control. PE, phycoerythrin. (i) Arg1-YFP<sup>+</sup>NK1.1 ST2–CD4– counts in E15.5 Il2rg<sup>−/−</sup> and Il2rg<sup>−/−</sup> intestines (n = 4, 6 mice per group). ***P < 0.0001 (unpaired Student’s t-test). (j) Left, Id2<sup>+</sup> expression in Arg1-YFP<sup>+</sup>NK1.1 ST2–CD4– cells isolated from E15.5 Arg1<sup>YFP</sup> and il2<sup>−/−</sup> intestine; plots were gated on CD45<sup>−</sup>NK1.1 ST2–CD4– cells. Data are representative of four (a,e), three (b,d,j) or two (f–i) independent experiments. Two to five mice were used per experiment for representative flow cytometry plots. Numbers in flow cytometry plots indicate frequencies. Am cyan, *Anemonia majano* cyan.
cells express LTi-like cell surface markers, we dissected cryptopatches from the small intestine and analyzed them by flow cytometry. YFP+ cells isolated from cryptopatches were IL-7Rα−c-Kit−CD45+CD3−, consistent with the profile of LTi-like cells (Fig. 1e). Furthermore, YFP+ cells in cryptopatches expressed the LTi transcription factor RORγt as determined by RORγt antibody staining in Rag2−/− mice (Fig. 1d). RORγt+ cells that did not express Arg1 were also present in cryptopatches, which indicated heterogeneity among cryptopatch ILCs. These results indicate that in addition to ILC2s, Arg1 marks a subset of intestinal LTi-like cells in adult cryptopatches.

**Innate lymphoid cells express Arg1 in the fetal gut**

LTi-like cells in the adult intestinal cryptopatches have been proposed to be analogous to fetal LTi cells at the developing PP, as both of these cell types are RORγt dependent and cluster at VCAM-1+ intestinal sites that support B cell accumulation.12–14 We therefore set out to determine whether Arg1 is expressed in the fetal intestine during PP organogenesis. In the mouse embryo, LTi cells first begin forming PP anlagen between E15.5 and E16.5 in the proximal small intestine.15 At E15.5, we detected in the intestine hematopoietic YFP+ cells that expressed αβ, IL-7Rα, c-Kit and low amounts of CD11b, but not CD11c, CD3, CD19 or NKP46 (Fig. 2a,b). To determine whether these were LTi cells, we crossed Arg1YFP reporter mice to Rorc(γt)CreRosa26loxSTOP-RFP fate-mapping mice, in which cells that express Rorc(γt) are permanently marked with RFP after Cre-mediated excision of a loxP-flanked STOP sequence. Intestinal flow cytometry confirmed that RORγt fate-mapped (RORγt(fm)+) LTi cells expressed Arg1 (Fig. 2c). Smaller subsets of RORγt(fm)+ cells were Arg1-YFP+NK1.1+ and Arg1-YFP+CD11b+ (Fig. 2c and data not shown). These results indicate that RORγt+ LTi cells express Arg1 in both the fetal and adult intestine.

In addition to marking RORγt(fm)+ LTi cells, Arg1-YFP also marked RORγt(fm)− ILCs in the fetal intestine (Fig. 2c). These RORγt(fm)− cells were grouped into three populations on the basis of surface marker and transcription factor expression: NK1.1+ T-bet+ cells, ST2+GATA-3hi ILC2s and a population that lacked NK1.1 and ST2 expression, which we refer to here as Arg1YFP+RNT− (Arg1YFP+RORγt(fm)−NK1.1−ST2−) cells (Fig. 2c,d). YFP expression in fetal ILC2s validated our published finding that Arg1 expression is a constitutive feature of this cell type.12 To determine whether the NK1.1+T-bet+ cells were ILC1s, we characterized these cells in neonatal and adult mice. Arg1-YFP+NK1.1+ cells were present in the liver and spleen after birth, although the percentage of NK1.1+ cells that expressed YFP decreased with age (Supplementary Fig. 1a). These cells lacked expression of RORγt and Eomesodermin and were present in 20-d-old Rag2−/− mice, which indicates that they were not ILC3s, classical NK cells or invariant natural killer T (iNKT) cells (Supplementary Fig. 1b–d). In the adult liver and spleen, Arg1-YFP+RORγt−NK1.1+ ST2+ cells were detected in the fetal intestine at E15.5, confirming the existence of this population (Fig. 2e).

