Notch, Id2, and RORγt sequentially orchestrate the fetal development of lymphoid tissue inducer cells

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Lymphoid tissue development is initiated during embryogenesis by the migration of lymphoid tissue inducer (LTi) cells from the fetal liver to the periphery, where they induce the formation of lymph nodes and Peyer's patches. In the fetal liver, a subset of common lymphoid progenitors (CLPs) that expresses the integrin α4β7 gives rise to LTi cells, a process strictly dependent on the expression of the transcriptional repressor Id2 and the nuclear hormone receptor retinoic acid–related orphan receptor γ t (RORγt). In this study, we show that Id2 and RORγt are sequentially up-regulated during LTi cell development, matching two waves of differentiation with opposite requirements for Notch signaling. Both the expression of Id2 and Notch are required for the generation of α4β7$^+$ RORγt$^-$ fetal progenitors, but Notch subsequently blocks progression to the RORγt$^+$ stage and final maturation of LTi cells. Notch is therefore a necessary switch to engage the LTi developmental pathway, but needs to be turned off later to avoid diversion to the T cell fate.

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Abbreviations used: CLP, common lymphoid progenitor; DL, Delta-like; ILC, innate lymphoid cell; Lin, lineage; LT, lymphotoxin; LTi, lymphoid tissue inducer; RORγt, retinoic acid–related orphan receptor γ t.

The fetal development of secondary lymphoid tissue is reminiscent of the inflammatory process and is initiated by the interaction of hematopoietic lymphoid tissue inducer (LTi) cells with stromal lymphoid tissue organizers (Mebius, 2003). This process involves the interaction of integrin α4β7 expressed by LTi cells with the addressin MadCAM-1 expressed by high endothelial venules in the lymph node anlagen (Mebius et al., 1996). Once recruited, LTi cells induce the activation of specialized stromal cells through lymphotoxin (LT) α1β2 and its receptor LTβR (Honda et al., 2001). As a consequence, activated stromal cells up-regulate the expression of the adhesion molecules ICAM-1 and VCAM-1 and the structural chemokines CCL21, CCL19, and CXCL13. These factors are crucial for the recruitment to the developing lymphoid tissue of CCR7$^+$ and CXCR5$^+$ LTi cells, and later, of lymphocytes and DCs.

The development of LTi cells requires expression of the nuclear hormone receptor retinoic acid–related orphan receptor γ t (RORγt; Sun et al., 2000; Eberl et al., 2004). In the absence of RORγt, mice lack lymph nodes and Peyer's patches. RORγt is also required for the generation of cells expressing the proinflammatory cytokines IL-17 and IL-22, including CD4$^+$ Tαβ cells (Ivanov et al., 2006; termed Th17 cells), invariant NKT cells (Michel et al., 2008), Tγδ cells (Ivanov et al., 2006), and the recently described innate lymphoid cells (ILCs), which mostly reside in the intestinal lamina propria (Satoh-Takayama et al., 2008; Luci et al., 2009; Cupedo et al., 2009; Cella et al., 2009; Sanos et al., 2009; Sawa et al., 2011). LTi cells are RORγt$^+$ ILCs, and they share the expression of IL-17 or IL-22 with RORγt$^+$ cells (Takatori et al., 2009). Recently, it has been shown that the fetal RORγt$^+$ ILCs, mostly LTi cells, express high levels of IL-17 and IL-22 (Sawa et al., 2010, 2011). However, the role of these proinflammatory cytokines in the development of lymphoid tissues remains to be established. It is possible that the expression of RORγt induces a proinflammatory program in lymphoid cells that might, inevitably, include IL-17 and IL-22.

Fetal LTi cells are derived from common lymphoid progenitors (CLPs) residing in the liver and defined as lineage (Lin)$^-$ c-Kit$^+$ IL-7Rα$^+$ cells

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(Mebius et al., 2001). A subset of Lin$^-$ c-Kit$^+$ IL-7Rα^+ cells expressing the integrin $\alpha4\beta7$ generates T cells, NK cells, DCs, and LTi cells under appropriate culture conditions, but not B cells (Yoshida et al., 2001). More recently, using *Rorc(γt)-Gfp*^{TG} reporter mice (Lochner et al., 2008), it was shown that LTi cells derive from fetal liver Lin$^-$ $\alpha4\beta7^+$ cells expressing intermediate or high levels of RORγt, whereas another subset of RORγt$^+$ ILCs expressing the pan-NK marker NKp46 derives from Lin$^-$ RORγt$^+$ liver cells that do not express $\alpha4\beta7$ (Sawa et al., 2010).

In addition to RORγt, the development of LTi cells requires the expression of the transcriptional repressor Id2 (Yokota et al., 1999; Eberl et al., 2004), which regulates lineage specification through inhibition of E-box proteins. Indeed, deficiency in the E-box protein E2A can restore both LTi and NK cell development in Id2-deficient mice (Boos et al., 2007). Another lineage decision maker, Notch, is involved in the development of LTi cells (Lee et al., 2011; Possot et al., 2011). During T cell development, Notch is required in early thymocyte progenitors to block B cell potential (Radtke et al., 1999). At the next stage, when B cell potential is lost, Notch engagement is required to repress NK cell and myeloid cell potentials and maintain the T cell fate (Schmitt et al., 2004; Feyerabend et al., 2009). LTi cells arise from fetal liver progenitors that have lost B cell potential but retained T cell potential (Yoshida et al., 2001). Thus, it is possible that Notch plays a role in the early steps of specification of the LTi cell lineage.

Using *Rorc(γt)-Gfp*^{TG} reporter mice, we have determined the progression of fetal CLPs to Id2$^+$ cells, RORγt$^+$ cells, and mature LTi cells, as well as the role of Notch in this differentiation pathway. We find that Notch signaling is required to engage the LTi developmental pathway and generate $\alpha4\beta7^+$ RORγt$^-$ progenitors expressing Id2. However, Notch signaling eventually has to be blocked to avoid the development of T cells and to allow the expression of RORγt and the terminal differentiation of LTi cells.

