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TNFα-dependent development of lymphoid tissue in the absence of RORγt^{+} lymphoid tissue inducer cells

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Lymphoid tissue often forms within sites of chronic inflammation. Here we report that expression of the proinflammatory cytokine tumor necrosis factor α (TNFα) drives development of lymphoid tissue in the intestine. Formation of this ectopic lymphoid tissue was not dependent on the presence of canonical RORgt^{+} lymphoid tissue–inducer (LTi) cells, because animals expressing increased levels of TNFα but lacking RORgt^{+} LTi cells (*TNF/Rorc(gt)*$^{-/-}$ mice) developed lymphoid tissue in inflamed areas. Unexpectedly, such animals developed several lymph nodes (LNs) that were structurally and functionally similar to those of wild-type animals. TNFα production by F4/80^{+} myeloid cells present within the anlagen was important for the activation of stromal cells during the late stages of embryogenesis and for the activation of an organogenic program that allowed the development of LNs. Our results show that lymphoid tissue organogenesis can occur in the absence of LTi cells and suggest that interactions between TNFα-expressing myeloid cells and stromal cells have an important role in secondary lymphoid organ formation.

INTRODUCTION

Lymphoid organs are critical for generation of adaptive immune response. Secondary lymphoid organs (SLO) are formed at predefined areas during embryogenesis, whereas tertiary lymphoid organs (TLO) are formed after birth in tissues with ongoing inflammatory processes.[1,2] Both secondary and TLO have lymphocytes that are topologically segregated and diverse sets of myeloid and stromal cells. In addition, they have specialized vasculature such as high endothelial venules (HEV) and a lymphatic network.

The two major cell types involved in lymph node organogenesis are the hematopoietic lymphoid tissue–inducer (LTi) cells and non-hematopoietic lymphoid tissue stromal "organizer cells" (LTo).[1] Clustering of LTi and LTo cells is an essential step in lymph node development.[3] Animals that are deficient in the nuclear retinoid orphan receptor (ROR)γ, encoded by the *Rorc* gene, or the negative regulator of basic helix-loop-helix protein signaling Id2 (inhibitor of DNA-binding 2), lack LTi cells and therefore fail to form lymph nodes (LNs) and Peyer's patches (PP).[4–6] The current model for the development of lymphoid organs posits that LTi cells originate in the fetal liver from common lymphoid progenitors and that they migrate to the sites where the LNs are formed (lymph node anlagen).[1,7] At these sites, binding of the tumor necrosis factor α (TNFα) family ligand receptor activator of nuclear factor (NF)-κB (RANKL) to its receptor RANK induces the differentiation and survival of LTi cells and trigger expression of lymphotoxin *a*1*b*2 (LTα1β2) on their surface.[3,8–11] A key step in the development of LNs is the engagement of LTα1β2 expressed by LTi cells to its receptor LTβR on mesenchymal organizer cells.[12,13] This interaction promotes upregulation of intercellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1) and mucosal addressin cell adhesion molecule (MAdCAM-1) on the surface of LTo cells[14,15] and the expression of the chemokines C-C motif chemokine ligand 19 (CCL19), CCL21, and C-X-C motif chemokine ligand 13 (CXCL13).[7]

Animals genetically deficient in LT-alpha and LTβR do not form LNs or PP.[10,12,16] Furthermore, genetic deletion of

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molecules in the LTβR signaling pathway (NF-κB non-canonical pathway) such as NF-κB-inducible kinase (NIK)[17] and RelB[18] precludes LN formation. Although the role of LTα1β2/LTβR is firmly established in the process of lymphoid organogenesis, the role of other members of the TNFα superfamily is unclear.

Female mice injected *in utero* with LTβR-immunoglobulin (Ig) fusion protein retain cervical and mesenteric LN (mLNs) but fail to form other LNs.[19,20] However, simultaneous treatment of LTβR-Ig fusion protein and anti-TNFR1 antibody, or LTβR-Ig plus anti-TNFα antibodies, prevents development of all LNs,[21] which suggests that TNFα has a role in mLN organogenesis. However, TNFα or TNF-R1-deficient mice have all LNs, including mLNs, but they fail to form B-cell follicles. These results suggest that TNFα activity in lymphoid organogenesis may be secondary to other TNFα members, such as LT. However, simultaneous deficiency of TNFR1 and RelA abrogates the development of all LNs, despite the presence of a normal complement of LTα1β2$^+$ LTi cells.[22] Thus, the role of TNFα in lymphoid organogenesis remains poorly defined.

