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An essential function for the nuclear receptor RORγt in the generation of fetal lymphoid tissue inducer cells

Gérard Eberl[1,5], Shana Marmon[1], Mary-Jean Sunshine[1,2], Paul D Rennert[3], Yongwon Choi[4] & Dan R Littman[1,2]

Lymphoid tissue inducer (LTi) cells are associated with early development of lymph nodes and Peyer's patches. We show here that during fetal life the nuclear hormone receptor RORγt is expressed exclusively in and is required for the generation of LTi cells. RORγt$^+$ LTi cells provide essential factors, among which lymphotoxin-$\alpha_1\beta_2$ is necessary but not sufficient for activation of the mesenchyma in lymph node and Peyer's patch anlagen. This early LTi cell–mediated activation of lymph node and Peyer's patch mesenchyma forms the necessary platform for the subsequent development of mature lymphoid tissues.

Development of the murine lymphatic system begins at embryonic day 10.5 (E10.5; 10.5 days after conception) with the invagination of endothelial cells from veins and the formation of lymphoid sacs[1]. It is not known which events initiate the localized development of lymph nodes or Peyer's patches. However, LTi cells, characterized as CD4$^+$CD3$^-$ and interleukin 7 receptor-α (IL-7Rα)-positive cells, are among the first hematopoietic cells to be detected in lymph node and Peyer's patch anlagen[2–6]. These cells are found in spleen, blood and lymph node anlagen by E12.5 and in Peyer's patch anlagen by E16, but are present in only very low numbers after birth. The absence of IL-7Rα$^+$ cells and Peyer's patches in mice deficient in IL-7Rα or Jak3 kinase, required for IL-7Rα-mediated signaling, has led to the suggestion that IL-7Rα$^+$ cells are 'Peyer's patch inducers'[4,5,7]. In addition, purified CD4$^+$CD3$^-$ cells induce the development of Peyer's patches or nasal-associated lymphoid tissue when transferred into mice otherwise deficient in Peyer's patches or nasal-associated lymphoid tissue[8,9]. These data show that LTi cells are involved in the development of lymph nodes, Peyer's patches and nasal-associated lymphoid tissue.

The appearance of LTi cells in lymph node and Peyer's patch anlagen correlates with a lymphotoxin-α (LT-α)-dependent increase in local expression of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1)[5,10]. LTi cells express the membrane-bound LT$\alpha_1\beta_2$ (refs. 3,5), whereas mesenchymal cells purified from Peyer's patch anlagen express VCAM-1, ICAM-1 and LTβR[11], the receptor for LT$\alpha_1\beta_2$. This indicates that LTi cells and mesenchymal cells interact in lymph node and Peyer's patch anlagen through LT$\alpha_1\beta_2$ and LTβR to induce further development of lymph nodes and Peyer's patches. In accordance with a central function for this interaction[12], lymph nodes and Peyer's patches are absent in mice deficient in LTα[13], LTβR[14] or factors required for signaling downstream of LTβR[15–17].

LTi cells, as well as lymph nodes and Peyer's patches, are absent from mice deficient in the inhibitory transcription factor Id2 (ref. 18) or the retinoic acid–related orphan receptors (RORs) RORγ and RORγt[19,20]. RORγ and RORγt are two isoforms that differ in their N-terminal sequence, encoded by alternative 5′ exons within the *Rorc* locus. Whereas RORγ mRNA is detected in many adult tissues, including liver, lung, muscle, heart and brain, RORγt mRNA and protein has been found only in immature double-positive CD4$^+$CD8$^+$ thymocytes[21–23]. The isoform expressed in LTi cells and required for lymph node and Peyer's patch development has not been described. To study the function of the individual RORγ isoforms in lymph node and Peyer's patch development, we generated mice in which the gene encoding enhanced green fluorescent protein (EGFP) was targeted to the first exon of the gene encoding RORγt (called *Rorc*(γt) here). We found that during fetal life, RORγt was expressed exclusively in CD4$^+$ and CD4$^-$ LTi cells. This restricted fetal expression of RORγt in LTi cells allowed us to visualize early lymph node and Peyer's patch development and to assess the function of different factors in the generation and recruitment of LTi cells into lymph node and Peyer's patch anlagen. The absence of LTi cells in RORγt-deficient mice allowed us to evaluate directly the function of LTi cells in lymph node and Peyer's patch development.

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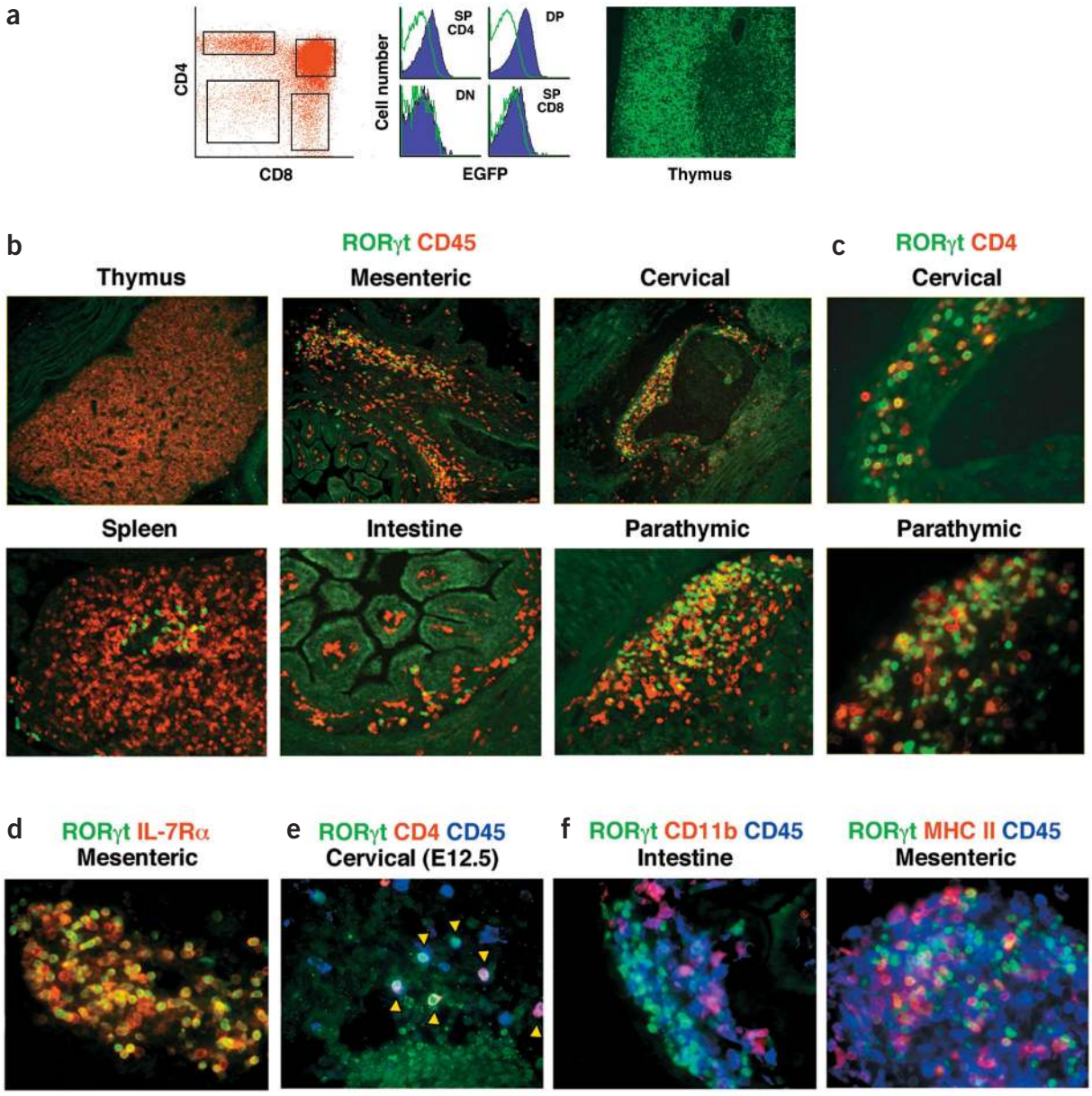