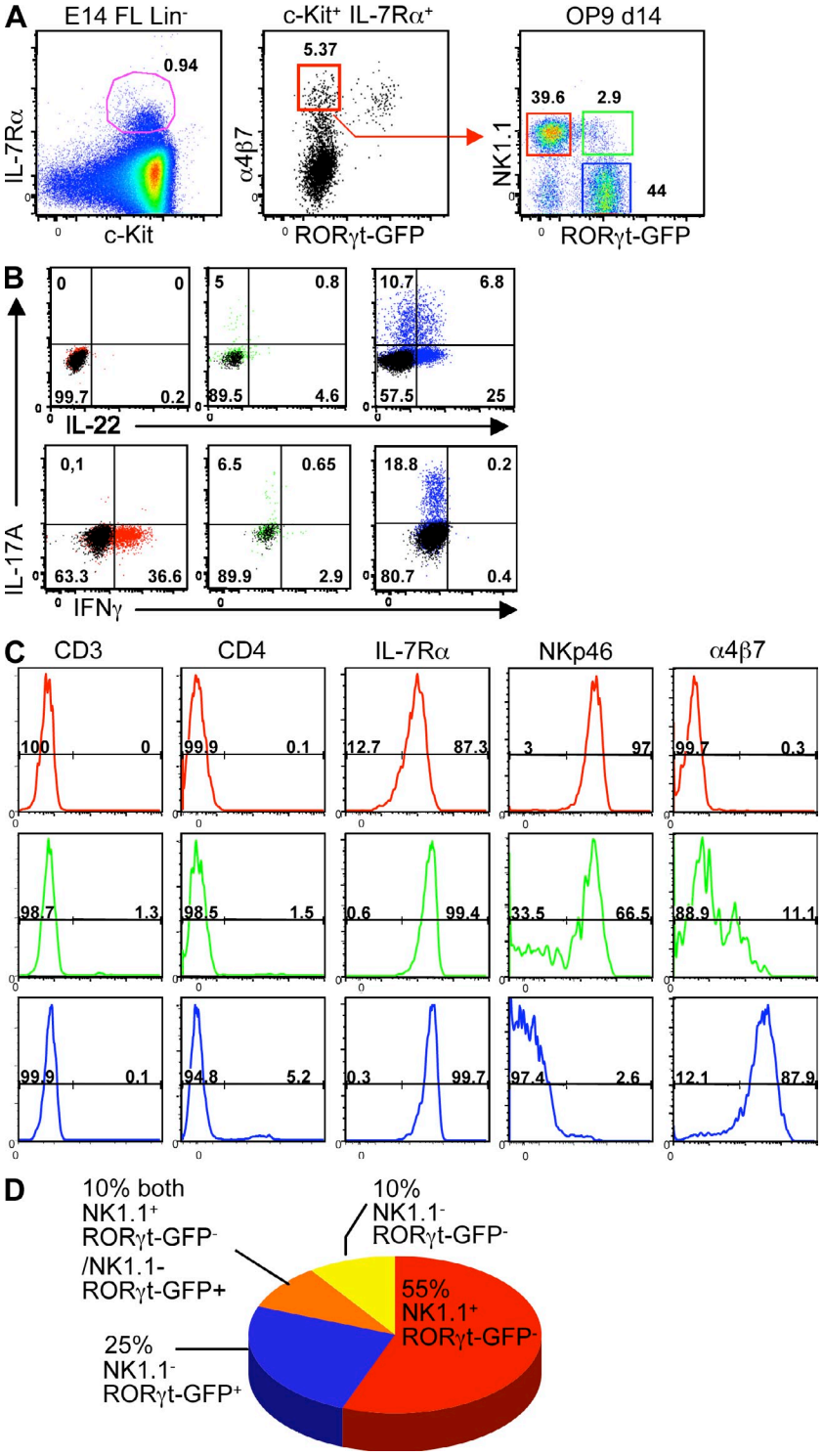


Figure 1. Generation of RORγt$^+$ cells from fetal liver $\alpha4\beta7^+$ RORγt$^-$ progenitors. (A) Flow cytometry analysis of Lin$^-$ cells from the fetal liver of E14 *Rorc(γt)-Gfp*^{TG} mice. Lin$^-$ c-Kit$^+$ IL-7Rα^+ $\alpha4\beta7^+$ RORγt$^-$ cells were sorted and cultured for 14 d on OP9 stromal cells in the presence of IL-7 and SCF. Data are representative of at least three independent experiments performed with four to six fetuses each. (B) Production of IL-17, IL-22, and IFN-γ by NK1.1$^+$ RORγt$^-$ cells (red), NK1.1$^+$ RORγt$^+$ cells (green), and NK1.1$^-$ RORγt$^+$ cells (blue), as indicated in A, after 3 h of stimulation with PMA and ionomycin. Data are representative of three independent experiments. (C) Cultures described in A were analyzed for the expression of surface markers. Data are representative of at least three independent experiments. (D) Pie chart summarizing the phenotype of 52 clones cultured individually from single fetal liver $\alpha4\beta7^+$ RORγt$^-$ cells on OP9 stromal cells for 14 d.

RESULTS

Generation of RORγt⁺ cells from fetal liver α4β7⁺ RORγt⁻ progenitors

Using *Rorc(γt)-Gfp*^{TG} reporter mice (Lochner et al., 2008), flow cytometry analysis of E14 fetal liver showed that Lin⁻ c-Kit⁺ IL-7Rα⁺ cells (or CLPs) include three distinct cell subsets as defined by the expression of integrin α4β7 and RORγt (Fig. 1 A). Whereas expression of integrin α4β7 defines a subset of progenitors for T cells, NK cells, and LTi cells (Yoshida et al., 2001), expression of both integrin α4β7 and RORγt restricts progenitors to an LTi cell fate, both in vitro and in vivo (Sawa et al., 2010). To define the potential of α4β7⁺ RORγt⁻ cells to generate RORγt⁺ cells, Lin⁻ c-Kit⁺ IL-7Rα⁺ α4β7⁺ RORγt⁻ cells were sorted from the fetal liver and cultured for 14 d in the presence of OP9 stromal cells. In these conditions, α4β7⁺ RORγt⁻ cells generated mostly NK1.1⁺ RORγt⁻ cells and NK1.1⁻ RORγt⁺ cells (Fig. 1 A). Like conventional NK cells, NK1.1⁺ RORγt⁻ cells expressed NKp46 and produced IFN-γ, but not IL-17 or IL-22 (Fig. 1, B and C). In contrast, and similar to LTi cells, NK1.1⁻ RORγt⁺ cells expressed integrin α4β7 and IL-7Rα, IL-17, and IL-22, but not NKp46 or IFN-γ (Sawa et al., 2011). We have previously described that the fetal liver harbors a subset of Lin⁻ RORγt⁺ precursors to IL-22–producing NKp46⁺ RORγt⁺ cells that expresses low levels of c-Kit and IL-7Rα, but not α4β7 (Sawa et al., 2010). In agreement with these findings, only few NKp46⁺ RORγt⁺ cells were generated from fetal liver α4β7⁺ RORγt⁻ cells. However, these cells produced low amounts of IL-17, but no IL-22 or IFN-γ, and thus appeared to be distinct from IL-22–producing NKp46⁺ RORγt⁺ cells.

To assess the differentiation potential of α4β7⁺ RORγt⁻ cells at the single-cell level, individual cells were grown on OP9 stromal cells. After 14 d, 55 and 25% of the clones were either RORγt⁻ NK1.1⁺ or RORγt⁺ NK1.1⁻, respectively, whereas only 10% of the clones contained both RORγt⁺ NK1.1⁻ and RORγt⁻ NK1.1⁺ cells (Fig. 1 D). These data indicate that fetal liver α4β7⁺ RORγt⁻ cells include distinct precursors to NK cells and LTi cells that remain to be characterized.

In vitro versus in vivo generated LTi cells

To validate our in vitro culture system to study the development of LTi cells from CLPs, we compared RORγt⁺ NK1.1⁻ generated in vitro from α4β7⁺ RORγt⁻ precursors, to LTi cells isolated from fetal liver and gut. To ease the description of the data, we will term CLPs stage I, α4β7⁺ RORγt⁻ cells stage II, α4β7⁺ RORγt⁺ cells stage III (Fig. 2 A),