**Figure 3** Fetal Arg1-YFP+RNT− cells aggregate at the developing PP anlage. (a) YFP+ cell counts from upper (1), middle (2) and lower (3) sections of the E14.5–15 small intestine (n=7 mice per group) ∗p<0.05, ∗∗p<0.01, ∗∗∗p≤0.001 (one-way ANOVA followed by Tukey’s test). (b) YFP+ cells at the PP anlage in the E16.5 intestine. VCAM-1+ marks activated stromal cells, and sections were counterstained with DAPI. (c) Arg1 (YFP) and RORγt(fm) (RFP) expression at the anlage of E16.5 Arg1YFP+Rorc(γt)Cre Rosa26loxSTOP-RFP double reporter mice. Light blue arrowheads point at examples of YFP+RFP− cells. (d) Identification of the anlage in intact E16.5 Arg1YFP+Rorc(γt)Cre Rosa26loxSTOP-RFP intestines. (e) Ratio of YFP+ populations to EpCAM+ cells in dissected anlagen and adjacent sites (n=10 mice per group). ∗∗∗p<0.001, NS, not significant (p>0.05; paired Student’s t-test). (f) Arg1 (YFP) and RORγt(fm) (RFP) expression in sections of E16.5 intestines from Lta−/− (left) and Lta−/− images are representative of PP anlagen, while Lta−/− images are representative of sections through the proximal half of the intestine (n=3, 4 mice per group). Dotted white lines indicate the anti-mesenteric side of each intestine. (g) Arg1 (YFP) expression in sections of E16.5 intestines from Rorc(γt)−/− (left) and Rorc(γt)−/− (right) littermates (n=3, 4 mice per group). (h) Expression of CCR7 and CXCR5 in Arg1-YFP+RNT− cells and Arg1-YFP+RORγt(fm)+ LTi cells from whole intestines (left) or dissected anlagen (right). Data are representative of three (d–f) or two (g–h) independent experiments or are pooled from two independent experiments (a,e).
Additionally, these cells were present in LTI-deficient Rorc(γt)−/− E15–E15.5 intestines, which indicates that they were not LTI cells (Fig. 2f). To directly compare the two RORγt reporters, we generated Arg1YFP;Rorc(γt)GFP;Rorc(γt)Cre;Rosa26loxSTOP-RFP triple-reporter mice. In these mice, more than 95% of intestinal Arg1-YFP+RORγt-GFP+ LTI cells were marked by RFP at E15.5 and E16.5 (Fig. 2g), and more than 95% of Arg1-YFP+RNT− cells identified by fate mapping were GFP+ at E15.5 (Fig. 2h), which indicates that the two RORγt reporters have similar efficiency in marking Arg1-YFP+ LTI cells and in identifying Arg1-YFP+RNT− cells in the fetal intestine. Similar to other ILC populations, Arg1-YFP+RNT− cells (estimated by staining for Arg1-YFP+NK1.1+ST2+CD4− cells) were dependent on the common γ-chain (encoded by Il2rg), expressed the transcription factor Id2 and were absent in Id2−/− animals (Fig. 2i,j). Thus, Arg1-YFP+RNT− cells are an ILC population in the fetal mouse intestine with unique lineage specificity. Taken together, our results indicate that NK1.1+ILC1s, ST2+ ILC2s, RORγt+ ILC3s and an uncategorized RNT−ILC population express Arg1 in the fetal intestine.

Fetal Arg1-YFP+RNT− cells aggregate at the developing PP

As Arg1-YFP+RNT− cells were present in the fetal intestine during PP development, we next determined whether these cells were interacting with developing lymphoid organs. In the fetus, the most proximal PP begins to develop between E15.5 and E16.5, with PPs distal to the first site developing sequentially over the next few days. At E14.5–E15, before PP development begins, Arg1-YFP+ LTI and RNT− cells were most abundant in the upper and middle portions of the small intestine, which indicates that these ILCs are locally positioned before the development of proximal PP anlagen (Fig. 3a and Supplementary Fig. 2). At E16.5, after the start of PP development, Arg1-YFP+ cells were present at the first developing VCAM-1+ PP anlage (Fig. 3b) and consisted of both RORγt(fm)+ LTI cells and RORγt(fm)− ILCs (Fig. 3c). To quantify Arg1-YFP+RORγt(fm)− populations at the anlage, we identified ILC clusters on the basis of RFP expression in whole intestines and dissected them for flow cytometric analysis (Fig. 3d). At E16.5, the first developing PP contained significantly more Arg1-YFP+RNT− and Arg1-YFP+NK1.1+ cells than sites immediately adjacent to the developing organ, on the basis of the ratio of YFP cells to CD45− epithelial cell adhesion molecule (EpCAM)+ cells (Fig. 3e). In contrast, Arg1-YFP+ST2+ ILC2s did not accumulate at the anlage. These data indicate that multiple ILC populations, including Arg1-YFP+RNT− cells, accumulate with LTI cells at the developing PP.

The PP anlage is formed when stromal cells at the anti-mesenteric side of the intestine are activated at discrete sites by LTαβ+ hematopoietic cells. To test whether fetal Arg1-YFP+RNT− accumulation at the anlage was dependent on stromal activation, we assessed intestines from E16.5 Lta−/− mice, which are unable to form the LTαβ+ heterotrimer, for YFP+ aggregates in consecutive sections of the proximal half of the small intestine. RFP+ LTI cells and YFP+ RFP− cells were enriched along the anti-mesenteric side of the intestine in both Lta−/− and Lta+− littermates (Fig. 3f). However, aggregated clustering of YFP+ cells was dependent on Lta, which indicates that factors induced by LTαβ in stromal cells are required for Arg1-YFP+RNT− cell accumulation at the PP anlage. LTI cells express LTαβ, and to test whether this population was required for Arg1-YFP+RNT− aggregates, sections of LTα-deficient Rorc(γt)−/− intestines were assessed for YFP+ clusters. E16.5 Rorc(γt)−/− intestines lacked YFP+ aggregates in consecutive sections throughout the small intestine (Fig. 3g), which indicates that Arg1-YFP+RNT− cells cluster after LTI cells activate stromal cells at the anlage. Finally, as LTαβ activates expression of the chemokines CXCL13 and CCL19 in intestinal stromal cells in situ and LTI cells migrate toward these chemokines in vitro, we tested whether these factors could also recruit Arg1+RNT− cells. Compared to LTI cells, which express the chemokine receptors CXCR5 and CCR7, Arg1-YFP+RNT− cells did not express these receptors even after restricting our analysis to cells isolated from PP anlagen (Fig. 3h). We conclude that Arg1-YFP+RNT− cells accumulate at the PP anlage in a CCR7− and CXCR5− independent manner after LTβR signaling is activated in stromal cells.