Here we used *TNF*$^{\Delta ARE/+}$ mice, a well-established model of human inflammatory disease, to study the role of TNFα in lymphoid organogenesis. These animals express increased levels of TNFα under basal conditions, due to mutation in the 3′ region of the *Tnfa* gene that causes higher stability of its mRNA and, consequently, increased levels of TNFα protein.[23] Intercross of *TNF*$^{\Delta ARE/+}$ mice with *Rorc(γt)*$^{-/-}$ mice led to the generation of *TNF/Rorc(γt)*$^{-/-}$ mice. Surprisingly, *TNF/Rorc(γt)*$^{-/-}$ mice developed TLO and several SLO (mesenteric, axillary and cervical LN and others) despite the lack of the classical RORγt$^+$ LTi cells. Development of LNs was mechanistically linked to activation of stromal cells by TNFα produced by myeloid cells present in the anlagen and expression of molecules involved in lymphoid organogenesis. These results establish that lymphoid organogenesis can occur in the absence of *Rorc* if there is increased TNFα signaling.

RESULTS

Increased expression of TNFα promotes development of TLO in the absence of LTi cells

Two types of lymphoid aggregates can be identified in the intestine of adult mice: isolated lymphoid follicles and TLO. Isolated lymphoid follicles are genetically programmed clusters of B cells present at the base of the villi that require RORγt$^+$ LTi cells and LTβR signaling for their formation.[5,24–26] TLO are composed by large clusters of B220$^+$ cells that contain CD3$^+$ lymphocytes and are formed in response to infection or inflammation.[27,28] To further define the role of LTi cells and TNFα in the formation of lymphoid aggregates in the intestine, we examined the presence of these structures in the ileum of *TNF*$^{\Delta ARE/+}$ mice. The inflammatory infiltrates in the ileum are composed of neutrophils, macrophages, and T cells that are distributed throughout the submucosa and muscular layers and sometimes reach the serosa. Large mononuclear aggregates rich in B cells, or TLO, are also found in the terminal ileum of the *TNF*$^{\Delta ARE/+}$ mice.[29] To determine whether the formation of these aggregates is dependent on RORγt$^+$ LTi cells, we crossed *Rorc(γt)*$^{-/-}$ mice with *TNF*$^{\Delta ARE/+}$ mice to generate *TNF/Rorc(γt)*$^{-/-}$ mice. Histological analysis of the terminal ileum of age-matched wild-type (WT), *Rorc(γt)*$^{-/-}$, *TNF/Rorc(γt)*$^{+/+}$ and *TNF/Rorc(γt)*$^{-/-}$ mice at 16–20 weeks of age showed that *TNF/Rorc(γt)*$^{+/+}$ and *TNF/Rorc(γt)*$^{-/-}$ mice, but not WT or *Rorc(γt)*$^{-/-}$ mice, had marked submucosal inflammation, vilus blunting, patchy transmural inflammation, and lymphoid aggregates (**Figure 1a**). The lymphoid aggregates in *TNF/Rorc(γt)*$^{+/+}$ and *TNF/Rorc(γt)*$^{-/-}$ mice contained large clusters of B220$^+$ B cells and few CD3$^+$ T cells (**Figure 1b,c**), which were absent in *Rorc(γt)*$^{-/-}$ mice. These results indicate that TLO can be formed in the ileum in the absence of RORγt$^+$ LTi cells.

TNF/Rorc(γt)$^{-/-}$ mice develop SLO

Rorc is essential for the development of SLO.[5] As expected, no LNs were found in the *Rorc(γt)*$^{-/-}$ mice examined