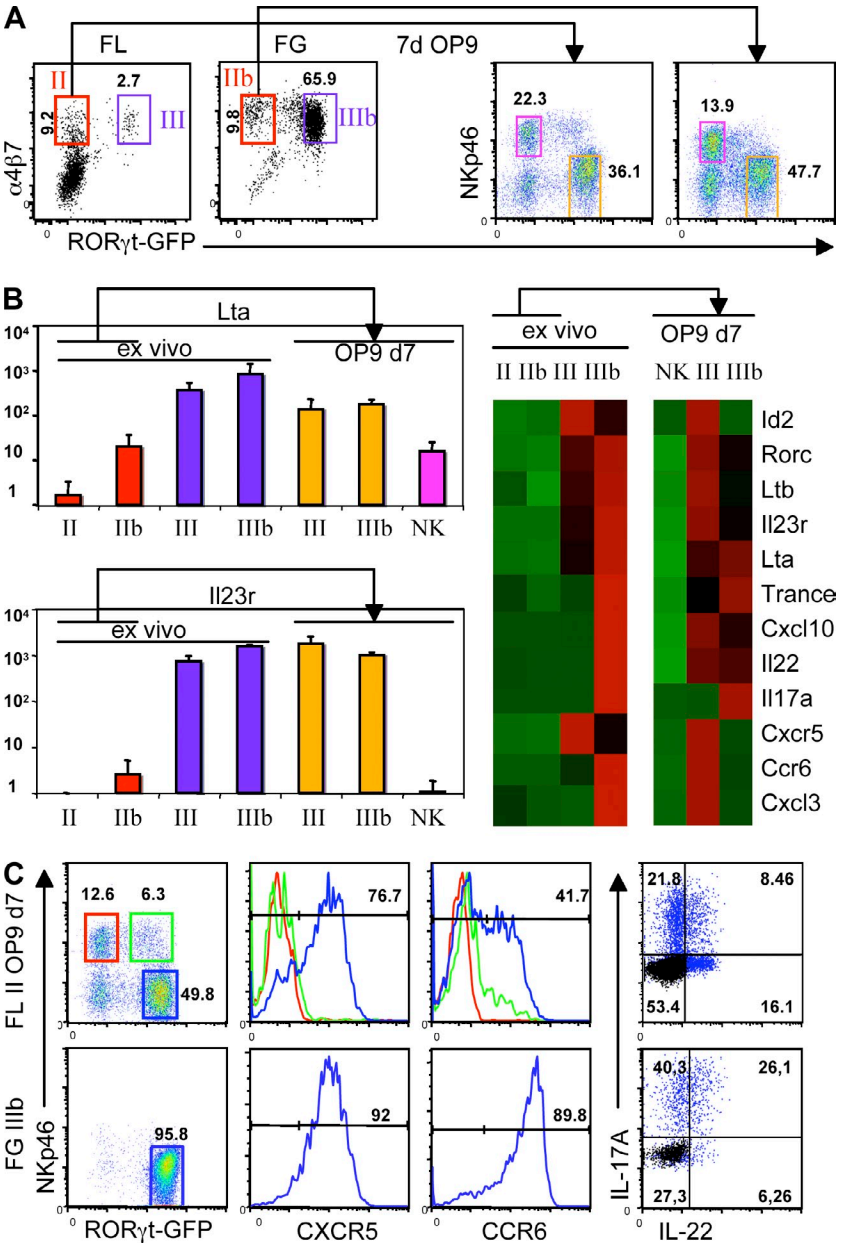


Figure 2. In vitro– versus in vivo–generated LTi cells. Fetal liver (FL) and fetal gut (FG) cells were isolated from E14 *Rorc(γt)-Gfp*^{TG} mice. (A) Flow cytometry analysis of Lin⁻ c-Kit⁺ IL-7Rα⁺ cells from the fetal liver and gut of E14 *Rorc(γt)-Gfp*^{TG} mice. CLPs, α4β7⁺ RORγt⁻ cells, and α4β7⁺ RORγt⁺ cells are labeled stage I, II, and III, respectively; the appearance of "b" with these designations indicates that cells were isolated from the gut. Stage II/IIb (red) and stage III/IIIb (blue) cells were sorted and cultured and/or analyzed for gene expression. After 7 d of culture on OP9 cells, stage III/IIIb cells (gold) and NKp46⁺ RORγt⁻ (NK) cells (pink) were sorted and analyzed for gene expression. (B, left) Relative expression of *Lta* and *Il23r* in the indicated cell populations. Data are from three independent experiments. Error bars are SD. (right) Heat map of qPCR analysis from in vivo–isolated stage II to IIIb cells, and in vitro–generated NK cells, stage III, and IIIb cells. Data are the mean of at least three independent samples. (C) Flow cytometry analysis of cultured fetal liver stage II cells (top row) and ex vivo–isolated fetal gut stage IIIb cells (bottom row). Data are representative of at least three independent experiments.

as first suggested by Yoshida et al. (2001). Stage II α4β7$^+$ RORγt$^-$ cells were isolated from fetal liver (II) and fetal gut (IIb) and cultured for 7 d in the presence of OP9 stromal cells. The expression of *Lta* and *Ltb*, coding for the LTα and LTβ required for the development of lymphoid tissues (De Togni et al., 1994; Rennert et al., 1996), was strongly up-regulated in RORγt$^+$ cells (III and IIIb) generated from stage II cells, and reached levels comparable to RORγt$^+$ cells isolated from fetal liver (III) and gut (IIIb; Fig. 2 B). Similar results were obtained for other genes expressed by LTi cells, such as *Il23r*, which codes for the IL-23 receptor and induces IL-22 production (Sawa et al., 2011); *Trance*, which is required for the terminal differentiation of lymph node anlagen (Kim et al., 2000); and *Cxcr5*, which is required for the recruitment of LTi cells to lymph node and Peyer's patch anlagen (Förster et al., 1996; Ansel et al., 2000). *Id2* (Yokota et al., 1999) and, to some extent, *Ccr6* (Sawa et al., 2010) were also expressed. At the protein level, LTi cells that were generated in vitro or in vivo expressed comparable amounts of CXCR5, IL-17, and IL-22 (Fig. 2 C). Together, these data show that RORγt$^+$ cells generated in vitro from α4β7$^+$ RORγt$^-$ cells (II) express an array of factors that characterizes LTi cells at levels comparable to LTi cells isolated from fetal tissues.

Sequential expression of Id2 and RORγt confers LTi function

To assess the functional maturation of precursor cells along the differentiation pathway from CLPs to LTi cells, CLPs (I), α4β7$^+$ RORγt$^-$ cells (II) and α4β7$^+$ RORγt$^+$ cells (III) were isolated from E14 fetal liver and compared with homologous cell subsets in the E14 fetal intestine (IIb and IIIb; Fig. 3 A). Notably, Lin$^-$ c-Kit$^+$ IL-7Rα$^+$ cells from the intestine harbored few cells with a stage II phenotype and a majority of α4β7$^+$ RORγt$^+$ cells with a stage III phenotype. The gene expression pattern of these cells was determined for several transcription factors, cytokines, and chemokines relevant to LTi cell differentiation and function (Fig. 3 B). Whereas CLPs (stage I) expressed *Pax5*, *Ebf-1*, and *E2a*, synonymous with B cell potential (Bain et al., 1994; Lin and Grosschedl, 1995; Nutt et al., 1999), this pattern was lost in subsequent stages, concomitant with the up-regulation of *Runx1*, *Id2*, and *Tox*, which are all required for LTi cell development (Yokota et al., 1999; Aliahmad et al., 2010; Tachibana et al., 2011). Expression of genes encoding proinflammatory cytokines and chemokines, such as *Il17*, *Il22*, *Cxcl1*, *Cxcl2*, *Cxcl3*, *Cxcl10*, *Lta*, *Tnfa*, and *Trance*, was highest in gut RORγt$^+$ cells (IIIb), as was expression of *Il23r* and *Cxcr5*. Interestingly, the receptor to IL-25, *Il17rb*, is an inhibitor of the

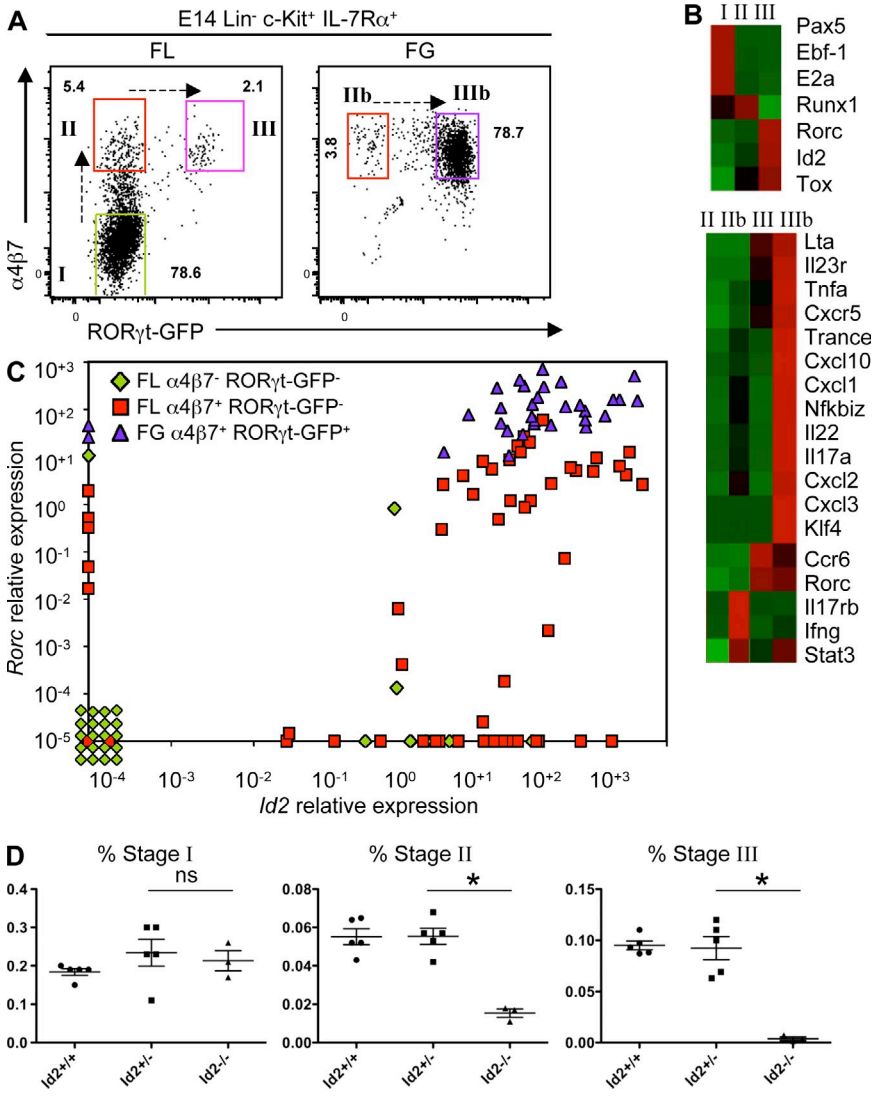


Figure 3. Sequential expression of Id2 and RORγt confers LTi function. (A) Flow cytometry analysis of Lin$^-$ c-Kit$^+$ IL-7Rα$^+$ cells from the fetal liver (FL) and fetal gut (FG) of E14 *Rorc(γt)-Gfp*^{TG} mice. The dot plot on the left was duplicated from Fig. 1 A to illustrate the gating strategy. Dashed arrows suggest the lineage relationship between cell subsets I–III. The data are representative of at least three independent experiments performed with four to six fetuses each. (B) Heat map of qPCR analysis of freshly isolated cells. Data are from at least three independent samples for each subpopulation. (C) Single-cell qPCR analysis of *Id2* (x axis) and *Rorc* (y axis) expression in 32 fetal liver stage I cells (green), 64 fetal liver stage II cells (red), and 32 fetal gut stage IIIb cells (blue). (D) The frequency of fetal liver stage I, II, and III cells in Id2-sufficient or -deficient mice was measured by flow cytometry. The data were pooled from three independent experiments with five mice per group, except for Id2$^{-/-}$ mice with three mice per group. Statistical significance was assessed by the unpaired Student's *t* test. *, P < 0.001. Error bars are SD.