Although multiple ILC populations expressed Arg1 at the developing PP, Arg1 expression was not required for normal numbers of PPs or PP follicles as assessed in Vav1Cre;Arg1fl/fl adult mice, in which loxP sites inserted in Arg1 ( floxed, or fl) disrupt the gene in a Cre-mediated manner in hematopoietic cells (Supplementary Fig. 3). Mice deficient in hematopoietic Arg1 also had normal B and T cell...
compartmentalization in the PP, inguinal lymph node and spleen (data not shown). Thus, hematopoietic Arg1 expression is not required for secondary lymphoid tissue development.

**Arg1-YFP+RNT− cells differentiate into mature ILCs**

Before PP development, at E13.5, Arg1-YFP+RNT− cells were the most abundant YFP+ ILC lineage in the fetal intestine (Fig. 4a). As the frequency of YFP+ cells that were RORγt(fm)+ LTi cells increased over the next 3 d, the percentage of RNT− cells decreased, which suggests that Arg1-YFP+RNT− cells in the fetal intestine are ILC precursors that undergo differentiation into ILC lineages. To test this possibility, we assessed transcription factor expression in Arg1-YFP+RNT− cells by flow cytometry. In comparison to adult bone marrow ILC precursors, which express GATA-3 but not T-bet and RORγt, Arg1-YFP+RNT− cells in the E15.5 intestine expressed all three transcription factors at varying amounts (Fig. 4b). Because the RORγt antibodies used in these experiments (B2D clone) can also bind to the RORγ isoform, we confirmed that in Arg1-YFP+ILCs these antibodies were specifically interacting with RORγt using cells from fetal Arg1-YFP+RNT−/− intestines (Supplementary Fig. 4).

Even though the transcription factors expressed by Arg1-YFP+RNT− cells indicated that this population was heterogeneous, these cells lacked the distinct protein expression profiles associated with mature ILC lineages. Arg1-YFP+RNT− cells that expressed RORγt protein were distinct from RORγt(fm)+ LTi cells in that they expressed more GATA-3 and T-bet (Fig. 4c). Additionally, fetal

![Figure 5](image-url)

**Figure 5** Arg1-YFP+RNT− cells differentiate into mature ILCs. (a) Purity of sorted Arg1-YFP+RNT− cells (right) compared to unsorted cells (left). (b) Populations detected after culturing Arg1-YFP+RNT− cells for 20 h. Left, cultured YFP+ cells from Arg1-YFP single-reporter mice; right, YFP+RNT− cells from Arg1-YFP(Rorc(t))Cre Rosa26STOP-RFP double-reporter mice. (c) Expression of CD3 and NKp46 by RORγt(fm)+ cells after 6 d of culture. Shaded histograms represent isotype controls. (d) Transcription factors expressed by RORγt(fm)+ NK1.1+ and RORγt(fm)–NK1.1+ CD25+ cells after 6 d of culturing Arg1-YFP+RNT−CD25+ cells with OP9 cells. (e) Inducible T-cell costimulator (ICOS) expression in NK1.1–RORγt(fm)+CD25− cells (red) compared to NK1.1+ (green) and RORγt(fm)+ (blue) populations at day 6 of culture. (f) CD5, CD19 and CD11b expression at day 6 of culture. (g) Examples of gates used to identify populations in single cell cultures at day 6. Left, combined files of single wells from a 96-well plate. Right, examples of individual wells from single cell cultures. Plots (top left, bottom left and right) show different ILC populations from three separate wells. (h) Cell populations isolated from wells from single cell cultures at day 6 (left) and breakdown of wells that contained two populations (right). Undetermined (UD) cells did not express markers used to identify other lineages. Data are representative of four (a–b), three (g) or two (c–f) independent experiments or are pooled from three independent experiments of 96 wells each (h). Cells from 3–7 mice were sorted for each representative flow cytometry plot. Numbers in flow cytometry plots indicate frequencies.
Arg1-YFP\(^{+}\)RNT\(^{-}\) cells expressed more CD45 than did ROR\(^{\gamma}\)T\(^{+}\) LTI cells and did not express the surface trimer LT\(^{a}\)T\(^{b}\) (Fig. 4d). Similarly, Arg1-YFP\(^{+}\)RNT\(^{-}\) cells that expressed T-bet were distinct from Arg1-YFP\(^{+}\)NK1.1\(^{+}\) ILC1s on the basis of higher expression of GATA-3 and ROR\(^{\gamma}\)T in the Arg1-YFP\(^{+}\)RNT\(^{-}\) population (Fig. 4e). Therefore, Arg1-YFP\(^{+}\)RNT\(^{-}\) cells in the fetal intestine express multiple transcription factors associated with known ILC lineages, but their surface markers and transcription factor expression profiles indicate that they do not consist of mature ILC1s or ILC3s. Because we found that a small percentage of Arg1-YFP\(^{+}\)RNT\(^{-}\) cells expressed CD25 (Supplementary Fig. 5a) and may represent an ILC2 precursor–like cell, we tested whether this could account for variation in transcription factor expression. Although Arg1-YFP\(^{+}\)RNT\(^{-}\)CD25\(^{+}\) cells were T-bet\(^{-}\) and ROR\(^{\gamma}\)T\(^{-}\), this did not account for the heterogeneity in total Arg1-YFP\(^{+}\)RNT\(^{-}\) cells (Supplementary Fig. 5b,c). Collectively, these data indicate that intestinal Arg1-YFP\(^{+}\)RNT\(^{-}\)CD25\(^{-}\) cells are a heterogeneous population of cells that does not consist of known mature ILC subsets.