Figure 1 In the fetus, RORγt is exclusively expressed in LTi cells. (**a**) Left: CD4 and CD8 expression in thymocytes from 6- to 8-week-old *Rorc*(γt)$^{+/GFP}$ mice. Boxed areas indicate cell populations analyzed for EGFP expression. Center: blue histograms, thymocytes from *Rorc*(γt)$^{+/GFP}$ mice; green lines, thymocytes from *Rorc*(γt)$^{+/+}$ mice. SP, single-positive; DP, double-positive; DN, double-negative. Right: staining of *Rorc*(γt)$^{+/GFP}$ thymus with anti-EGFP. Cortex and medulla were defined by nuclear DAPI staining (data not shown). Data are representative of at least five independent experiments. (**b**–**f**) Sections from whole *Rorc*(γt)$^{+/GFP}$ fetuses at age E16.5 (**b**–**d**,**f**) or E12.5 (**e**) were stained as indicated by color of lettering above sections. Thymus, spleen, intestine and lymph node anlagen in designated regions are shown here. Green autofluorescence was used to visualize tissue structure. Arrowheads in **e** point to EGFP^{+} cells. Pink cells in **f** are stained for both red (CD11b or MHC class II) and blue (CD45). Original magnifications: **b**, ×100 (top) and ×200 (bottom); **c**–**f**, ×400. Sections are representative of at least ten individual sections and three to ten independent experiments.

RESULTS

Generation of *Rorc*(γt)-EGFP 'knock-in' mice

The mouse *Rorc* locus on chromosome 3 encodes both the RORγ and RORγt isoforms (**Supplementary Fig. 1** online)[22,24]. An alternative first exon (exon 1γt), used in expression of RORγt, is located approximately 2 kb downstream of exon 2γ, and is spliced to the common exon 3γ. Using homologous recombination in embryonic stem cells, we inserted the coding sequence of EGFP into exon 1γt directly downstream of the ATG translational start codon (**Supplementary Fig. 1** online). The translational stop codon of the coding sequence for EGFP was retained, but no additional splice or polyadenylation sites were introduced into exon 1γt. The *loxP*-flanked neomycin-resistance (*neo*^{r}) cassette, used to select targeted embryonic stem cells, was excised *in vivo* by crossing heterozygous *Rorc*(γt)$^{+/GFPneo}$ founder mice with 'Cre-deleter' mice (**Supplementary Fig. 1** online). For subsequent experiments, we used only heterozygous *Rorc*(γt)$^{+/GFP}$ mice to analyze expression of the *Rorc*(γt) gene and homozygous *Rorc*(γt)$^{GFP/GFP}$ mice to study the phenotype of RORγt-deficient mice.

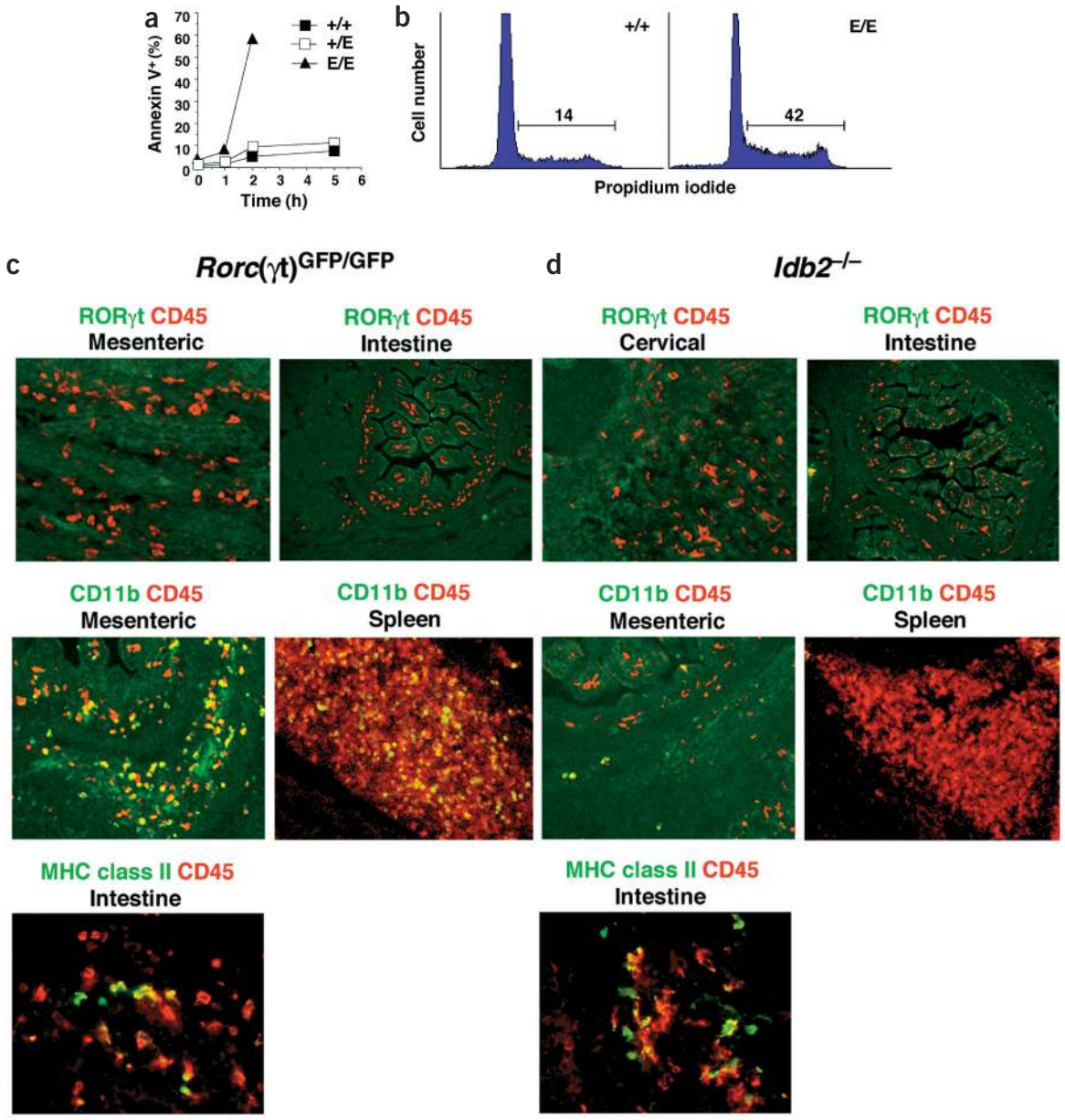

Figure 2 A specific absence of LTi cells in RORγt-deficient fetuses. (**a**) Thymocyte survival requires RORγt. Thymocytes from wild-type *Rorc*(γt)$^{+/+}$ (+/+), heterozygous *Rorc*(γt)$^{+/GFP}$ (+/E) and homozygous *Rorc*(γt)$^{GFP/GFP}$ (E/E) mice were cultured (times, horizontal axis) and stained with Annexin V to detect apoptotic cells. (**b**) Thymocytes from *Rorc*(γt)$^{+/+}$ (+/+) and *Rorc*(γt)$^{GFP/GFP}$ (E/E) mice were fixed and stained with propidium iodide to quantify the amount of DNA per cell. Data are representative of three independent experiments. Numbers above brackets indicate percent cells in S and G2/M phases of the cell cycle. (**c**,**d**) Both RORγt and Id2 are required for the generation and/or survival of LTi cells, but Id2 is also required for the generation of fetal CD11b^{+} cells. Sections from *Rorc*(γt)$^{GFP/GFP}$ or *Idb2*$^{-/-}$*Rorc*(γt)$^{+/GFP}$ E16.5 whole fetuses were stained as indicated by color of lettering above sections. Lymph node and Peyer's patch anlagen in the designated regions, as well as spleen, are shown. Original magnifications: intestine, ×100 (top row) and ×400 (bottom row); other panels, ×200. Sections are representative of at least ten individual sections and three independent experiments.