IL-17 pathway (Kleinschek et al., 2007) and is expressed in gut α4β7$^+$ RORγt$^-$ cells (IIb) but not at the following stage. These data show that functional maturation of LTi cells is achieved after their migration from the liver to peripheral tissues.

Whereas *Id2* was clearly expressed in α4β7$^+$ RORγt$^+$ cells (III and IIIb), we assessed its expression at the single-cell level to determine whether it preceded or was concomitant with *Rorc* expression in the LTi cell lineage. Whereas few α4β7$^-$ RORγt$^-$ cells (I) expressed *Id2* or *Rorc*, most α4β7$^+$ RORγt$^-$ cells (II) expressed *Id2* and half expressed varying levels of *Rorc* (Fig. 3 C), indicating that α4β7$^+$ RORγt$^-$ cells (II) are a heterogeneous population of cells not yet fully committed to the LTi cell lineage. In contrast, most gut α4β7$^+$ RORγt$^+$ cells (IIIb) expressed both *Id2* and high levels of *Rorc*, altogether indicating that *Id2* expression precedes full expression of *Rorc* in the LTi cell lineage. Notably, the level of *Rorc* transcripts detected in α4β7$^+$ RORγt$^-$ (GFP$^-$) cells (II) was, on average, 10–100-fold lower than in RORγt$^+$ (GFP$^+$) cells (III), which probably explains the lack of GFP detection in stage II cells.

To assess whether Id2 was required to progress from α4β7$^+$ RORγt$^-$ cells (II) to α4β7$^+$ RORγt$^+$ cells (III), Id2-deficient *Rorc(γt)-Gfp*^{TG} reporter mice were generated. We measured the frequency of CLPs (I), α4β7$^+$ RORγt$^-$ cells (II), and RORγt$^+$ cells (III) among Lin$^-$ cells in the liver and gut of newborn mice. The frequency of both α4β7$^+$ RORγt$^-$ cells (II) and RORγt$^+$ cells (III) was markedly reduced in the liver (Fig. 3 D) and gut (unpublished data) of Id2-deficient compared with Id2-sufficient littermates, indicating that Id2 expression plays a critical role in the generation of α4β7$^+$ LTi cell precursors. In contrast, the α4β7$^-$ RORγt$^+$ precursors to NKp46$^+$ RORγt$^+$ cells (Sawa et al., 2010; Fig. 4 A) were not affected by the absence of Id2 (Fig. 4 B), even though NKp46$^+$ RORγt$^+$ cells fail to develop in Id2-deficient mice (Satoh-Takayama et al., 2010). These data also show that α4β7$^-$ RORγt$^+$ precursors to NKp46$^+$ RORγt$^+$ cells cannot derive from α4β7$^+$ RORγt$^-$ (II) precursors to LTi cells, as the former, but not the latter, are present in Id2-deficient mice.

Notch signaling plays opposite roles at successive stages of LTi cell differentiation

Notch proteins and their ligands play fundamental roles in lineage specification. It has recently been reported that the absence of Notch signaling in the hematopoietic lineage strongly decreases the generation of NKp46$^+$ RORγt$^+$ cells, and to a lesser extent, of CD4$^+$ LTi cells (Lee et al., 2011). However, Possot et al. (2011) reported that the development of fetal Lin$^-$ c-Kit$^+$ IL-7Rα$^+$ cells expressing CXCR6, half of which express RORγt, are not affected by the absence of Notch signaling. We therefore assessed more details of the role of Notch signaling in the differentiation pathway of LTi cells.

First, fetal liver CLPs (I) were grown on OP9 or OP9 cells expressing the Notch ligand Delta-like-1 (OP9-DL1; Fig. 5 A). As expected, the absence of Notch signaling in cultures with OP9 cells resulted in the generation of mostly B cells from CLPs (I). Few α4β7$^+$ RORγt$^-$ cells (II) could be detected, whereas a small but sizeable population of α4β7$^+$ RORγt$^+$ cells (III) was generated. In contrast, and as expected, the generation of B cells was blocked in the presence of Notch signaling in cultures with OP9-DL1. Furthermore, a large population of α4β7$^+$ RORγt$^-$ cells (II), but few α4β7$^+$ RORγt$^+$ cells (III), was generated in these conditions, indicating that Notch signaling is required to generate α4β7$^+$ RORγt$^-$ cells (II) from CLPs (I). Next, α4β7$^+$ RORγt$^-$ cells (II) were purified from these cultures and grown further on OP9 or OP9-DL1 cells (Fig. 5 B). In the absence of Notch signaling, α4β7$^+$ RORγt$^-$ cells (II) generated a majority of α4β7$^+$ RORγt$^+$ cells (III) cells and few T cells, as expected. In the presence of Notch signaling, half of the cells derived from α4β7$^+$ RORγt$^-$ cells (II) were T cells. Furthermore, none of the RORγt$^+$ cells generated in these conditions expressed α4β7$^+$, indicating that these cells were T cell precursors rather than LTi cells.

Similar results were obtained when both CLPs (I) and α4β7$^+$ RORγt$^-$ cells (II) were isolated from fetal liver and

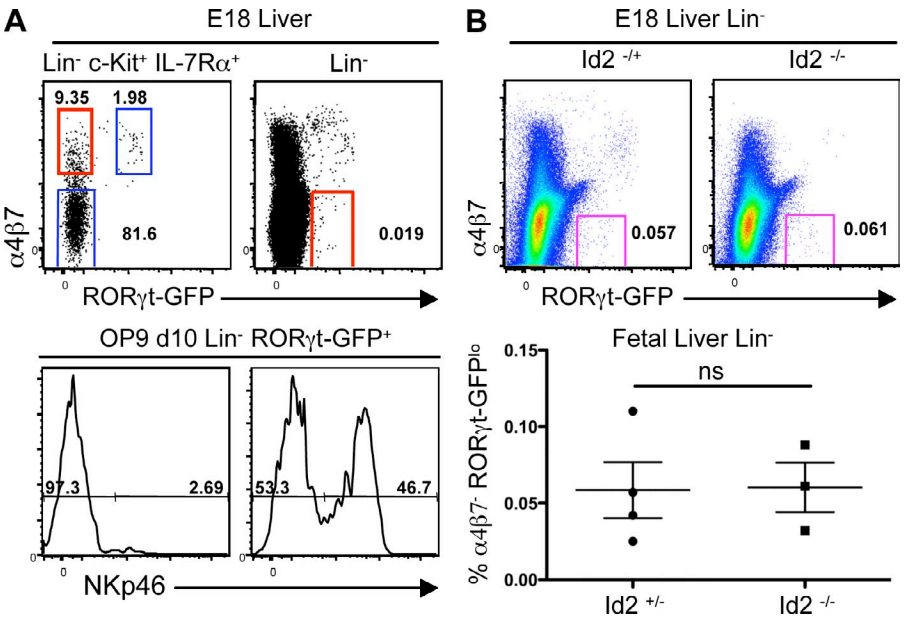


Figure 4. LTi cells and NK22 develop from distinct precursors. (A) RORγt-E18 fetal liver stage II cells (red gate; left) or Lin$^-$ α4β7$^-$ RORγt^{low} cells (red gate; right) were cultured on OP9 cells for 10 d, and the resulting Lin$^-$ RORγt$^+$ cells were assessed for NKp46 expression by flow cytometry (bottom). Data are representative of at least three independent experiments. (B) Lin$^-$ cells were isolated from the fetal liver of Id2-sufficient or -deficient mice, and the frequency of α4β7$^-$ RORγt^{low} cells was measured by flow cytometry. Data are from three independent experiments with three mice per group. Statistical significance was assessed by the unpaired Student's *t* test. Error bars are SD.