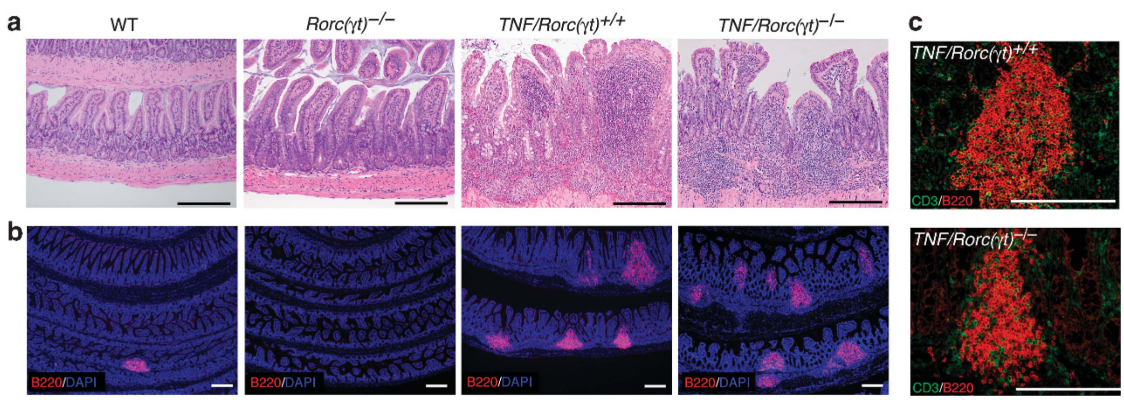

Figure 1 Tertiary lymphoid organs are formed in the ileum of *TNF/Rorc(γt)*$^{-/-}$ mice. (**a**) Representative hematoxylin and eosin sections of the Ileum of wild-type (WT), *Rorc(γt)*$^{-/-}$, *TNF/Rorc(γt)*$^{+/+}$, and *TNF/Rorc(γt)*$^{-/-}$ mice at 16 weeks. Notice the presence of inflammatory infiltrates in the ileum of *TNF/Rorc(γt)*$^{+/+}$ and *TNF/Rorc(γt)*$^{-/-}$ mice. (**b**) Ileum sections of indicated mice were stained with anti-B220 antibody to visualize B-cell aggregates and 4,6-diamidino-2-phenylindole (DAPI) for nuclear staining. Small B-cell clusters were found in the ileum of WT but were absent in the ileum of *Rorc(γt)*$^{-/-}$ mice. (**c**) Overexpression of tumor necrosis factor α (TNFα) induced the formation of large B-cell clusters with few T cells in the ileum of *TNF/Rorc(γt)*$^{+/+}$ and *TNF/Rorc(γt)*$^{-/-}$ mice. Bars = 250 μm, n = 4/group.

(**Figure 2a**). However, we were surprised to find that 100% of the *TNF/Rorc(γt)*$^{-/-}$ mice had fully developed mLNs that were grossly indistinguishable from those found in WT mice. Axillary (**Figure 2b**), cervical (**Figure 2c**), brachial, inguinal, para-aortic, and peripancreatic LN were also present at lower frequency (**Figure 2d**). Mediastinal and popliteal LN, as well as PP, were not observed in these animals.

To further characterize the structure of the LNs present in *TNF/Rorc(γt)*$^{-/-}$ mice, we performed immunostaining. LNs of WT and *TNF/Rorc(γt)*$^{-/-}$ mice had segregated T- and B-cell areas, PNAd^{+} HEV, an extensive lymphatic network, ER-TR7^{+} LN stroma, and CD35$^{\text{bright}}$ follicular dendritic cells (**Figure 2e**). To determine if these LN were functional we immunized *TNF/Rorc(γt)*$^{+/+}$ and *TNF/Rorc(γt)*$^{-/-}$ mice