A segment encompassing exons 1γ–5γ of the RORγ mRNA was amplified by RT-PCR and detected in the liver of wild-type *Rorc*(γt)$^{+/+}$, mutant *Rorc*(γt)$^{+/GFP}$ and *Rorc*(γt)$^{GFP/GFP}$ mice, showing that insertion of the sequence encoding EGFP into exon 1γt did not affect the transcription of *Rorc*(γ) mRNA. In contrast, a segment encompassing exons 1γt–5γ of *Rorc*(γt) mRNA was amplified from the thymi of *Rorc*(γt)$^{+/+}$ and *Rorc*(γt)$^{+/GFP}$ mice, but not of *Rorc*(γt)$^{GFP/GFP}$ mice, as expected (**Supplementary Fig. 1** online). In accordance with these data, RORγt protein was detected in the thymus of *Rorc*(γt)$^{+/+}$ and *Rorc*(γt)$^{+/GFP}$ mice, but not of *Rorc*(γt)$^{GFP/GFP}$ mice. However, no RORγ protein could be detected in total liver samples of the different mouse lines, indicating very low expression of RORγ or that only a small fraction of liver cells express RORγ (**Supplementary Fig. 1** online).

In the fetus, only LTi cells express RORγt

We first analyzed EGFP expression, which reports *Rorc*(γt) transcription, in the thymi of adult *Rorc*(γt)$^{+/GFP}$ mice. Consistent with

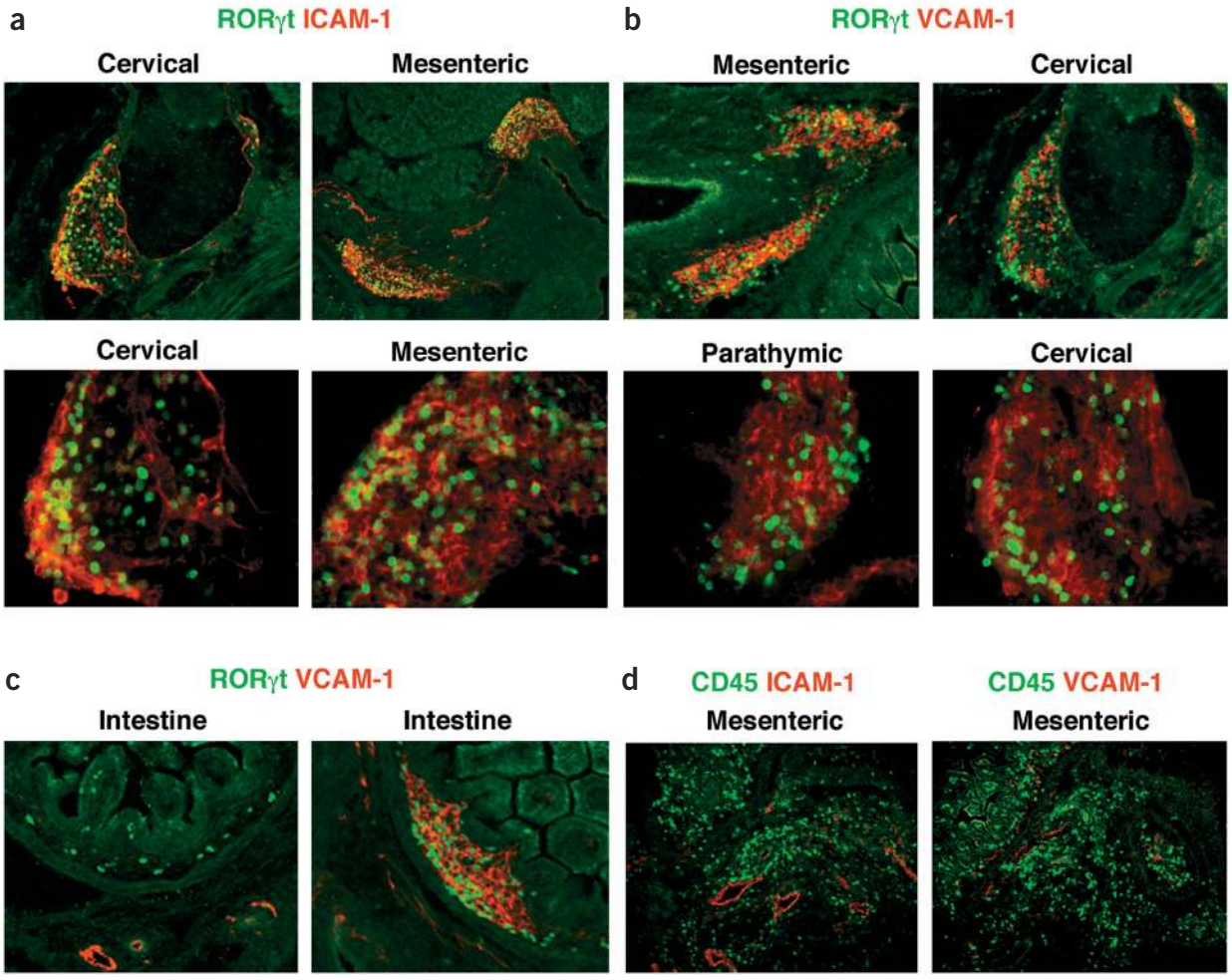

Figure 3 Lymph node and Peyer's patch anlagen have high expression of ICAM-1 and VCAM-1. (**a**,**b**) Sections from whole *Rorc*(γt)$^{+/GFP}$ E16.5 fetuses were stained as indicated by color of lettering above sections. Original magnifications: ×150 (top row except second panel from the left, ×100); ×400 (bottom row). (**c**) Small clusters of LTi cells do not have high expression of ICAM-1 or VCAM-1. Only staining for VCAM-1 is shown, but similar results were obtained for ICAM-1. Two different regions of the intestine of the same *Rorc*(γt)$^{+/GFP}$ E16.5 fetus are shown. Original magnification, ×200. (**d**) Absence of high ICAM-1 and VCAM-1 expression in the absence of LTi cells in *Rorc*(γt)$^{GFP/GFP}$ E16.5 fetuses. Sections are representative of at least twenty individual sections and five independent experiments. Original magnification, ×100.

previous results[22,23], EGFP was expressed in double-positive thymocytes but not double-negative thymocytes (**Fig. 1a**). We also found small amounts of EGFP in CD4$^+$ and CD8$^+$ single-positive thymocytes, although *Rorc*(γt) mRNA was not detected in these populations[22,23]. This result may be because of the long half-life of EGFP protein (>24 h), which would be detected in single-positive thymocytes even after cessation of transcription of the sequence encoding EGFP. In addition, expression of EGFP was confined to the thymic cortex, the resident site for double-positive and immature single-positive heat-stable antigen–high thymocytes (**Fig. 1a**, right). We obtained similar results with a monoclonal antibody (mAb) recognizing both RORγ isoforms[19] (data not shown).