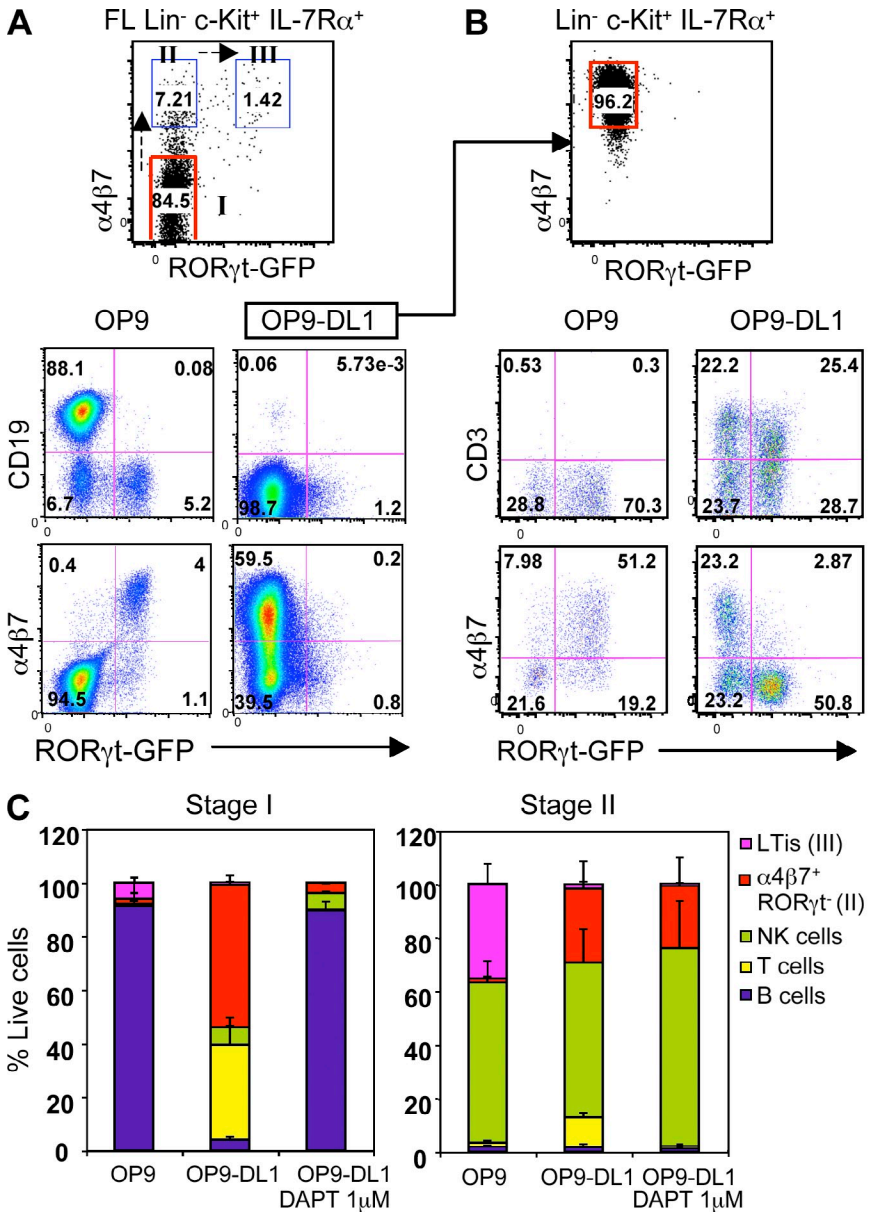


Figure 5. Notch signaling plays opposite roles at successive stages of LTi cell differentiation. (A) Stage I (Lin⁻ c-Kit⁺ IL-7Rα⁺ α4β7⁻) cells were sorted from fetal liver (FL) and cultured on OP9 or OP9-DL1. (B) After 5 d, stage II (Lin⁻ c-Kit⁺ IL-7Rα⁺ α4β7⁺) cells were sorted from OP9-DL1 cultures and transferred to OP9 or OP9-DL1 cultures for 7 d. Expression of CD19, CD3, α4β7, and RORγt was measured by flow cytometry. Data are representative of three independent experiments. (C) E14 fetal liver stage I and II cells were sorted and cultured on OP9 or OP9-DL1 treated with 1 μM γ-secretase inhibitor DAPT or vehicle. Bar graphs show the proportions of LTis (III), Lin⁻ α4β7⁺ RORγt⁻ (II), and NK, T, and B cells generated from stage I and stage II cells after 10 d of culture. Data are from three independent experiments. Error bars are SD.

cultured (Fig. 5 C). The differentiation of CLPs (I) to α4β7⁺ RORγt⁻ cells (II) could be blocked, as expected, by inhibiting the Notch signaling with the γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT; Sinha et al., 1999). In contrast, and surprisingly, the differentiation of α4β7⁺ RORγt⁻ cells (II) to α4β7⁺ RORγt⁺ cells (III) could not be rescued by blocking Notch signaling with DAPT, indicating that the generation of RORγt⁺ cells (III) is extremely sensitive to Notch signaling. Altogether, these data show that Notch signaling promotes the generation of α4β7⁺ RORγt⁻ cells (II) from CLPs (I) and engage CLPs into the LTi differentiation pathway rather than the B cell differentiation pathway. Subsequently, Notch signaling has to be terminated to generate LTi cells from α4β7⁺ RORγt⁻ cells (II). A small population of LTi cells could nevertheless be generated from CLPs in the absence of Notch signaling (Fig. 5 A), as if diverted from B cell differentiation through progression to α4β7⁺ RORγt⁺ cells (III). Finally, whether Notch signaling controls expression of *Id2* and *Rorc*, or whether these factors are required for LTi development independently of Notch, remains to be determined.

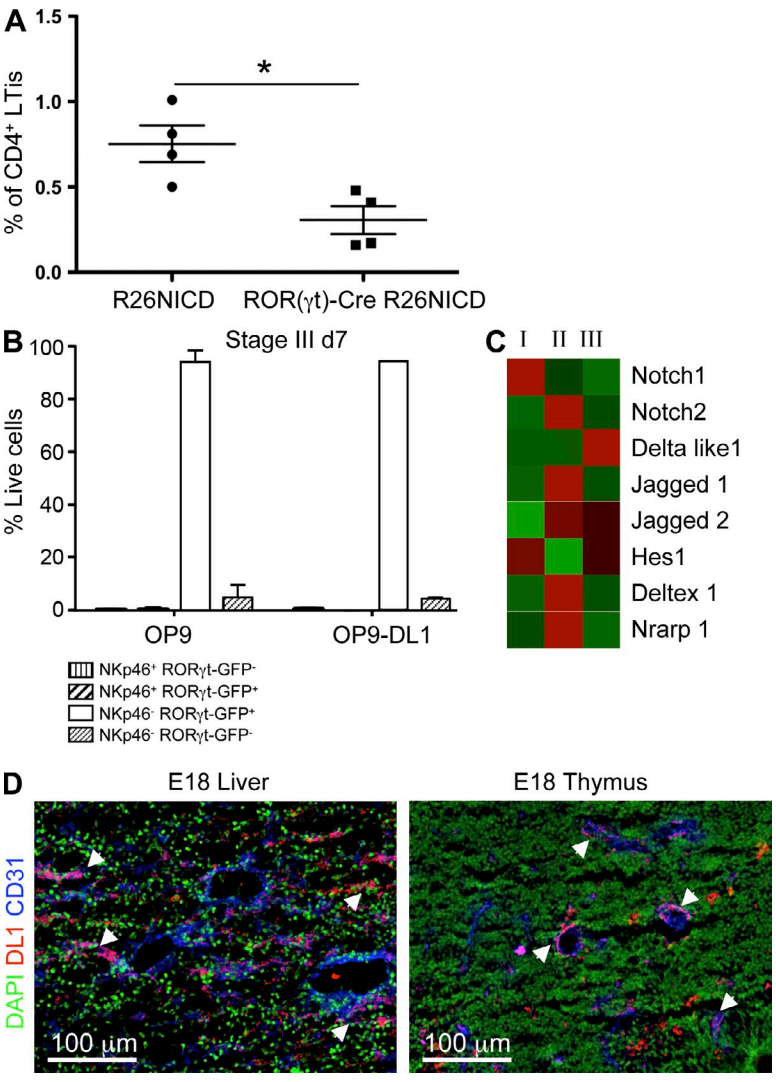
It has been shown recently that the deletion in the hematopoietic lineage of RBP-J_K, the main transcriptional mediator of Notch signaling (Jarriault et al., 1995), significantly reduces the generation of CD4⁺ LTi cells in vivo (Lee et al., 2011). This is in accordance with our results obtained in vitro showing that Notch signaling is required to generate α4β7⁺ RORγt⁻ cells (II) from CLPs (I). To confirm that Notch signaling blocks the subsequent differentiation of α4β7⁺ RORγt⁻ cells (II) to α4β7⁺ RORγt⁺ cells (III), we induced expression of the intracellular Notch domain (the active form of Notch receptors) in RORγt⁺ cells of Rorc(γt)-Cre^{TG} x $Rosa26\text{-}Stop^{Floxed}$-NICD mice. In accordance with the data obtained in vitro (Fig. 5), the proportion of CD4⁺ LTi cells was significantly reduced as compared with control littermates (Fig. 6 A). Of note, even though stage II cells do not express GFP reporting RORγt, they express low levels of transcripts for RORγt (Fig. 3 C) that may activate the expression of NICD. Nevertheless, to test for the possibility that Notch signaling also affects stage III cells, RORγt⁺ cells were cultured on OP9 or OP9-DL1 cells. After 7 d of culture, no difference was found in the number of stage III cells (Fig. 6 B).