It was reported that E14 PLZF\(^{hi}\) ILC progenitors in the fetal liver express ROR\(^{\gamma}\)T and T-bet RNA. To determine whether intestinal Arg1-YFP\(^{+}\)RNT\(^{-}\) cells are similar to liver ILC progenitors in terms of transcription factor expression, we assessed ROR\(^{\gamma}\)T and T-bet expression in E14.5 Lin\(^{-}\)IL-7R\(^{a}\)T\(^{a}\)FTH3\(^{-}\)CD3\(^{-}\)ST2\(^{-}\) fetal liver cells, a population that consists of progenitors and early committed ILCs. The GATA-3\(^{+}\) fraction of these cells, which contains ILC progenitors, was ROR\(^{\gamma}\)T\(^{-}\) and T-bet\(^{-}\), which indicates that ILC progenitors from the fetal liver are more homogenous than Arg1-YFP\(^{+}\)RNT\(^{-}\) cells from the fetal intestine (Supplementary Fig. 6).

To test whether Arg1-YFP\(^{+}\)RNT\(^{-}\) cells could differentiate into mature ILCs, we isolated cells from E15.5 intestines and cultured them in vitro with recombinant mouse IL-7 (Fig. 5a). After 20 h of culture, Arg1-YFP\(^{+}\)RNT\(^{-}\) cells had given rise to ROR\(^{\gamma}\)T\(^{+}\), ROR\(^{\gamma}\)T\(^{-}\)NK1.1\(^{+}\) and ST2\(^{+}\) cells (Fig. 5b). ROR\(^{\gamma}\)T\(^{+}\) cells that developed in culture did not express CD3 or NKp46 at day 6 (Fig. 5c), consistent with these cells being NK receptor–negative ILC3s. Because a subset of Arg1-YFP\(^{+}\)RNT\(^{-}\) cells expressed CD25 (Supplementary Fig. 5a), we excluded these cells by sorting and culturing Arg1-YFP\(^{+}\)RNT\(^{-}\)CD25\(^{-}\) cells from E15.5 intestines in subsequent experiments. An analysis of transcription factors after 6 d of culture with the mouse bone marrow stromal cell line OP9 indicated that Arg1-YFP\(^{+}\)RNT\(^{-}\)CD25\(^{-}\) cells gave rise to NK1.1\(^{+}\)ROR\(^{\gamma}\)T\(^{+}\) T-bet\(^{-}\)GATA-3\(^{-}\) ILC1s, CD25\(^{+}\)ICOS\(^{+}\)ROR\(^{\gamma}\)T\(^{+}\)T-bet\(^{-}\)GATA-3\(^{-}\) ILC2s, ROR\(^{\gamma}\)T\(^{-}\)GATA-3\(^{-}\) ILC3s and a small population of ROR\(^{\gamma}\)T\(^{-}\)GATA-3\(^{-}\) ex-ROR\(^{\gamma}\)T cells (Fig. 5d,e and data not shown). Day 6 cultures did not contain CD5, CD19 or CD11b\(^{+}\) populations (Fig. 5f). Although YFP and ST2 were expressed by cultured cells after 20 h, these proteins were not detected at day 6, which indicates that additional factors are required to maintain expression of Arg1 and IL-33R in fetal cells (data not shown). However, ILCs that developed in culture were functional, as these cells expressed signature cytokines associated with mature ILC subsets after 3 h of stimulation with phorbol ester (PMA) and ionomycin (Supplementary Fig. 7a).

To determine the frequency of E15.5 intestinal Arg1-YFP\(^{+}\)RNT\(^{-}\)CD25\(^{-}\) cells that are capable of differentiating into Arg1-YFP\(^{+}\) ILC lineages, we cultured single cells with the OP9 cell line. From day 10 cultures expressed high amounts of LT\(^{a}\)T\(^{b}\), which indicates that this population derived from Arg1-YFP\(^{+}\)RNT\(^{-}\) cells has the potential to function as LTI cells by activating stromal cells (Supplementary Fig. 7b).