We assessed expression of EGFP during fetal life by immunofluorescence histology on serial sections of whole *Rorc*(γt)$^{+/GFP}$ fetuses. We first analyzed E16.5 fetuses. At that stage, no mature B cells are yet produced[25] and T cells can only be found in the thymus. We identified lymph node anlagen by the presence of large clusters of hematopoietic (CD45$^+$) cells in the immediate vicinity of veins[10], whereas we could not identify Peyer's patch anlagen on that basis because of the presence of CD45$^+$ cells throughout the lamina propria and the submucosal region of the intestine[4] (**Fig. 1b**). EGFP was expressed exclusively in tight clusters of cells found in lymph node anlagen, in the submucosal region of the intestine and around central vessels in the spleen (**Fig. 1b**). However, EGFP was not expressed in the thymi of E16.5 fetuses, in contrast to earlier findings based on the detection of *Rorc*(γt) mRNA[26]. This discrepancy could be explained by the presence of parathymic lymph node anlagen that may have been isolated together with the thymus and thus account for the detection of *Rorc*(γt) mRNA in thymus samples (data not shown). Most but not all RORγt$^+$ cells expressed CD4, whereas only rare cells expressed CD4 but not RORγt (**Fig. 1c**). In contrast, all RORγt$^+$ cells expressed IL-7Rα (**Fig. 1d**). These results are in accordance with previous work showing that both CD4$^+$IL-7RA$^+$ cells and CD4$^-$IL-7RA$^+$ cells are present in similar numbers in Peyer's patch anlagen and express similar sets of chemokine receptors[5,27]. Both the localization and the phenotype of CD4$^+$ and CD4$^-$ RORγt$^+$ cells identify these as LTi cells. We detected RORγt$^+$ cells as early as E12.5 in the immediate vicinity of vessels among small clusters of CD45$^+$ cells (**Fig. 1e**). From E12.5, RORγt$^+$

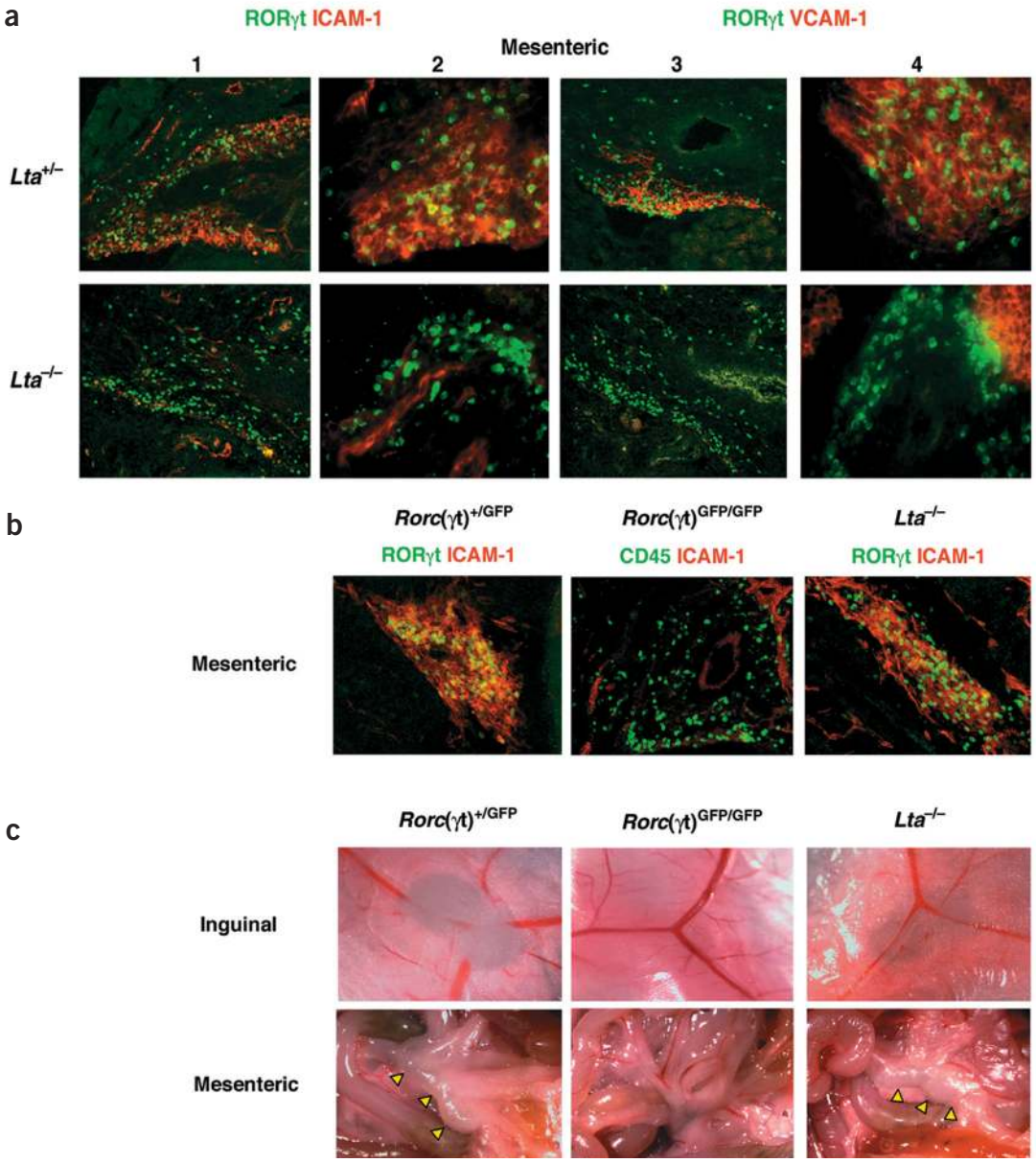

Figure 4 LT$\alpha_1\beta_2$ is necessary but not sufficient for the induction of lymph node and Peyer's patch development by LTi cells. (**a**) Absence of ICAM-1 and VCAM-1 induction, but normal recruitment of LTi cells in LTα-deficient mice. Sections from *Lta*$^{+/-}$*Rorc*(γt)$^{+/GFP}$ or *Lta*$^{-/-}$*Rorc*(γt)$^{+/GFP}$ E16.5 whole fetuses were stained as indicated by color of lettering above sections. Mesenteric lymph node anlagen is shown; similar results were obtained for other lymph node anlagen, including cervical and sacral lymph node anlagen. Original magnifications, ×150 (columns 1 and 3) and ×400 (columns 2 and 4). Bottom right, the liver appears as bright red autofluorescent tissue in the upper right quadrant. Sections are representative of at least ten individual sections and three independent experiments. (**b**) Induction of ICAM-1 expression by LTβR activation in LTα-deficient but not RORγt-deficient fetuses. Pregnant mothers were treated at fetal age E14 and E15.5 with an agonist mAb to LTβR. Sections of *Rorc*(γt)$^{+/GFP}$, *Rorc*(γt)$^{GFP/GFP}$ or *Lta*$^{-/-}$*Rorc*(γt)$^{+/GFP}$ E16.5 whole fetuses were stained as indicated by color of lettering above sections. No ICAM-1$^+$ clusters were detected in treated *Rorc*(γt)$^{GFP/GFP}$ fetuses, whereas all lymph node anlagen present in *Lta*$^{-/-}$*Rorc*(γt)$^{+/GFP}$ fetuses expressed ICAM-1. Similar results were obtained for VCAM-1 expression. Original magnification, ×200. Data are representative of at least ten individual sections and three independent experiments. (**c**) Induction of lymph node development by LTβR activation in LTα-deficient but not RORγt-deficient fetuses. Pregnant mothers were treated at fetal age E14 and E16 with an agonist mAb to LTβR. The presence of lymph nodes and Peyer's patches was assessed in offspring (genotypes, above sections) at 4 weeks of age. All lymph nodes and Peyer's patches were present in LTα-deficient offspring, whereas all lymph nodes and Peyer's patches were absent from *Rorc*(γt)$^{GFP/GFP}$ offspring. Similar results were obtained when the mAb was injected at E15 and E17. Arrowheads point to the mesenteric lymph nodes. Data are representative of three independent experiments.