In terms of the expression of Notch and Notch targets, *Notch1* was expressed in CLPs (I), *Notch2* was expressed in α4β7⁺ RORγt⁻ cells (II), and both were down-regulated, but not absent, in α4β7⁺ RORγt⁺ cells (III; Fig. 6 C). In addition,

expression of the Notch target *Hes1* was highest in CLPs (I), whereas the Notch-induced *Deltex1* and *Nrarp*, which also negatively regulate the Notch signaling pathway (Izon et al., 2002; Yun and Bevan, 2003), were expressed in $\alpha4\beta7^+$ RORγt^- cells (II), but not in LTi cells. These data support a stage-specific role of Notch signaling in LTi cell development. Finally, microniches of stromal cells expressing DL1 could be visualized in the fetal liver (Fig. 6 D), in accordance with earlier results obtained on the expression of Notch ligands by stromal cell subsets (Harman et al., 2005). Thus, in addition to the regulated expression of Notch in precursors of LTi cells, compartmentalized expression of Notch ligands in the fetal liver may allow for transient Notch signaling during LTi cell differentiation in vivo.

Thymic $\alpha4\beta7^+$ cells generate LTi-like cells in the absence of Notch signaling

The fetal thymus harbors a large population of $\alpha4\beta7^+$ RORγt^- cells (II) among Lin$^-$ c-Kit$^+$ IL-7Rα^+ cells, as well as a small population of $\alpha4\beta7^+$ RORγt^+ cells (III; Fig. 7). RORγt^+ LTi-like cells induce the maturation of medullary thymic epithelial cells (mTECs) that express *Aire* (Autoimmune regulator) and are involved in the negative selection of self-reactive T cells (Rossi et al., 2007). We therefore tested whether fetal thymic $\alpha4\beta7^+$ RORγt^- cells (II) could generate $\alpha4\beta7^+$ RORγt^+ cells (III) in our culture system. In the absence of Notch signaling, up to 60% of cells were $\alpha4\beta7^+$ RORγt^+ cells (III) after 14 d of culture. When $\alpha4\beta7^+$ RORγt^- cells (II) were grown in the presence of Notch signaling however, a large proportion of T cells but few $\alpha4\beta7^+$ RORγt^+ cells were generated, as expected. Thus, the fetal thymus harbors Lin$^-$ $\alpha4\beta7^+$RORγt^- cells (II) that have the potential to generate LTi-like cells, but this potential is repressed in the fetal thymic environment, which expresses high levels of DL1 and DL4 (Koch et al., 2008; Hozumi et al., 2008). The low numbers of LTi-like cells that are nevertheless generated in the thymic environment might require microniches that do not express Notch ligands for their development from $\alpha4\beta7^+$ RORγt^- cells (II). We could visualize such thymic micro-niches, devoid of DL1 (Fig. 6 C), in accordance with earlier results obtained on DL1 and DL4 expression (Fiorini et al., 2008; Koch et al., 2008). Whether thymus-derived LTi cells or $\alpha4\beta7^+$ precursors can emigrate from the thymus and contribute to the pool of LTi cells in the periphery is an interesting possibility that remains to be assessed.



DISCUSSION

LTi cells are members of the larger family of ILCs that were recently shown to play major roles in intestinal immunity and homeostasis. A first subset, termed natural killer cells, was first described 35 yr ago (Kiessling et al., 1976) and replicates the cytokine expression profile of Th1 cells. A second subset, termed nuocytes, or natural helper cells, replicates

Figure 6. Sustained Notch signaling blocks the development of LTi cells in vivo. (A) Hematopoietic cells were isolated from the gut of newborn mice expressing Notch intracellular domain (NICD) under the control of the RORγt locus (*Rorc(γt)-Cre* x R26NICD) or control mice (R26NICD). The frequency of CD4$^+$ LTis among CD3$^-$ NKp46$^-$ cells was measured by flow cytometry. Data are pooled from two independent experiments with four mice per group. Statistical significance was assessed by the unpaired Student's *t* test. *, $P < 0.001$. Error bars are SD. (B) Stage III cells were sorted from the fetal liver and cultured on OP9 or OP9-DL1 for 7 d. The proportion of each cell subsets, defined by the expression of NKp46 and RORγt-GFP, was analyzed by flow cytometry. Data are from four independent experiments. Error bars are SD. (C) Heat map of qPCR analysis for Notch, Notch ligands, and Notch target genes in fetal liver stage I, II, and III cells. qPCR analysis was performed using at least three independent samples for each cell subset. (D) Immunostaining for CD31 (blue) and DL1 (red) of fetal liver and thymus (E18). Pictures were taken at 200x magnification. Examples of micro-niches expressing DL1 are indicated by white arrowheads.

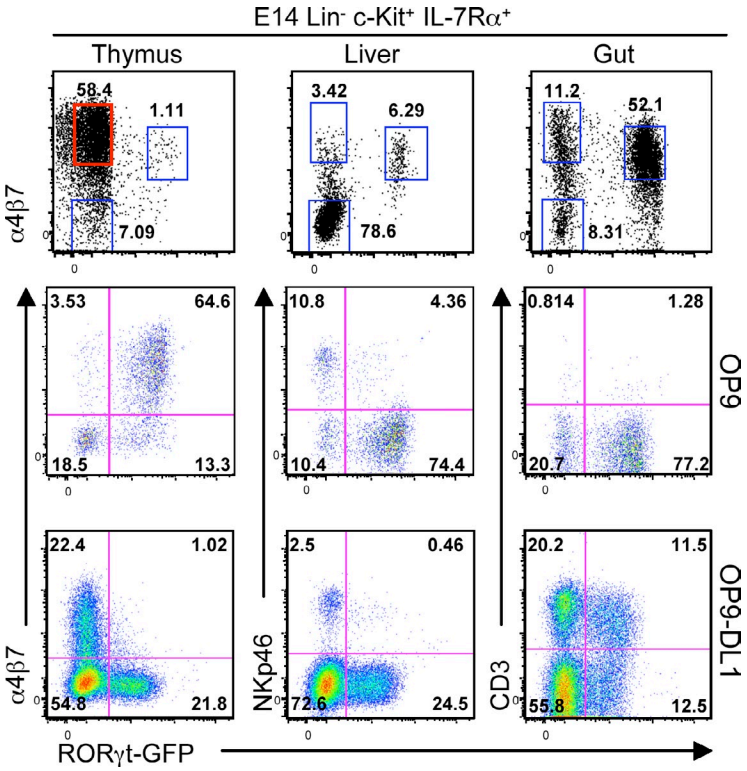


Figure 7. Thymic Lin⁻ α4β7⁺ cells generate LTi-like cells in the absence of Notch signaling. Lin⁻ c-Kit⁺ IL-7Rα²⁺ cells were isolated from the thymus, liver, and gut of E14 *Rorc(γt)-Gfp*^{TG} mice (top row), and expression of α4β7 and RORγt-GFP was analyzed by flow cytometry. Stage II cells (red) were then sorted from the thymus and cultured on OP9 (middle row) or OP9-DL1 (bottom row) stromal cells for 14 d, and further analyzed by flow cytometry. Data are representative of at least three independent experiments.