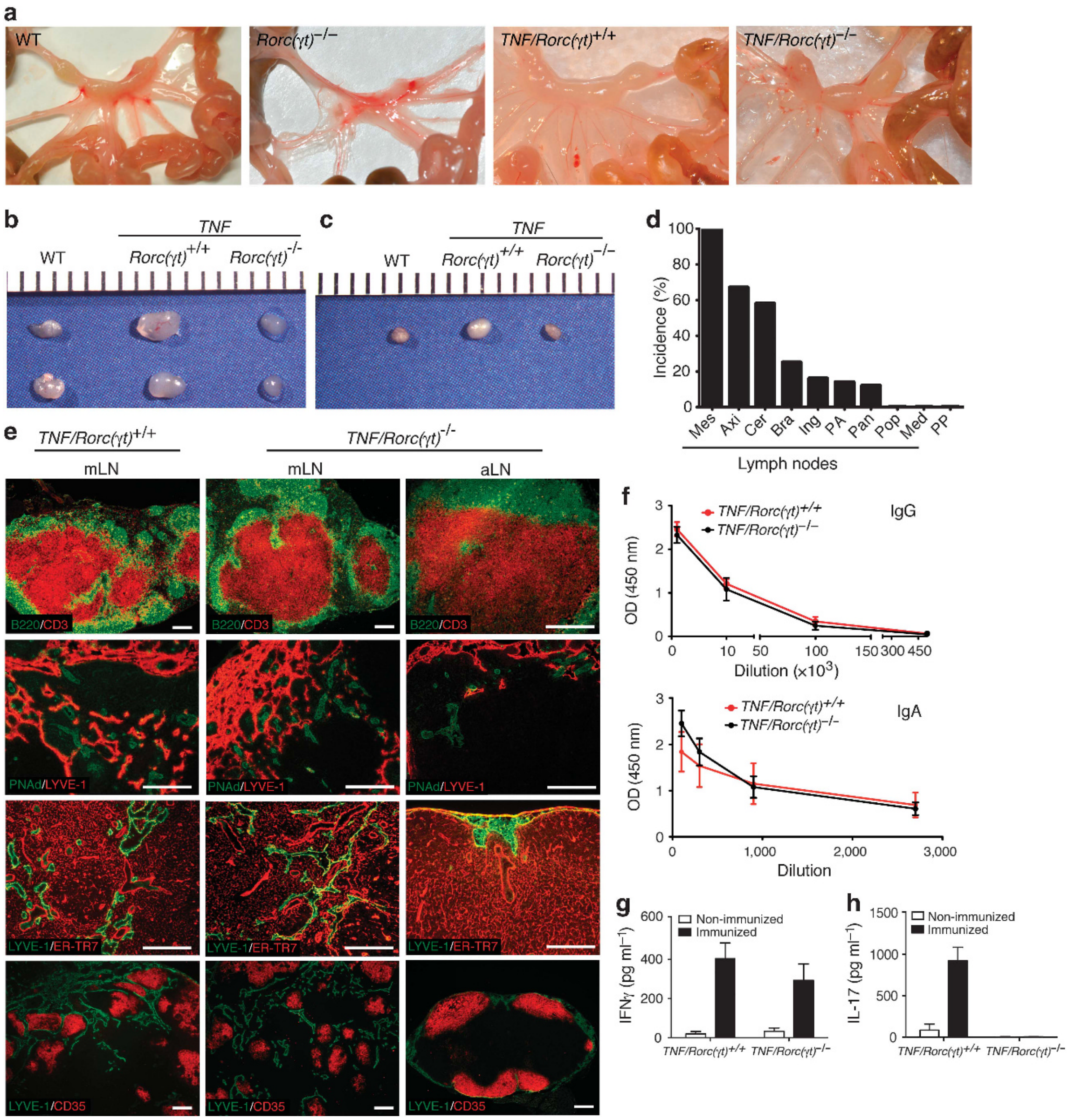

Figure 2 Increased expression of tumor necrosis factor α (TNFα) induces the development of secondary lymphoid organs in the absence of retinoid orphan receptor (ROR)γt^{+} lymphoid tissue-inducer (LTi) cells. (**a**) Photograph of the mesentery of wild-type (WT), *Rorc(γt)*$^{-/-}$, *TNF/Rorc(γt)*$^{+/+}$, and *TNF/Rorc(γt)*$^{-/-}$ mice. Photograph of (**b**) axilary and (**c**) cervical lymph nodes of WT, *TNF/Rorc(γt)*$^{+/+}$ and *TNF/Rorc(γt)*$^{-/-}$ mice. (**d**) Incidence of mesenteric (Mes), axillary (Axi), cervical (Cer), brachial (Bra), inguinal (Ing), para-aortic (PA), peripancreatic (Pan), popliteal (Pop), mediastinal (Med) lymph nodes, and Peyer's patches (PP) formed in *TNF/Rorc(γt)*$^{-/-}$ mice ($n=80$). (**e**) Lymph nodes from *TNF/Rorc(γt)*$^{+/+}$ and *TNF/Rorc(γt)*$^{-/-}$ mice at 6 weeks of age were analyzed by immunostaining. Note segregation of T- and B-cell areas, presence of PNAd^{+} high endothelial venules and lymphatic vessels, normal distribution of ER-TR7^{+} meshwork, and CD35$^{\text{bright}}$ FDC in mesenteric and axillary lymph nodes (mLN and aLN, respectively) of *TNF/Rorc(γt)*$^{-/-}$ mice. These features were similar to the ones observed in the mLNs of *TNF/Rorc(γt)*$^{+/+}$ mice ($n=5$ mice/group). Bars = 250 μm. (**f**) Ovalbumin (OVA)-specific immunoglobulin G (IgG) and IgA measured in the serum of *TNF/Rorc(γt)*$^{+/+}$ ($n=5$) and *TNF/Rorc(γt)*$^{-/-}$ ($n=4$) obtained after five rounds of immunization. (**g**) Interferon (IFN)-γ and (**h**) interleukin (IL)-17 levels in the supernatants of cultured mLN cells after 7 rounds of OVA immunization.

orally with ovalbumin (OVA) and cholera toxin seven times at 1-week intervals and assessed OVA-specific antibody serum titers by enzyme-linked immunosorbent assay (**Figure 2f**). The serum levels of OVA-specific IgA and IgG were similar between *TNF/Rorc(γt)*$^{+/+}$ and *TNF/Rorc(γt)*$^{-/-}$ mice, indicating that both strains responded to oral immunization with OVA. We next examined whether cells from the mLNs could produce cytokines after immunization. mLNs were collected and cultured with media alone or with 50 μg ml^{-1} of OVA. Supernatants were harvested 72 h later, and interferon-γ and interleukin (IL)-17 were measured by enzyme-linked immunosorbent assay. As shown in **Figure 2g**, similar levels of interferon-γ were produced by mesenteric LN cells of *TNF/Rorc(γt)*$^{+/+}$ and *TNF/Rorc(γt)*$^{-/-}$ mice. As expected, IL-17 was not detected in *TNF/Rorc(γt)*$^{-/-}$ cells as RORγt is required for IL-17 production (**Figure 2h**).[30] Collectively, these results indicate that increased expression of TNFα can drive the formation of SLO in the absence of RORγt^{+} LTi cells.