cell numbers increased up to birth (E20) and decreased thereafter within 2 d to barely detectable amounts (data not shown). We obtained similar results when we stained fetus sections with a mAb to both RORγt and RORγ (data not shown).

When we attempted to identify the CD45$^+$ hematopoietic cells surrounding the clusters of RORγt$^+$ cells, we found that a fraction of these cells (10–20%) expressed CD11b (**Fig. 1f**), a β_2 integrin confined to cells of the natural killer, polymorphonuclear and myeloid

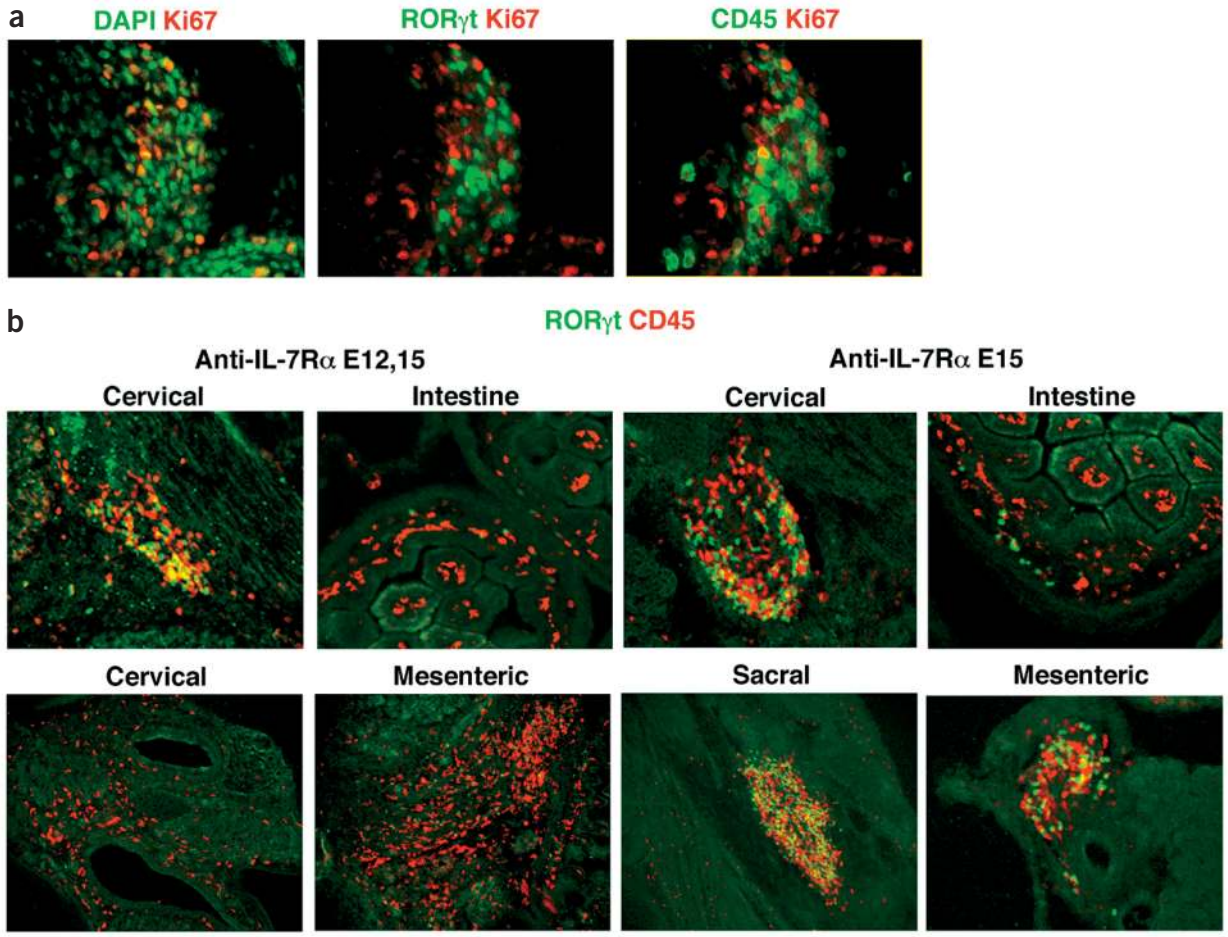

Figure 5 LTi cells do not proliferate, but are recruited to lymph node and Peyer's patch anlagen. (**a**) LTi cells do not proliferate in lymph node or Peyer's patch anlagen. Sections from whole *Rorc*(γt)$^{+/GFP}$ E16.5 fetuses were costained for nuclei (DAPI), EGFP, CD45 and Ki67. For clarity, only stainings for two markers are shown together on the same panel. A parathymic lymph node anlage is shown. Original magnification, ×400. Similar results were obtained in other lymph node anlagen, as well as in Peyer's patch anlagen. Sections are representative of at least ten individual sections and three independent experiments. (**b**) Blockade of IL-7Rα early in fetal development inhibits the accumulation of LTi cells. Sections from whole *Rorc*(γt)$^{+/GFP}$ E16.5 fetuses, obtained from pregnant mothers treated at E12 and E15, or at E15 only, with a blocking mAb to IL-7Rα, were stained as indicated by color of lettering above sections. Only rare lymph node anlagen, such as the small cervical lymph node anlage at top left, were present in fetuses treated at E12 and E15. Original magnifications, ×250 (top and bottom right) and ×100 (remaining bottom).

lineages[28]. However, CD45$^+$ cells present in lymph node and Peyer's patch anlagen did not express additional markers of the myeloid lineage, such as Gr-1 and CD11c (data not shown). A larger fraction (approximately 50%) of CD45$^+$ cells, as well as 25–50% of RORγt$^+$ cells, expressed major histocompatibility complex (MHC) class II proteins[3] (**Fig. 1f**), usually expressed on antigen-presenting cells. The relevance of MHC class II expression in lymph node and Peyer's patch anlagen of E16.5 fetuses is not apparent, as at this stage CD4$^+$ T cells are found only in the thymus.

RORγt is required for the generation of LTi cells

We first examined whether lack of the RORγt isoform accounts for the previously described increased cell cycle progression and susceptibility to apoptosis of thymocytes deficient in both the RORγ and RORγt isoforms[19]. Consistent with an essential function for RORγt in thymocyte development, thymocytes from *Rorc*(γt)$^{GFP/GFP}$ mice underwent apoptosis in culture within 2–3 h, whereas most thymocytes from heterozygous *Rorc*(γt)$^{+/GFP}$ or wild-type mice survived in culture for more than 5 h (**Fig. 2a**). Moreover, 42% of the freshly isolated RORγt-deficient thymocytes were in the S – and G2/M phases of the cell cycle, compared with 14% of wild-type thymocytes (**Fig. 2b**).