the cytokine expression profile of Th2 cells and expands shortly after helminth infection (Moro et al., 2010; Neill et al., 2010; Saenz et al., 2010). A third subset, which includes LTi cells and NK22 cells, expresses $LT\alpha_1\beta_2$ and IL-22, which are both involved in cross talk with epithelial cells to deliver antiapoptotic signals and induce the expression of antimicrobial peptides (Spits and Di Danto, 2011). This latter subset is characterized by the expression of RORγt and, as with Th17 cells (Ivanov et al., 2006), requires RORγt for its development (Eberl et al., 2004). In addition to RORγt, the development of LTi cells from CLPs requires the transcription factors Runx1, Id2, and Tox (Yokota et al., 1999; Aliahmad et al., 2010; Tachibana et al., 2011), as well as development of a committed precursor expressing the integrin α4β7 (Sawa et al., 2010). Here, we show that Notch signaling and Id2 are sequentially engaged to generate α4β7⁺ precursors, and that Notch signaling must then be terminated to up-regulate RORγt and generate LTi cells.

We find that Notch signaling is required to generate α4β7⁺ precursors to LTi cells. It is unclear whether Notch signaling is involved in the expression of integrin α4β7, or whether it plays a role in the generation of precursor cells that up-regulate α4β7. It has been reported that Notch1 activates β1 integrins through the GTPase R-ras (Hodkinson et al., 2007), but regulation of integrin expression is not documented. Another possibility for the emergence of α4β7⁺ precursors in the presence of Notch signaling is blockade of the B cell developmental pathway. When cultured in the absence of Notch, CLPs generate mostly B cells. This is similar to the development of B cells in thymi repopulated with Notch1-deficient bone marrow precursors (Radtke et al., 1999). However, it remains to be assessed whether in the absence of Notch signaling, α4β7⁺ precursors fail to develop because of an intrinsic requirement for activated Notch or because of niche competition with B cells. Furthermore, we also find that Id2 is required for the generation of α4β7⁺ precursors. As Id proteins block the activity of E-box proteins required to engage the B cell lineage (Sun, 1994), these data suggest that engagement of the B cell lineage is preferentially engaged over the LTi cell lineage in the absence of Id2.

In the subsequent step of LTi cell differentiation, Notch signaling impairs the generation of LTi cells from α4β7⁺ precursors. Similar proportions of RORγt⁺ cells are generated in the presence or absence of Notch signaling (Fig. 5 B). However, RORγt⁺ cells generated in the presence of Notch signaling do not express α4β7, as LTi cells do, and up-regulate CD3ε. Similarly, immature CD4⁻CD8⁻ double negative (DN) thymocytes express high levels of integrin α4β7 at the CD44⁺ DN1 and DN2 stages (Peaudecerf et al., 2011). However, expression of integrin α4β7 is down-regulated in the subsequent stages of T cell development, as they acquire expression of RORγt at the DN4 CD25⁻CD44⁻ stage. RORγt expression is maintained in CD4⁺CD8⁺ double positive (DP) cells, where it is required to induce the anti-apoptotic molecule Bcl-x_L and increase the lifespan of DP thymocytes during selection (Sun et al., 2000). Therefore, Notch signaling does not block expression of RORγt, but rather favors the development of T cells over LTi cells. In accordance with this view, few LTi cells develop within the thymus. However, thymic α4β7⁺ precursors generate a significant proportion of LTi cells when cultured in the absence of Notch signaling (Fig. 7).

Our results are in apparent conflict with a recent study by Possot et al. (2011). These authors report that, when cultured on irradiated fetal liver explants, fetal liver CLPs generate LTi cells in the absence of Notch signaling. However, we also find that LTi cells can be generated from CLPs in the absence of Notch signaling, although at low efficiency (Fig. 5 A). We suggest that in the absence of Notch signaling, low numbers of α4β7⁺ precursors are generated that nevertheless proceed to LTi cells as this developmental step is rescued in the absence of Notch signaling. A similar situation exists in the thymus of RORγt-deficient mice. Even though DP thymocytes require RORγt for survival, a low number of T cell precursors survive selection and proceed to the more mature CD4⁺ or CD8⁺ single-positive stage when RORγt is no longer required and expressed

(Sun et al., 2000). It is also possible that a small fraction of CLPs is already primed by Notch ligands in vivo and no longer require Notch signaling in vitro to up-regulate the expression of $\alpha4\beta7$ and generate LTis cells. In support of our general conclusion that Notch signaling is required for the generation of LTi cells, Lee et al. (2011) report a significant reduction in $CD4^+$ LTi cells when Notch signaling is absent in hematopoietic cells.

$NKp46^+$ $ROR\gamma t^+$ (NK22) cells, which are closely related to LTi cells, do not derive from $\alpha4\beta7^+$ precursors, but from $\alpha4\beta7^-$ precursors that express low levels of $ROR\gamma t$ (Sawa et al., 2010). Interestingly, Id2 is required for the generation of $\alpha4\beta7^+$ precursors, but not for the generation of these $\alpha4\beta7^-$ $ROR\gamma t^{low}$ precursors (Fig. 4), demonstrating that $\alpha4\beta7^+$ precursors to LTi cells do not generate $\alpha4\beta7^-$ $ROR\gamma t^{low}$ precursors to $NKp46^+$ $ROR\gamma t^+$ cells. Nevertheless, both LTi cells and $NKp46^+$ $ROR\gamma t^+$ cells fail to develop in Id2-deficient mice (Yokota et al., 1999; Satoh-Takayama et al., 2010), indicating that Id2 is required at a subsequent developmental step from $\alpha4\beta7^-$ $ROR\gamma t^{low}$ precursors to $NKp46^+$ $ROR\gamma t^+$ cells. It also remains to be assessed whether Notch signaling is required for the generation of $\alpha4\beta7^-$ $ROR\gamma t^{low}$ precursors. CLPs generate $\alpha4\beta7^-$ $ROR\gamma t^{low}$ cells both in the absence and presence of Notch signaling (Fig. 5 A), but the potential of these cells to generate $NKp46^+$ $ROR\gamma t^+$ cells remains to be assessed. Lee et al. (2011) report that the generation of $NKp46^+$ $ROR\gamma t^+$ cells is severely impaired in the absence of Notch signaling, demonstrating that Notch signaling is required for the generation of both types of LTi cells and $NKp46^+$ $ROR\gamma t^+$ cells.

LTi cells in the fetal liver express low levels of proinflammatory chemokines, cytokines, and cytokine receptors compared with LTi cells in the fetal intestine, suggesting that the functional maturation of LTi cells is not achieved within the liver, but in lymph node and Peyer's patch anlagen. Such in situ maturation of LTi cells has been previously suggested by Yoshida et al. (2002). Specifically, TRANCE is required for the development of lymph nodes, but not Peyer's patches (Kim et al., 2000), whereas IL-7 is essential for the development of Peyer's patches (Adachi et al., 1998), but not for most lymph nodes (Mebius, 2003). Both cytokines induce the up-regulation of $LT\alpha_1\beta_2$ by LTi cells, which is required for the development of lymphoid tissues (Luther et al., 2003; Yoshida et al., 2002). Furthermore, LTi cells isolated from TRAF6-deficient mice, which cannot signal through TRANCE-R, up-regulate $LT\alpha_1\beta_2$ when treated with IL-7 (Yoshida et al., 2002). In addition, TRAF6-deficient mice develop lymph nodes when treated with IL-7, showing that TRANCE provided locally by lymph node anlagen is normally required for final maturation of LTi cells. On another note, expression of integrin $\alpha4\beta7^+$ is required for the migration of LTi cells to lymph nodes anlagen (Mebius et al., 1996). As integrin $\alpha4\beta7^+$ is already expressed on $ROR\gamma t^-$ LTi cell precursors, maturity of LTi cells may not be a prerequisite for their emigration from the fetal liver and colonization of lymphoid tissue anlagen. This is probably also true for the emigration of LTi cell precursors from the bone marrow after birth, as no $ROR\gamma t^+$ cells could be identified in this compartment. Interestingly, whereas the ligand of integrin $\alpha4\beta7$, MadCAM-1, is expressed on high endothelial venules of lymph node and Peyer's patch anlagen in the fetus, it is restricted to the intestinal compartment after birth (Mebius et al., 1996). This may account for the enrichment of $ROR\gamma t^+$ ILCs in the intestinal lamina propria after birth, and for the presence of LTi cell cluster, or cryptopatches, exclusively in that compartment.