F4/80^{+}CD11b^{+} cells are the source of TNFα in the mLN anlagen

Our results suggested that a RORgt-independent cell type could have a role in the formation of SLO in *TNF/Rorc(γt)*$^{-/-}$ mice. To start addressing this hypothesis, we first examined the cellular composition of the mLNs of *Rorc(γt)*$^{-/-}$ and *TNF/Rorc(γt)*$^{-/-}$ mice at P0.5-P1. Very few lymphocytes were present to the mLN anlagen of *TNF/Rorc(γt)*$^{-/-}$ mice at this stage (**Figure 3a**). F4/80^{+}, NK1.1^{+}, and CD11c^{+} cells were the most abundant CD45^{+} leukocytes present in the mLN anlagen of *Rorc(γt)*$^{-/-}$ and *TNF/Rorc(γt)*$^{-/-}$ mice, but their relative proportions were comparable. CD11c^{+} cells in the mLNs of *Rorc(γt)*$^{-/-}$ and *TNF/Rorc(γt)*$^{-/-}$ mice did not express c-Kit (**Figure 3b**) and thus were distinct from the c-Kit^{+}CD11c^{+} lymphoid tissue initiator cells shown to be important in the formation of PP.[31] Further flow cytometric analyses showed that the F4/80^{+} cells comprised two populations: F480^{hi}/CD11b^{low}/MHC II^{neg}/CD11c^{-} and F4/80^{low}/CD11b^{hi}/MHC II^{pos}/CD11c^{+} cells (**Figure 3c**). These results indicate that there were no marked differences in the type and relative number of leukocytes in the mLN anlagen of *Rorc(γt)*$^{-/-}$ and *TNF/Rorc(γt)*$^{-/-}$ mice at P0.5-P1.

We asked next whether TNFα was expressed in the mLNs during development. TNFα expression was detected in the mLN anlagen of WT mice at steady state during embryogenesis (see **Supplementary Figure S1b** online). Next, we compared the levels of TNFα mRNA in mLN anlagen of *Rorc(γt)*$^{-/-}$ and