Whereas heterozygous *Rorc*(γt)$^{+/GFP}$ mice developed lymph nodes and Peyer's patches normally, RORγt-deficient mice lacked all lymph nodes and Peyer's patches, as well as LTi cells. We detected no EGFP$^+$ cells in sections of E16.5 *Rorc*(γt)$^{GFP/GFP}$ fetuses (**Fig. 2c**), although EGFP$^+$ double-positive thymocytes were present in adult mice. *Rorc*(γt)$^{GFP/GFP}$ mice had fewer double-positive cells (30–50% that of wild-type mice) and expressed twice as much EGFP as did thymocytes from heterozygous *Rorc*(γt)$^{+/GFP}$ mice (data not shown). These data demonstrate that RORγt is necessary for the generation of LTi cells. As RORγt was exclusively expressed in LTi cells in the fetus, these results also demonstrate that LTi cells are necessary for the development of lymph nodes and Peyer's patches. In contrast, clusters of hematopoietic CD45$^+$ cells were still present in E16.5 *Rorc*(γt)$^{GFP/GFP}$ fetuses, as were CD11b$^+$ and MHC class II$^+$ cells (**Fig. 2c**), showing that RORγt is not involved in their generation.

Mice deficient in Id2, an inhibitor of basic helix-loop-helix transcription factors, also lack all lymph nodes, Peyer's patches and LTi cells[18]. We were unable to find RORγt$^+$ cells in sections of E16.5 Id2-deficient *Rorc*(γt)$^{+/GFP}$ fetuses (**Fig. 2d**). However, in addition to LTi cells, Id2-deficient mice also lacked CD11b$^+$ cells, whereas clusters of hematopoietic CD45$^+$ cells and MHC class II$^+$ cells were still present (**Fig. 2d**). These data show that Id2 is required for the generation not only of LTi cells but also of other fetal cells, including CD11b$^+$ cells. The absence of fetal CD11b$^+$ cells may account for the greatly reduced number of natural killer cells and splenic CD8α$^+$ dendritic cells, as well as the lack of Langerhans cells, in adult Id2-deficient mice[18,29].

LTα$_1$β$_2$ is necessary but not sufficient for LTi cell function

Earlier reports have shown that LTi cells colocalize in lymph node and Peyer's patch anlagen at sites where ICAM-1 and VCAM-1 are expressed by mesenchymal cells[5,10,11]. Both adhesion molecules had low expression throughout the *Rorc*(γt)$^{+/GFP}$ E16.5 fetuses on most endothelia (**Fig. 3**). However, ICAM-1 and VCAM-1 were most abundantly expressed in the thymus (data not shown) and in lymph node and Peyer's patch anlagen (**Fig. 3a–c**). High expression of ICAM-1 and VCAM-1 was not induced in emerging lymph node and Peyer's patch anlagen with low numbers of LTi cells (**Fig. 3c**). In the absence of LTi cells in *Rorc*(γt)$^{GFP/GFP}$ fetuses, we found no expression of ICAM-1 and VCAM-1 in putative lymph node and Peyer's patch anlagen (**Fig. 3d**). This result shows that LTi cells are required for the activation of mesenchyma in lymph node and Peyer's patch anlagen.

LTα and its receptor, LTβR, are essential in lymph node and Peyer's patch development[13,14]. Little or no expression of VCAM-1 is detected in the intestine and lymph node anlagen of LTα-deficient mice, even though IL-7Rα$^+$ cells are still present[5,10]. Consistent with these observations, the number of LTi cells present in lymph node anlagen of LTα-deficient *Rorc*(γt)$^{+/GFP}$ E16.5 fetuses was normal, but large amounts of ICAM-1 and VCAM-1 failed to be induced (**Fig. 4a**). In the fetus, LTi cells are thought to be the main if not the only producers of LTα$_1$β$_2$, the ligand of LTβR[5,11,30]. Activation of the LTβR with an agonist mAb was previously shown to rescue lymph node and Peyer's patch development in LTα-deficient mice[12]. Consistent with that earlier report, the agonist mAb induced ICAM-1 and VCAM-1 expression in lymph node anlagen and restored lymph node development in LTα-deficient mice. In contrast, this mAb failed to do so in RORγt-deficient mice (**Fig. 4b,c**). These results demonstrate that LTi cells induce lymph node and Peyer's patch development by activating the LTβR signaling pathway in mesenchymal cells, and that additional LTi cell-derived factors are required and remain to be identified.

Factors involved in recruitment of LTi cells

Accumulation of LTi cells in lymph node and Peyer's patch anlagen results from local expansion or from active recruitment. In sections of *Rorc*(γt)$^{+/GFP}$ E16.5 fetuses stained for the proliferation marker Ki67, we found no proliferating LTi cells, and only very rare CD45$^+$ hematopoietic cells were Ki67$^+$ (**Fig. 5a**). In contrast, substantial numbers of CD45$^-$RORγt$^-$ cells stromal cells did proliferate. This indicates that LTi cells are recruited to lymph node and Peyer's patch anlagen.

Many factors known to be required for lymph node and Peyer's patch development may function in the recruitment of LTi cells. In *Rorc*(γt)$^{+/GFP}$ fetuses, it is possible to visually assess the function of these factors early in lymph node and Peyer's patch development. Mice deficient for BLC (CXCL13) or for its receptor, CXCR5, lack most lymph nodes and Peyer's patches, with the exception of mesenteric and deep cervical lymph nodes[31,32]. Consistent with these data, LTi cells in BLC-deficient mice were present in normal numbers only in mesenteric and cervical lymph node anlagen (data not shown). Mice deficient in IL-7Rα or Jak3, or treated with a blocking mAb to IL-7Rα, lack LTi cells in Peyer's patch anlagen and fail to develop Peyer's patches[5,7] but develop most lymph node anlagen[10,27]. In E16.5 *Rorc*(γt)$^{+/GFP}$ fetuses treated on E12 and E15 with a blocking mAb to IL-7Rα, clusters of LTi cells were absent from the intestine and lymph node anlagen, or present only in reduced numbers (**Fig. 5b**). In fetuses treated with mAb to IL-7Rα only on E15, clusters of LTi cells were present in Peyer's patch anlagen and the number of LTi cells in lymph node anlagen seemed normal (**Fig. 5b**). These data indicate that IL-7Rα is required early in the recruitment of LTi cells to lymph node and Peyer's patch anlagen, and that other factors may provide recruiting functions later during lymph node development. Mice deficient in the tumor necrosis factor (TNF) family cytokine TRANCE[33] or the intracellular adaptor TRAF6 (ref. 10), involved in the signaling downstream of the TRANCE receptor, have severely reduced numbers of LTi cells in mesenteric lymph node anlagen at birth. These mice fail to develop lymph nodes, whereas Peyer's patch development is not affected. In E16.5 fetuses treated on E12 and E15 with TRANCE receptor–immunoglobulin fusion protein, the number of LTi cells found in lymph node anlagen was generally reduced (**Supplementary Fig. 2** online). Finally, mice deficient in TNF or TNF-R-I (a receptor for both TNF and LTα$_3$) have reduced numbers of Peyer's patches[34,35]. Consistent with these data, we found only rare LTi cells in the intestine of E16.5 fetuses treated with TNF-R-I–immunoglobulin fusion protein (**Supplementary Fig. 2** online).