To determine whether Arg1 also marks ILC precursors in the adult mouse, we assessed adult bone marrow for YFP expression. Adult Lin\(^{-}\)Id2\(^{-}\)IL-7R\(^{a}\)T\(^{a}\)FTH3\(^{-}\)CD25\(^{-}\) ILC progenitors in bone marrow are distinguished from common lymphoid progenitors (CLPs) by Flt3 and from ILC2 precursors by CD25 (ref. 12). Whereas 80% of Lin\(^{-}\)Id2\(^{-}\)GFP\(^{+}\)IL-7R\(^{a}\)T\(^{a}\)FTH3\(^{-}\)CD25\(^{-}\) cells from E14.5–E15 fetal livers were Arg1\(^{+}\) (Fig. 6a,b), Lin\(^{-}\)IL-7R\(^{a}\)T\(^{a}\)FTH3\(^{-}\)Arg1-YFP\(^{+}\) cells in adult bone marrow consisted of only CD25\(^{-}\)ST2\(^{-}\) or ST2\(^{+}\) cells (Fig. 6c). Arg1\(^{YFP}\) and Arg1\(^{YFP}\)Id2\(^{GFP}\) mice confirmed that...
Arg1+ RNT− cells seem to have more restricted potential at the fetal intestine, and have the capacity to develop into ILC1s, ILC2s and ILC3s. These intestinal Arg1-YFP+ RNT− cells are associated with differentiation into specific lineages, providing a localized source of additional LTi cells as well as other ILC populations resident in the fetal gut. The fate of ILC precursors and the functions of their progeny during early life are intriguing areas for further investigation.

**DISCUSSION**

ILCs have crucial functions in organogenesis, homeostasis and immunity, but their development in the fetal mouse is not fully characterized. Here we show that Id2-dependent Arg1+ ftILCPs are present in the fetal intestine, and have the capacity to develop into ILC1s, ILC2s and ILC3s. These intestinal Arg1-YFP+ RNT− cells are heterogeneous and express varying amounts of GATA-3, T-bet and RORγ but do not consist of mature ILC populations on the basis of their surface markers and transcription factors expression profiles. In contrast, Id2+ multipotent ILC progenitors described in adult bone marrow do not express RORγ or T-bet [12, 13], and fetal liver ILC progenitors are more homogenous than intestinal Arg1-YFP+ RNT− cells. These data suggest that Arg1+ ftILCPs in the intestine may occupy an intermediate developmental step between undifferentiated GATA-3+ T-bet+ RORγ+ ILC precursors and mature ILC populations. Indeed, intestinal Arg1-YFP+ RNT− cells seem to have more restricted potential at the single-cell level than bone marrow and fetal liver progenitors [12, 13]. We found that most intestinal precursors give rise to single lineages, whereas bone marrow and fetal liver progenitors have been reported to produce multiple lineages at the single-cell level. These results support a model in which ILC precursors leave the fetal liver and enter other organs, where they exist in a transitional developmental stage before completing their differentiation into mature ILC lineages in tissues. Whether differential abundance of transcription factors expressed by Arg1+ YFP+ RNT− are associated with differentiation into specific lineages is unknown, as these intracellular proteins were identified only in fixed cells. Further studies are required to determine whether intestinal Arg1+ YFP+ RNT− cells consist of committed subsets or whether at this stage they are undergoing terminal lineage differentiation in response to local tissue factors.

During the analysis of Arg1+ lineages in the fetal intestine, we found that RORγ expression and protein expression did not completely overlap. This incomplete reporting is not due to inefficiency of Cre-induced loxP excision in Rorc(γt)−/− Rosa26STOP BFP animals, as Rorc(γt)GFP ‘knock-in–knockout’ reporter mice show similar patterns of expression. Instead, the lack of fluorescent protein expression in Arg1+ RNT− is regulated in ILC populations should provide insight into these differences. Whereas Arg1 expression in myeloid cells is induced by STAT6 or MyD88 activation, Arg1 expression in ILC2s occurs under homeostatic conditions [16–19, 27]. Arg1 has been identified as one of many GATA-3–dependent genes in ILC2s and ILC3s in vivo [28]. However, type 2 CD4+ T cells in the helminth-infected mouse lung and ILC precursors in naive adult mouse bone marrow are GATA-3 but do not express Arg1, which indicates that this transcription factor is not sufficient for expression of this enzyme in lymphoid cells [19]. Further studies will be required to identify additional factors that regulate Arg1 in different ILC populations.

In contrast to ILC precursors in the fetal liver, Arg1+ ILC precursors in the fetal intestine are a uniquely localized source of LTi cells at sites that require their rapid accumulation. Here we show that ILC precursors preferentially accumulate at the developing PP at E16.5 as compared to adjacent areas. CD45+ IL-7Rα+ SCA-1+ CD4+ cells have been found at the lymph node anlage as early as E13.5 (ref. 29). Whether this population contains precursors that give rise to multiple ILC lineages during lymph node development remains unknown. In the intestine, we determined that Arg1+ YFP+ RNT− accumulation at the PP was dependent on Lta and Rorc(γt), which indicates that this event occurs after LTi cells initiate organogenesis. Lta(β2) induces expression of adhesion molecules and chemokines at the PP anlage, and identifying which factors are required for accumulation will be crucial to test the effects of cell localization. Intestinal Arg1+ ILC precursors are unable to initiate the development of lymphoid organs because they lack Lta(β2) expression, but they may enhance the positive feedback loop once at the PP anlage by providing additional LTi cells on site. We suggest that this system reinforces stromal cell activation at each PP while maintaining normal numbers of lymphoid organs. By this model, stochastically differentiated LTi cells that induce PP development at E15.5–E16.5 initiate a LTβR-signaling program in stromal cells necessary for the aggregation of ftILCPs at the PP, thus providing a localized source of additional LTi cells as well as other ILC populations resident in the fetal gut.