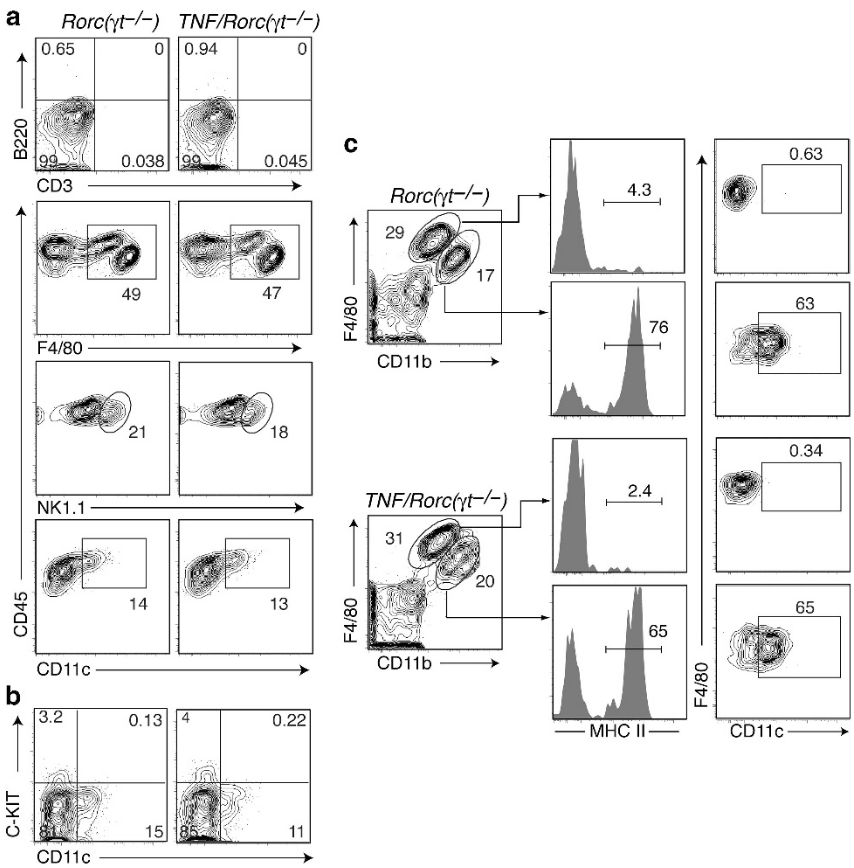

Figure 3 Phenotype of the cells present in the mesenteric lymph node (mLN) anlagen of *Rorc(γt)*$^{-/-}$ and *TNF/Rorc(γt)*$^{-/-}$ mice. (**a**–**c**) Cell suspensions from the mLN region of *RORγt*$^{-/-}$ and *TNF/Roc(γt)*$^{-/-}$ mice at P0.5-P1 stage were analyzed by flow cytometry for the expression of the indicated markers. Cells were gated on PI^{-}CD45^{+}. Representative plots of three independent experiments ($n = 2$–3/group). MHC, major histocompatibility complex.

TNF/Rorc(γt)$^{-/-}$ mice and found it to be upregulated in the latter at all embryonic and post-natal stages examined (see **Supplementary Figure S1a**). We then used flow cytometry to determine the cellular source of TNFα in the mLN anlagen. In WT mice, TNFα was detected in F4/80$^+$ myeloid cells as early as E15.5 in WT mLNs while TNFR1 was expressed in both myeloid and CD45$^-$ stromal cells (see **Supplementary Figure S1c**). Analysis of the mLN anlagen of P0.5-P1 *Rorc(γt)*$^{-/-}$ and *TNF/Rorc(γt)*$^{-/-}$ mice showed that TNFα was mainly produced by CD45$^+$F4/80$^+$ cells and not by other CD45$^+$ or stromal (CD45$^-$) cells (**Figure 4a**). A twofold increase in the production of TNFα by F4/80$^+$ cells was observed in the mLN anlagen of *TNF/Rorc(γt)*$^{-/-}$ mice. Further flow cytometric analyses showed that TNFα was expressed by both F4/80^{hi}/CD11b^{low} and F4/80^{low}/CD11b^{hi} cells (**Figure 4b**). Together, these results indicate that: (1) TNFα is physiologically expressed by F4/80$^+$ cells in the mLN anlagen of WT mice during embryogenesis, (2) that TNFα is expressed by F4/80$^+$ cells in both *Rorc(γt)*$^{-/-}$ mice *TNF/Rorc(γt)*$^{-/-}$ mice, and (3) that TNFα expression is increased during embryogenesis and early post-natal life in the mLNs of *TNF/Rorc(γt)*$^{-/-}$ mice compared with *Rorc(γt)*$^{-/-}$ mice.

TNFα does not bypass the requirement of ID2 for lymphoid organogenesis

Id2-deficient mice lack LTi cells,[6] NK cells,[6] and fetal CD11b$^+$ myeloid cells[5] in the LN anlagen and do not develop SLO. We had shown above that TNFα overexpression bypasses the requirement for *Rorc(γt)*$^+$ cells in SLO formation, thus we investigated next whether TNFα would bypass the requirement for *Id2* in LN organogenesis. To do so, we intercrossed TNF$^{\Delta ARE/+}$ mice with *Id2*$^{-/-}$ mice to generate *TNF/Id2*$^{-/-}$ mice (**Figure 5**). None of the *TNF/Id2*$^{-/-}$ mice examined at birth ($n=7$) had mLNs. We also examined the presence of F4/80$^+$ myeloid cells and found them to be present in the mLN anlagen of WT and *TNF/Id2*$^{+/-}$ mice but absent in *TNF/Id2*$^{-/-}$ mice (**Figure 5**, dashed lines), and in *Id2*$^{-/-}$ mice, in agreement with previous reports.[5] Myeloid cell migration to the mLN anlagen of *TNF/Id2*$^{-/-}$ mice was impaired and, strikingly, no LNs were formed in these mice. These results indicate that TNFα does not bypass the requirement for *Id2* in lymphoid organogenesis and suggest that TNFα-producing F4/80$^+$CD11b$^+$ cells or NK cells are important for the development of LNs in *TNF/Rorc(γt)*$^{-/-}$ mice.