DISCUSSION

Using a knock-in mouse model, we have shown here that during fetal life RORγt is expressed exclusively in LTi cells and that in its absence, LTi cells are not generated and lymph nodes and Peyer's patches do not develop. In lymph node and Peyer's patch anlagen, LTi cells induce activation of the local mesenchyma and subsequent lymph node and Peyer's patch development, by way of the LTα$_1$β$_2$-LTβR interaction and additional factors that remain to be defined. Moreover, accumulation of LTi cells into lymph node and Peyer's patch anlagen is the result of active recruitment by chemokines and cytokines, rather than local proliferation of LTi cells. Our data demonstrate that RORγt is required for lymph node and Peyer's patch development as a consequence of its specific and essential function in the generation of LTi cells.

The results demonstrate that LTi cells, previously characterized as CD3$^-$CD4$^+$CD45$^+$IL-7Rα$^+$ cells[2–6], are defined by the expression of RORγt. The absence of LTi cells and of lymph nodes and Peyer's patches in RORγt-deficient mice formally demonstrates that LTi cells are necessary for the development of lymph nodes and Peyer's patches. Like RORγt, the inhibitor of basic helix-loop-helix transcription factors Id2 is also required for the generation of LTi cells[18]. However, unlike RORγt, Id2 was also required for the appearance of fetal CD11b$^+$ cells. Moreover, Id2 is involved in various cell differentiation programs during embryonic development[36]. RORγt is thus the only molecule to our knowledge known to be expressed exclusively in fetal LTi cells.

The results also demonstrate that LTi cells interact with and activate mesenchymal cells present in lymph node and Peyer's patch anlagen, as suggested by earlier studies[37]. We assessed activation of

mesenchyma by the expression of the adhesion molecules ICAM-1 and VCAM-1. In E16.5 fetuses, these molecules were highly expressed only in lymph node and Peyer's patch anlagen, as well as in thymus and spleen, consistent with a central function for these adhesion molecules in lymph node and Peyer's patch development. A threshold number of LTi cells was required in this process, reflecting a possible 'community effect' of clustered LTi cells[33].

Mesenchymal cells express LTβR[11], whereas LTi cells express its ligand, membrane $LT\alpha_1\beta_2$ (refs. 3,5). The essential function of the $LT\alpha_1\beta_2$-LTβR interaction in lymph node and Peyer's patch development is shown by the absence of lymph nodes and Peyer's patches in LTα-deficient mice[13]. Lymph node and Peyer's patch development can be rescued in LTα-deficient mice by treatment of embryos with an agonist mAb to LTβR[12]. However, treatment of RORγt-deficient mice with this mAb did not rescue lymph node and Peyer's patch development. Similarly, the agonist mAb does not rescue lymph node development in TRANCE-deficient mice, and transgenic TRANCE expression (in cells distinct from LTi cells) does not rescue lymph node development in LTα-deficient mice[6]. These data show that the function of LTi cells in inducing lymph node and Peyer's patch development is not confined to providing $LT\alpha_1\beta_2$ for the activation of the mesenchyma. Other factors expressed by LTi cells, such as TRANCE[33], may be part of the necessary arsenal of factors produced by LTi cells during lymph node development. As TRANCE is not involved in Peyer's patch development, additional factors provided by LTi cells remain to be defined.

The accumulation of LTi cells in lymph node and Peyer's patch anlagen may result from active recruitment of circulating cells, or from expansion of resident LTi cells or $CD45^+$ precursor cells. In favor of the first possibility, we found that no LTi cells and only rare hematopoietic cells proliferated in lymph node and Peyer's patch anlagen. In addition, IL-7Rα, TRANCE, TNF-R-I and BLC (CXCL13) were all involved in the early recruitment of LTi cells to lymph node and Peyer's patch anlagen. The individual function of each of these factors is not apparent, and recent reports have shown that BCL (CXCL13) forms a redundant recruitment system with IL-7Rα and CCR7 for the development of particular lymph nodes[27,38].

Recruitment of LTi cells to lymph node and Peyer's patch anlagen may be favored by a positive feedback loop generated by activation of mesenchyma by LTi cells through the $LT\alpha_1\beta_2$-LTβR interaction[39]. Ligation of the LTβR induces expression of an array of chemokines in embryonic fibroblasts and in B cells, including BLC (CXCL13), ELC (CCL19) and SLC (CCL21)[40,41]. BLC and ELC are also expressed by $ICAM\text{-}1^+VCAM\text{-}1^+$ mesenchymal cells in Peyer's patch anlagen[11]. In addition, the receptors for BLC (CXCR5) and ELC and SLC (CCR7) are expressed by LTi cells and transduce chemotactic signals in LTi cells *in vitro*[11]. LTi cells may therefore migrate to lymph node and Peyer's patch anlagen in response to basal levels of chemokine expression by mesenchymal cells and cytokines. There, LTi cells activate the mesenchyma through the $LT\alpha_1\beta_2$-LTβR interaction, induce further chemokine expression and increase recruitment of LTi cells. However, even though the mesenchyma of lymph node and Peyer's patch anlagen failed to be activated in LTα-deficient fetuses, we found that the number of LTi cells present was not affected. This indicates that a positive feedback loop may operate efficiently only at later stages of lymph node and Peyer's patch development; that is, after E16.5. At this stage, a threshold number of LTi cells may be present to exert a 'community effect' and induce activation of mesenchyma. The $LT\alpha_1\beta_2$-LTβR interaction seems also to be required for prolonged recruitment or survival of LTi cells in lymph node anlagen, as few LTi cells are found at birth in lymph node anlagen of LTα-deficient mice[33].

In conclusion, the nuclear hormone receptor RORγt defines by its expression a single population of fetal cells, the LTi cells. The function of RORγt in the generation of LTi cells is not known. Previous findings on the requirement for RORγt in double-positive thymocytes indicate that it could control survival of LTi cells[19]. However, forced expression of the anti-apoptotic protein $Bcl\text{-}x_L$ in place of RORγt failed to rescue LTi cell generation (data not shown). It will therefore be useful to identify targets of RORγt in LTi cells. Analysis of such genes is likely to provide insights into the mechanisms of lymph node and Peyer's patch development.

METHODS

Generation of RORγt-EGFP mice. The mouse bacterial artificial chromosome (BAC) library ClTB (Research Genetics) was screened by PCR with primers amplifying a 280bp fragment containing *Rorc*(γ) exon 1. We identified one clone, I15, that contained all the *Rorc* locus, as determined by restriction enzyme mapping, and used it as the source of genomic DNA for the *Rorc* locus. The targeting vector used for homologous recombination in embryonic stem cells consisted of a short homology arm followed by EGFP, a *loxP*-flanked neo^r cassette (from pL2neo; **Supplementary Fig. 1** online), a long homology arm and the thymidine kinase gene, cloned into the pGEM-11Zf vector. The short arm consisted of a 1-kb fragment located immediately 5′ of the ATG translational start site of *Rorc*(γt) exon 1, and was amplified by PCR from BAC clone I15 with a *Nco*I site at its 3′ end. The EGFP-coding sequence (BD Biosciences Clontech) was fused to the short arm through the *Nco*I site. The long arm consisted of a 7.8-kb fragment located immediately 3′ of the ATG translational start site of *Rorc*(γt) exon 1 and extending to *Rorc*(γ) exon 5, and was amplified by PCR from BAC clone I15. Embryonic stem cells (E14.1; 129/Ola) were electroporated with 30 μg of the linearized targeting vector, and 2 of 200 G418-gancyclovir double-resistant colonies had the correctly targeted *Rorc*(γt) locus. Initial screening was done by PCR, amplifying a 1.1-kb fragment starting 100 bp 5′ of the short arm in the *Rorc*(γ) locus and ending at the 5′ end of EGFP. Correct insertion of the sequence encoding EGFP was also confirmed by Southern blot (**Supplementary Fig. 1** online). The neo^r-containing embryonic stem cells were injected into blastocyts and two chimeric males were obtained. Germline transmission of the mutant allele was obtained with one chimera and yielded heterozygos $Rorc(\gamma t)^{+/GFPneo}$ mice, which were then crossed with a mouse line expressing Cre under the control of a human cytomegalovirus minimal promoter (The Jackson Laboratory) to excise the neo^r cassette. The genotype of the $Rorc(\gamma t)^{+/GFPneo}$ and derived mice was established by PCR with primers surrounding *Rorc*(γt) exon 1: the 5′ primer was 5′-CCCCCTGCCCAGAAACACT-3′ and the 3′ primer was 5′-GGATGCCCCCATTCACTTACTTCT-3′ (**Supplementary Fig. 1** online).