**METHODS**

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

**ACKNOWLEDGMENTS**

The authors thank V. Nguyen (UCSF Flow Cytometry Core) and Z. Wang (Sabra Sorting Facility) for cell sorting, and D. Sheppard, D. Kioussis (MRC National Institute for Medical Research), D. Littman (New York University), E. Robey (University of California, Berkeley), H. Luche and H. Fehling (University Clinics Ulm, Germany) for providing mice for these studies. We also thank J. Cyster and L. Lanier for critical reading of the manuscript. Supported by the Howard Hughes Medical Institute (R.M.L.), the National Institutes of Health (AI026918, AI030663 and AI119944 to R.M.L.), the Sandler Asthma Basic Research Center at UCSF (R.M.L.) and the UCSF Biomedical Sciences (BMS) Graduate Program (J.K.B.).
AUTHOR CONTRIBUTIONS
J.K.B. and R.M.L. designed experiments and wrote the manuscript. J.K.B. conducted experiments and H.-E.L. provided reagents.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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

1. Eberl, G. et al. An essential function for the nuclear receptor RORγt in the generation of fetal lymphoid tissue inducer cells. Nat. Immunol. 5, 64–73 (2004).
2. Yoshida, H. et al. IL-7+ receptor α+ CD3− cells in the embryonic intestine induces the organizing center of Peyer’s patches. Int. Immunol. 11, 643–655 (1999).
3. De Togni, P. et al. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotaxin. Science 264, 703–707 (1994).
4. Füterer, A., Mink, K., Luz, A., Kosco-Vilbois, M.H. & Pfeffer, K. The lymphotaxin β receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. Immunity 9, 59–70 (1998).
5. Honda, K. et al. Distinct roles in lymphoid organogenesis for lymphotaxins α and β revealed in lymphotaxin β-deficient mice. Immunity 6, 491–500 (1997).
6. Koni, P.A. et al. Distinct roles in lymphoid organogenesis for lymphotaxins α and β revealed in lymphotaxin β-deficient mice. Immunity 6, 491–500 (1997).
7. Okuda, M., Tagawa, A., Wada, H. & Nishikawa, S. Distinct activities of stromal cells involved in the organogenesis of lymph nodes and Peyer’s patches. J. Immunol. 179, 804–811 (2007).
8. Spits, H. & Cupedo, T. Inverse lymphoid cells: emerging insights in development, lineage relationships, and function. Annu. Rev. Immunol. 30, 647–675 (2012).
9. Yokota, Y. et al. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. Nature 422, 702–706 (1999).
10. Moro, K. et al. Innate production of T recall cytokines by adipose tissue-associated c-Kit+ Sca-1+ lymphoid cells. Nature 463, 540–544 (2010).
11. Satoh-Takayama, N. et al. IL-7 and IL-15 independently program the differentiation of intestinal CD3-NKp46+ cell subsets from Id2-dependent precursors. J. Exp. Med. 207, 273–280 (2010).
12. Klose, C.S. et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. Cell 157, 340–356 (2014).
13. Constantinides, M.G., McDonald, B.D., Verhoef, P.A. & Bendelac, A. A committed precursor to innate lymphoid cells. Nature 508, 397–401 (2014).
14. Cherrier, M., Sawa, S. & Eberl, G. Notch, Id2, and RORγt sequentially orchestrate the fetal development of lymphoid tissue inducer cells. J. Exp. Med. 205, 729–740 (2012).
15. Sandler, N.G., Mentink-Kane, M.M., Cheever, A.W. & Wynn, T.A. Global gene expression profiles during acute pathogen-induced pulmonary inflammation reveal divergent roles for T1 and T2 responses in tissue repair. J. Immunol. 171, 3655–3667 (2003).
16. Herbert, D.R. et al. Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology. Immunity 20, 623–635 (2004).
17. Hesse, M. et al. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo granulomatous pathology is shaped by the pattern of l-arginine metabolism. J. Immunol. 167, 6533–6544 (2001).
18. Munder, M., Eichmann, K. & Modolell, M. Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by CD4+ T cells correlates with T1/T2 phenotype. J. Immunol. 160, 5347–5354 (1998).
19. Bando, J.K., Nussbaum, J.C., Liang, H.E. & Locksley, R.M. Type 2 innate lymphoid cells constitutively express arginase-1 in the naive and inflamed lung. J. Leukoc. Biol. 94, 877–884 (2013).
20. Reese, T.A. et al. Chitin induces accumulation in tissue of innate immune cells associated with allergy. Nature 447, 92–96 (2007).
21. Kanaori, Y. et al. Identification of novel lymphoid tissues in murine intestinal mucosa where clusters of c-kit+ IL-7Rα+ Thy1+ lympho-hemopoietic progenitors develop. J. Exp. Med. 184, 1449–1459 (1996).
22. Eberl, G. & Littman, D.R. Thymic origin of intestinal αβ T cells revealed by fate mapping of RORγt+ cells. Science 305, 248–251 (2004).
23. Eberl, G. Inducible lymphoid tissues in the adult gut: recapitulation of a fetal developmental pathway? Nat. Rev. Immunol. 5, 413–420 (2005).
24. Adachi, S., Yoshida, H., Kataoka, H. & Nishikawa, S. Three distinctive steps in Peyer’s patch formation of murine embryo. Int. Immunol. 9, 507–514 (1997).
25. Klose, C.S. et al. A T-bet gradient controls the fate and function ofCCR6-RORγt+ innate lymphoid cells. Nature 494, 261–265 (2013).
26. Vonarbourg, C. et al. Regulated expression of nuclear receptor RORγt confers distinct functional fates to NK cell receptor-expressing RORγt+ innate lymphocytes. Immunity 33, 736–751 (2010).
27. El Kasi, M.C. et al. Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. Nat. Immunol. 9, 1399–1406 (2008).
28. Yagi, R. et al. The transcription factor GATA3 is critical for the development of all IL-7Rα-expressing innate lymphoid cells. Immunity 40, 378–388 (2014).
29. van de Pavert, S.A. et al. Maternal retinooids control type 3 innate lymphoid cells and set the offspring immunity. Nature 508, 123–127 (2014).
ONLINE METHODS