NK cells are not critical for the development of mLNs in *TNF/Rorc(γt)*$^{-/-}$ mice

Because Id2-deficient mice have defective NK cell development,[6] it remained possible that NK cells had a role in the formation of SLO. To test this hypothesis, we first examined whether NK cells were present in the anlagen. As shown in **Figure 3**, NK cells were present in the mLN anlagen of *Rorc(γt)*$^{-/-}$ and *TNF/Rorc(γt)*$^{-/-}$ at P0.5-P1. To determine whether they had a role in SLO development, we depleted them

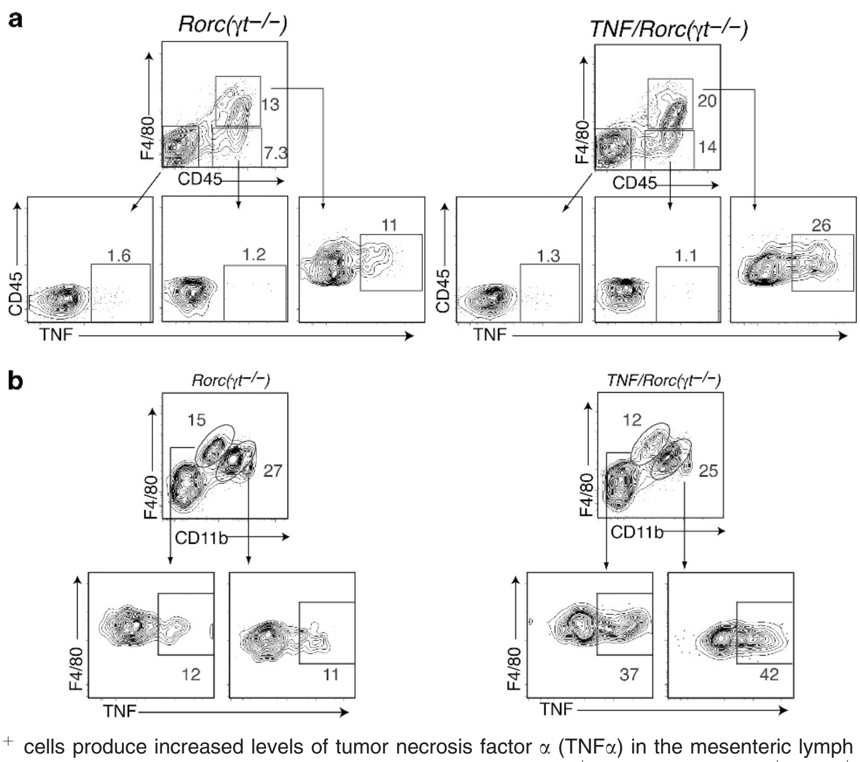

Figure 4 F4/80$^+$/CD11b$^+$ cells produce increased levels of tumor necrosis factor α (TNFα) in the mesenteric lymph nodes (mLNs) of *TNF/RORγt*$^{-/-}$ mice. (**a**) Flow cytometric analysis of TNF production by CD45$^-$, CD45$^+$F4/80$^-$, and CD45$^+$F4/80$^+$ cells isolated from the mLN region of *Rorc(γt)*$^{-/-}$, and *TNF/Roc(γt)*$^{-/-}$ mice at P0.5-P1 stage. (**b**) Analysis of TNF production by CD45$^+$F4/80^{hi}CD11b^{low} and CD45$^+$F4/80^{low}CD11b^{high} cells. Representative plot of two independent experiments, ($n=4$–5 animals/group).