Mice and treatments. LTα-deficient ($Lta^{-/-}$) mice and Id2-deficient ($Idb2^{-/-}$) mice[18] were crossed with $Rorc(\gamma t)^{+/GFP}$ mice to obtain $Lta^{-/-}Rorc(\gamma t)^{+/GFP}$ and $Idb2^{-/-}Rorc(\gamma t)^{+/GFP}$ mice. BLC-deficient mice have been described[31]. In blocking experiments, pregnant mothers were injected intravenously at E12 and E15 with 100 μg mAb or immunoglobulin fusion protein, unless indicated otherwise. All mice were bred and used in our specific pathogen–free animal facility according to the New York University School of Medicine Institutional Animal Care and Use Committee.

RT-PCR and immunoblotting. Total RNA was extracted from livers and thymi of 6- to 8-week-old mice, and cDNA was synthesized with the Reverse Transcription System from Promega. The PCR primer pairs specific for the two RORγ isoforms, as well as the PCR conditions and the blotting conditions of the PCR products, have been described[22]. For immunoblot, liver and thymus tissues were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Nonidet-P40, 0.5% deoxycholate, 0.1% SDS and 50 mM Tris-HCl, pH 8.0) supplemented with protease inhibitors and were sonicated. Equivalent amounts of protein were mixed with reducing loading buffer and separated by 12% SDS-PAGE. After transfer, membranes were incubated for 2 h at room temperature with 0.6 μg/ml of a mAb to RORγ[19] and washed and were further incubated for 1 h at room temperature with a horseradish peroxidase (HRP)-conjugated goat polyclonal antibody to armenian hamster (anti-armenian hamster; Jackson ImmunoResearch) at a dilution of 1:10,000.

Antibodies and immunoglobulin fusion proteins. The following mAbs were purchased from PharMingen: phycoerythrin-conjugated anti-I-E/A (M5/114.15.2), anti-CD4 (RM4-5), anti-ICAM-1 (3E2), anti-Ki67 (B56), CyChrome-conjugated anti-CD4 (H129.19), anti-CD11b (M1/70), allophycocyanin-conjugated anti-CD8α (53-6.7), anti-CD45.2 (104), anti-CD11c (HL3), biotin-conjugated anti-VCAM-1 (429) and purified anti-CD16/32 (2.4G2). Rabbit anti-GFP, fluorescein isothiocyanate–conjugated goat anti-rabbit, indocarbocyanine-conjugated goat anti-armenian hamster and Alexa Fluor 488–, Alexa Fluor 546– and Alexa Fluor 647–conjugated streptavidin were purchased from Molecular Probes. Biotin-conjugated and purified endotoxin-free monoclonal anti-IL-7Rα were purchased from eBioscience. The hamster mAb to RORγ and RORγt[19], the agonist mAb to LTβR, AF.H6 (ref. 12), and the immunoglobulin (Ig) fusion proteins LTβR-Ig, TNF-R-I-Ig[42] and TRANCE-R-Ig[43] have been described.

Flow cytometry. Single-cell suspensions were prepared from thymus, spleen, lymph nodes, bone marrow and liver. Total liver cells were resuspended in a 40% isotonic Percoll solution (Pharmacia) and underlaid with an 80% isotonic Percoll solution. Centrifugation for 20 min at 2,000*g* yielded the mononuclear cells at the 40–80% interface. Cells were washed twice with PBS-F (PBS containing 2% FCS), preincubated with mAb 2.4G2 to block Fcγ receptors, then washed and incubated with mAb conjugates for 40 min in a total volume of 100 μl PBS-F. Cells were washed, resuspended in PBS-F and analyzed on a FACScalibur flow cytometer (Becton-Dickinson). For cell cycle analysis of thymocytes, cells were fixed in 70% ethanol 30 min at 4 °C and washed with PBS-F, and 5×10^5 cells were incubated for 5 min at 37 °C with 12.5 μg/ml of propidium iodide (Sigma) and 50 μg/ml of RNAse A in 100 μl STE buffer (100 mM Tris base, 100 mM NaCl and 5 mM EDTA at pH 7.5). Cells were then washed, resuspended in PBS-F and analyzed.

Thymocyte survival assay. Thymocytes were isolated and cultured in DMEM supplemented with DMEM containing 10% FCS, 10 mM HEPES, 50 μM β-mercaptoethanol and 1% glutamine. After various periods of time, cells were stained with Annexin V (Pharmingen) and 1 μg/ml of propidium iodide to exclude dead cells and were analyzed by flow cytometry.

Immunofluorescence histology. Adult tissues or whole embryos were washed for several hours in PBS before being fixed overnight at 4 °C in a fresh solution of 4% paraformaldehyde (Sigma) in PBS. The samples were then washed 1 d in PBS, incubated in a solution of 30% sucrose (Sigma) in PBS until the samples sank, embedded in optimal cutting temperature compound 4583 (Sakura Finetek), frozen in a bath of hexane cooled with liquid nitrogen and stocked at –80 °C. Blocs were cut with a Microm HM500 OM cryostat (Microm) at a thickness of 8 μm (tissues) or 12 μm (embryos), and sections were collected onto Superfrost/Plus slides (Fisher Scientific). Slides were dried 1 h and processed for staining or were stocked at –80 °C. For staining, slides were first hydrated in PBS-XG (PBS containing 0.1% Triton X-100 and 1% normal goat serum; Sigma) for 5 min and were blocked for 1 h at room temperature with 10% goat serum and a 1:100 dilution of monoclonal anti-Fc receptor 2.4G2 in PBS-XG. Endogenous biotin was blocked with a biotin blocking kit (Vector Laboratories). Slides were then incubated overnight at 4 °C with primary polyclonal antibody or conjugated mAb (in general, at a 1:100 dilution) in PBS-XG, washed three times for 5 min each with PBS-XG, incubated for 1 h at room temperature with secondary conjugated polyclonal Ab or streptavidin, washed once, incubated for 5 min at room temperature with 4′,6-diamidino-2-phenylindole-2HCl (DAPI; Sigma), washed three times for 5 min each wash and mounted with Fluoromount-G (Southern Biotechnology Associates). Slides were examined with a Zeiss Axioplan 2 fluorescence microscope equipped with a charge-coupled device camera and were processed with Slidebook v3.0.9.0 software (Intelligent Imaging).

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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