Mice. Arg1°F/F (YARG) mice have been described20. Rorc(γt)GFP, IdzG2FP, Idz2γr, Arg1fluorescent transgenic mice were provided by D. Littman (New York University)22. Rosa26flo×STOP-RFP mice were provided by E. Robey (University of California, San Francisco) and D. Kioussis (MRC National Institute for Medical Research) provided VanCr mice20. Rorc(γt)Cre transgenic mice were provided by B. Gudas (University of Pennsylvania) and D. Littman (New York University)22. Rosa26flo×STOP-RFP mice were provided by E. Robey (University of California, San Francisco) and D. Kioussis (MRC National Institute for Medical Research) provided VanCr mice20. Rorc(γt)Cre transgenic mice were provided by B. Gudas (University of Pennsylvania) and D. Littman (New York University)22.

Tissue dissociation. Small intestines from adult mice were flushed with PBS and PPs were removed. Intestines were cut into 5-5 mm pieces and digested with collagenase VIII (Sigma) and DNase I (Roche Diagnostics) at 37 °C for four rounds of 35 min incubations. In experiments with VanCr mice, Cre was also expressed by adult mice to prevent germline RFP expression. Fetal mice were genotyped by tail PCR and flow cytometry of spleens. In experiments with VanCr mice, Cre was also expressed by adult mice to prevent germline RFP expression. Fetal mice were genotyped by tail PCR and flow cytometry of spleens.

Flow cytometry. Rat anti-mouse CD4 (RM4-5), rat anti-mouse CD11b (M1/70), Armenian hamster anti-mouse CD11c (HL3), rat anti-mouse CD19 (ID3), rat anti-mouse CD25 (7D4), rat anti-mouse B220 (RB6-8C5), rat anti-mouse CXCR5 (2G8), rat anti-mouse/human IL-5 (TRFK5) and rat anti-mouse IFN-γ (XMGI.2) antibodies were purchased from BD PharMingen; rat anti-mouse c-kit (2B8), rat anti-mouse CD3 (17A2), rat anti-mouse CD5 (53-7.3), rat anti-mouse CD25 (eBioTD4), anti-rat mouse CD127 (A7R34), rat anti-mouse Nkp46 (29A1.4), rat anti-mouse LAM (DATK32), rat anti-mouse CCR7 (4B12), rat anti-human/mouse GATA-3 (TWA), rat anti-mouse RORγt (mouse anti-NK1.1 (PK136), rat anti-mouse IL-17A (eBio1B7), rat anti-mouse IL-22 (IL22OP) and rat anti-mouse EOMES (Dan1Imag) antibodies were purchased from eBioscience; rat anti-mouse CD45 (30-F11), rat anti-mouse Ter119 (TER-119), mouse anti-human/mouse T-bet (2B10), Armenian hamster anti-human/mouse/ICOS (C398.4A), rat anti-mouse CD25 (PC61) and rat anti-mouse Flk3 (A2F10) antibodies were purchased from BioLegend; and rat anti-mouse ST2 (D18) antibodies were purchased from MD Biosciences. PE-cy7-conjugated streptavidin was purchased from BD Biosciences and APC-conjugated streptavidin was purchased from eBioscience. Live/dead (Invitrogen) or DAPI was used to exclude dead cells. Cells were sorted with an Aria II or MoFlo before intracellular staining owing to loss of GFP after fixation and reduced RFP detection after permeabilization. In experiments where transcription factors were assessed in Arg1°F/F RNT− cells, Arg1°F/F RFP+ cells (consisting of RNT−, NK1.1+ and ST2+ cells) were sorted to 99% purity into a single tube before intracellular staining. Arg1°F/F RORγt(fm)+ cells were sorted in parallel. In fetal liver experiments, Lin− cells were defined as lacking CD3, CD4, CD5, CD19, NK1.1, Ter119, Gr-1 and CD11b. In adult bone marrow experiments, Lin− cells were defined as lacking CD3, CD4, CD5, CD19, NK1.1, Ter119, Gr-1 and B220. Transcription factors were analyzed using the Foxp3/Transcription Factor Staining Buffer Set from eBioscience. In day 6 culture experiments where RFP detection was required after intracellular staining for transcription factors, cells were fixed in 2% PFA for 2 min and washed with PBS before fixation with reagents from the Transcription Factor Staining Buffer set. Cytokine production was assessed using Cytofix/Cytoperm reagents from BD Biosciences. For LTβRβ2 detection, sorted or cultured cells were blocked with donkey anti-mouse Fab fragments (Jackson Immunoresearch) before staining with LTβR-ig. Cells were stained with biotin-conjugated donkey anti-mouse IgG (Jackson Immunoresearch) and blocked with mouse serum before incubating cells with antibodies and APC-conjugated streptavidin. Counting beads were used to determine total cell numbers (CountBright absolute counting beads, Invitrogen). Flow cytometry was performed using an LSR II (BD Biosciences).