In sum, we show that the sequential engagement of Notch, Id2, and $ROR\gamma t$ drives the generation of LTi cells from CLPs. Intriguingly, lymph nodes and Peyer's patches develop in a programmed mode only in mammals, whereas lymphoid tissues in other animals develop in response to infection or injury (Eberl, 2007). The induced mode of lymphoid tissue development is best illustrated in the case of intestinal isolated lymphoid follicles (ILFs), which develop from cryptopatches during bacterial colonization of the gut (Bouskra et al., 2008). Thus, did mammals reprogram expression of Notch, Id2, and $ROR\gamma t$ during CLP differentiation to generate LTi cells? Was the reprogramming of $ROR\gamma t$ sufficient? As $ROR\gamma t$ is also required for the differentiation of Th17 cells and other types of IL-17–producing T cells, the investigation for further parallels between the development of LTi cells and of IL-17–producing T cells should be very informative.

MATERIALS AND METHODS

Mice. BAC transgenic mice *Rorc(γt)-Egfp*^{TG} (Lochner et al., 2008) are on a C57BL/6 background, whereas Id2-deficient mice (provided by P. Vieira, Institut Pasteur, Paris, France; Yokota et al., 1999), BAC transgenic *Rorc(γt)-Cre*^{TG} mice (Eberl and Littman, 2004), and *Rosa26-Stop*^{Floxed}*-NICD* mice (provided by M. Cohen-Tannoudji, Institut Pasteur, Paris, France; Murtaugh et al., 2003) are maintained on a mixed 129 x C57BL/6 background. All mice were kept in specific pathogen–free conditions, and all animal experiments were approved by the committee on animal experimentation of the Institut Pasteur and by the French Ministry of Agriculture.

Antibodies. The following mAbs were purchased from eBioscience: Alexa eFluor 780–conjugated anti-CD45.2 (104), biotinylated anti-B220 (RA3-6B2), biotinylated or PE-Cy7–conjugated anti-CD19 (MB19-1), biotinylated or PE-conjugated anti-CD11c (N418), biotinylated anti-Ter119 (TER-119), biotinylated anti-Gr1 (RB6-8C5), biotinylated or APC-Cy7–conjugated anti-NK1.1 (PK136), biotinylated or Pacific blue–conjugated anti-CD3e (500A2), PerCp-Cy5.5 or Pacific blue–conjugated anti-CD4 (RM4-5), APC-conjugated anti–IL-7Rα (A7R34), PE-conjugated anti-α4β7 (DATK32), APC-conjugated anti–IL-17A (eBio17B7), PE-conjugated anti–IL-22 (1H8PWSR), and APC-conjugated anti–IFN-γ (XMG1.2). APC-Cy7–conjugated anti–c-Kit (2B8) was purchased from SouthernBiotech. PE-conjugated anti-CCR6 (FAB590P) and biotinylated anti-NKp46 was obtained from R&D Systems. Biotinylated anti-DL1 (HMD1-3) was purchased from BioLegend. APC-conjugated anti-CD31 (MEC 13.3) was purchased from BD. Alexa Fluor 647–conjugated anti–rat antibody was purchased from Invitrogen.

Flow cytometry and FACS sorting. Fetal liver and thymus were dissociated by mechanical shearing in PBS containing 0.5% bovine serum and 2 mM EDTA, and then filtered through a 100-μM mesh. Fetal gut was cut into small pieces and digested with 1 mg/ml collagenase D (Roche) and 1 U/ml DNase 1 (Invitrogen) for 30 min at 37°C. After homogenization, the supernatant was filtered through a 100-μM mesh. Dead cells and debris were removed using a Percoll gradient. Fetal liver was depleted from Lin^+ cells using autoMACS (after incubation with biotinylated antibodies specific for Ter119, B220,

CD19, NK1.1, NKp46, CD11c, and CD3) and anti-biotin beads (Miltenyi Biotec). Fluorochrome-conjugated streptavidin was used for detection of the remaining Lin^+ cells. Mononuclear cell suspension obtained from liver, thymus, and gut were incubated with Fc block before surface staining for CD19, CD3, CD4, c-Kit, IL-7Rα, α4β7, and NKp46. For the surface staining of CCR6 and CXCR5, cells were first incubated with 10% FCS containing DME at 37°C for 30 min in the presence of antibody, followed by staining at 4°C for other surface markers. Cells were analyzed on a FACSCanto (BD) or a CyAn (DAKO) flow cytometer, followed by analysis with FlowJo software (Tree Star). Cell sorting was performed using FACSAria (BD).

Intracellular cytokine staining. For detection of cytokine production by intracellular staining, cells were stimulated for 3 h in DME containing 50 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich). For the last 1.5 h, 10 µg per ml brefeldin A (Sigma-Aldrich) was added to the culture medium. After cell surface staining for c-Kit, CD4, NKp46, and CD3ε, cells were fixed with 2% PFA (Sigma-Aldrich) and permeabilized with 0.5% saponin (Sigma-Aldrich). Intracellular staining was performed using antibodies against GFP, IL-17A, IL-22, and IFN-γ.

Immunofluorescence histology. Tissues were embedded in OCT compound 4583 (Sakura Finetek) and frozen in a bath of isopentane cooled with liquid nitrogen and stored at −80°C. Frozen blocks were cut into 10-µm-thick sections, fixed for 5 min in acetone at −20°C, air-dried, and processed for blocking and staining. Blocking was performed with 10% bovine serum in PBS and 100 µg/ml of hamster IgG for 1 h at room temperature, and endogenous biotin was blocked using the avidin biotin-blocking kit (Vector Laboratories). Slides were incubated with primary antibodies in PBS containing 1% bovine serum overnight at 4°C, and then washed 3 times for 5 min with PBS 1% bovine serum. Slides were then incubated with Alexa Fluor 647–conjugated anti–rat antibody and cyanine 3–conjugated streptavidin for 1 h at room temperature, washed once, incubated with DAPI (Sigma-Aldrich) for 5 min at room temperature, washed 3 times for 5 min, and mounted with Fluoromount-G (SouthernBiotech). Slides were examined with an AxioImager M1 fluorescence microscope (Carl Zeiss) equipped with a charge-coupled device camera, and images were processed with AxioVision software (Carl Zeiss).

Cell culture. Cell were FACS-sorted directly into complete Opti-MEM culture medium (Invitrogen) containing 10% FCS (Lonza), β2-mercaptoethanol, penicillin, and streptomycin, and seeded into flat-bottom 96-well plates coated with OP9 or OP9-DL1 stromal cells (provided by A. Cumano, Institut Pasteur, Paris, France;) in the presence of 10 ng/ml mouse rIL-7 and 10 ng/ml mouse rSCF (PeproTech). After 7–14 d, cultures were recovered with Cell Dissociation Buffer (Invitrogen), and 10,000–50,000 $CD45.2^+$ cells were analyzed by flow cytometry. For cloning of E14 fetal liver precursors, cells were directly sorted at 1 cell per well into flat-bottom 96-well plates coated with OP9 stromal cells in the presence of 25 ng/ml mouse rIL-7 and 25 ng/ml mouse rSCF. After 14 d of culture, clones were recovered as described above and analyzed by flow cytometry.

Quantitative PCR. To isolate mRNA, cells were directly FACS-sorted into RLT buffer (QIAGEN) supplemented with β2-mercaptoethanol. Total RNA was prepared using RNeasy micro kit (QIAGEN) according to the manufacturer's instructions. Concentration and integrity was assessed using the Bioanalyzer (Agilent). Linear amplification of mRNA was performed using the MessageBooster cDNA kit (Epicentre). cDNA was synthesized by reverse transcription using Superscript III (Invitrogen). Detection of specific gene expression was performed using primers from Superarray Biosciences and SYBR green (QIAGEN). Gene expression was normalized to *Gadph* and *Hprt* in each sample. For single-cell PCR, cells were directly sorted at 1 cell per well into PCR plates containing cell Direct RT-PCR master mix (Invitrogen). Specific target amplification and detection was performed using TaqMan primers and probes specific for *Gapdh*, *Hsp90ab*, *Id2*, or *Rorc* (Applied Biosystems). In Fig. 3 C, 21 out of 32 stage I cells, and 7 out of 64 stage II cells, were negative for both *Id2* and *Rorc* mRNA.

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