Immunohistochemistry. Samples were fixed with 4% PFA (Electron Microscopy Sciences) in PBS for 2 h, and then left in PBS overnight. Tissues were then incubated in 30% sucrose for 2 h before they were frozen in OCT compound (Sakura). Frozen tissue blocks were sectioned at 8 μm (adult gut) or 7 μm (fetal gut) using a Leica CM3050-S cryostat. In experiments with Lta−/− and Rorcγt−/− mice, intestines from both genotypes were frozen in the same block for direct comparison. Serial sections were taken of the proximal half of the small intestine (where the first PP develops), and every section was analyzed for YFP+ aggregates. Mice were genotyped before tissues were frozen, and thus the investigator was not blinded to sample genotypes.

Sections were incubated with 3% H2O2, 0.1% NaN3 in PBS for 45 min to quench endogenous peroxidase, and then blocked with rat anti-mouse CD16/CD32, 1% mouse serum and 1% rat serum for 1 h. Endogenous biotin and avidin-binding sites were blocked with a Biotin/Avidin Blocking Kit (Vector Labs). Slides were incubated with biotin-conjugated goat anti-GFP (Abcam), biotin-conjugated rabbit anti-RFP (Abcam) or biotin-conjugated rat anti-mouse VCAM-1 biotin (eBioscience) for 1–2 h followed by HRP-conjugated streptavidin (PerkinElmer) and FITC- (PerkinElmer) or A555-conjugated tyramide (Invitrogen). Sections that were stained with two biotinylated antibodies were treated with H2O2, NaN3 and avidin/biotin blocking reagents before each antibody incubation. In other experiments, sections were stained with rat anti-mouse RORγt (BD, eBioscience). DAPI was added to sections for 5 min to visualize nuclei.

Cell culture. Cells were sorted on an Aria II (BD Biosciences) with doublets excluded, and cultured with 10 ng/ml recombinant mouse IL-7 (R&D Systems) in RPMI 1640 medium (supplemented with HEPE, FCS, sodium pyruvate, 2-mercaptoethanol, streptomycin-penicillin and 1-glutamine) for 20 h, 6 d or 10 d at 2×105 cells per well. In other experiments, 1.5 × 106 cells or single cells (doublets excluded) were sorted into 96-well plates containing 1.2 × 104 irradiated OP9 cells per well (American Type Culture Collection), 10 ng/ml recombinant IL-7 and 10 ng/ml rSCF (R&D Systems). Media was replenished on the third day and wells were analyzed by flow cytometry on day 6. In experiments where cytokine production by ILCs was assessed, day 10 cultured ILCs were removed from OP9 co-cultures and stimulated for 3 h with 50 ng/ml PMA (Sigma) and 500 ng/ml ionomycin (Sigma). Brefeldin A (BioLegend) was added to the culture during the last 1.5 h. After activation, ILC1s were identified as CD45RORγt(fm)+NK1.1+ cells, ILC2s as CD45RORγt(fm)+NK1.1+ICOS+ cells, ILC3s as CD45RORγt(fm)+NK1.1+ cells and ex-ROTYt cells as CD45RORγt(fm)+NK1.1+ cells.
Statistical analysis. Data were analyzed with Prism 4 (GraphPad Software) using the two-tailed unpaired or paired Student’s t-test or one-way ANOVA followed by Tukey’s test. The unpaired Student’s t-test was used to compare cell numbers from two different mouse genotypes; the paired Student’s t-test analysis was used to compare cell counts from paired samples originating from the same mouse; and one-way ANOVA analysis was used in the comparison of three or more samples. Each independent experiment was conducted with 3–7 mice per group. In experiments determining cell localization within the intestine, repeats were pooled to determine reproducibility. Mice were not given different experimental treatments and thus were not subjected to randomization.

30. de Boer, J. et al. Transgenic mice with hematopoietic and lymphoid specific expression of Cre. Eur. J. Immunol. 33, 314–325 (2003).
31. Luche, H., Weber, O., Nageswara Rao, T., Blum, C. & Fehling, H.J. Faithful activation of an extra-bright red fluorescent protein in “knock-in” Cre-reporter mice ideally suited for lineage tracing studies. Eur. J. Immunol. 37, 43–53 (2007).