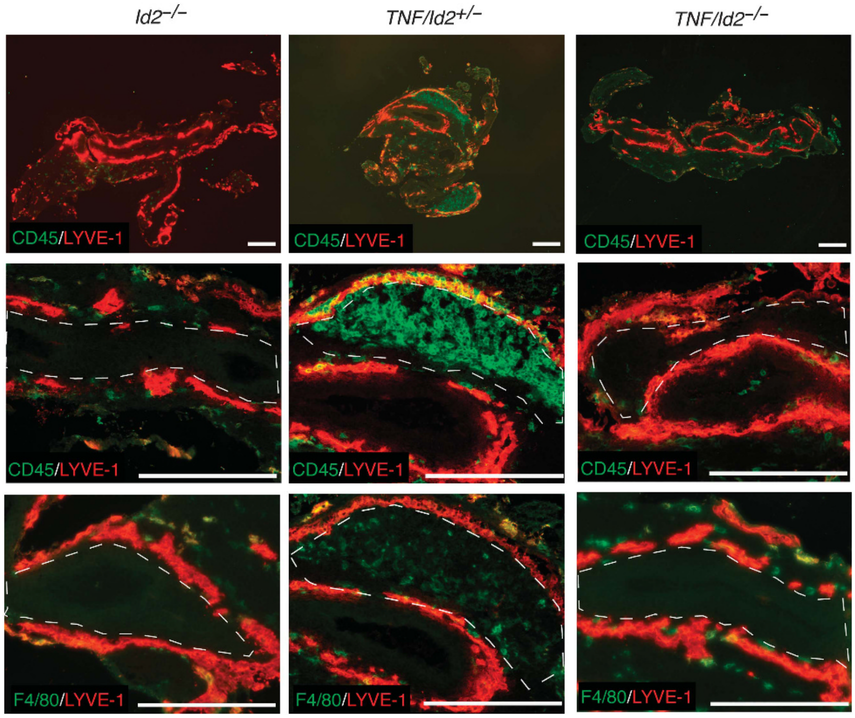

Figure 5 *TNF/Id2*$^{-/-}$ mice lack F4/80$^+$ cells in the mLN anlagen and do not develop secondary lymphoid organs. Mesenteric lymph node (mLN) region of *TNF/Id2*$^{+/-}$ and *TNF/Id2*$^{-/-}$ at P0.5-P1 stained with CD45, F4/80, and LYVE-1 antibodies. Notice the absence of F4/80$^+$ cells in the mLN region of *Id2*$^{-/-}$ and *TNF/Id2*$^{-/-}$ mice (dashed lines). Representative staining ($n = 3$/group). Bars = 250 µm.

from *TNF/Rorc(γt)*$^{-/-}$ mice. To do so, we injected pregnant mothers at E15 and E18 with 200 µg of isotype control or with the anti-NK monoclonal antibody PK136, which depletes NK cells *in vivo*. *TNF/Rorc(γt)*$^{-/-}$ offspring received additional injection of 100 µg of control or PK136 on days 0, 3, 6, and 9. On day 15, the mLNs were collected, and the number of NK cells and the formation of mLNs were examined (see **Supplementary Figure S2a,b**). Treatment of *TNF/Rorc(γt)*$^{-/-}$ mice with anti-PK136 caused a complete reduction in the number of NK cells in the mLNs (see **Supplementary Figure S2a**) but did not prevent the normal development of mLNs (see **Supplementary Figure S2b**). These results indicate that NK cells do not contribute significantly to SLO formation in *TNF/Rorc(γt)*$^{-/-}$ mice and suggest that the F4/80$^+$ cells are the important cells in the process, as they are the sole source of TNFα in the *TNF/Rorc(γt)*$^{-/-}$ LN anlagen.

TNFα triggers expression of several genes involved in lymphoid organogenesis

To determine how TNFα expression by myeloid cells could contribute to LN organogenesis, we compared the transcriptomes of the mLN anlagen of *Rorc(γt)*$^{-/-}$ and *TNF/Rorc(γt)*$^{-/-}$ mice at post-natal day 1 (P1), using the Ilumina gene arrays (**Figure 6a,b**). Consistent with a TNFα-driven signature, the highest expressed genes in the mLNs of *TNF/Rorc(γt)*$^{-/-}$ mice were acute-phase response genes (*Saa3* and *Serpina-3g*). Expression levels of several genes involved in lymphoid organogenesis, such as *Cxcl13*, *LTb*, *Relb*, *Ccl19*, and *Madcam-1*, were increased in the mLNs of *TNF/Rorc(γt)*$^{-/-}$ mice. Expression of macrophage-related genes (*Lyz2*, *Lyz1*, *csfr1*, *Mmp9*), MHC molecules (H2-M2), and chemokines (*Ccl5*, *Cxcl10*, and *Cxcl16*) were also increased in the mLNs of *TNF/Rorc(γt)*$^{-/-}$ mice. To validate and extend these findings, we performed quantitative PCR analysis (**Figure 6c**). Expression levels of *Cxcl13*, RANKL, and *Ltb*, were confirmed to be upregulated in the mLNs of *TNF/Rorc(γt)*$^{-/-}$ animals at different stages of post-natal development. Interestingly, transcripts for *LTa* were significantly upregulated in the mLNs of *TNF/Rorc(γt)*$^{-/-}$ mice after P1 but not at earlier time points. These results indicate that increased expression of TNFα promotes expression of genes involved in lymphoid tissue organogenesis during embryogenesis.

TNFα induces stromal cell maturation

Maturation of mesenchymal stromal cells into organizer cell is a key step in lymphoid organogenesis.[1] The existing evidence suggests that activation of the stromal cells is mediated by interaction of LTα1β2 present on the RORγt$^+$ LTi cells with LTβR expressed on stromal cells. This interaction leads to upregulation of ICAM-1, VCAM-1, and MAdCAM-1 expression on the surface of the stromal cells.[14,32] To examine whether stromal maturation to "organizer" cells could occur in the absence of LTi cells, we analyzed the presence of ICAM-1^{hi}VCAM-1^{hi} cells in the mLN region of *Rorc(γt)*$^{-/-}$ and *TNF/Rorc(γt)*$^{-/-}$ (**Figure 7a**) and WT (**Figure 7b**) mice by flow cytometry. Cells were gated in the CD45$^-$ stromal cell population. ICAM-

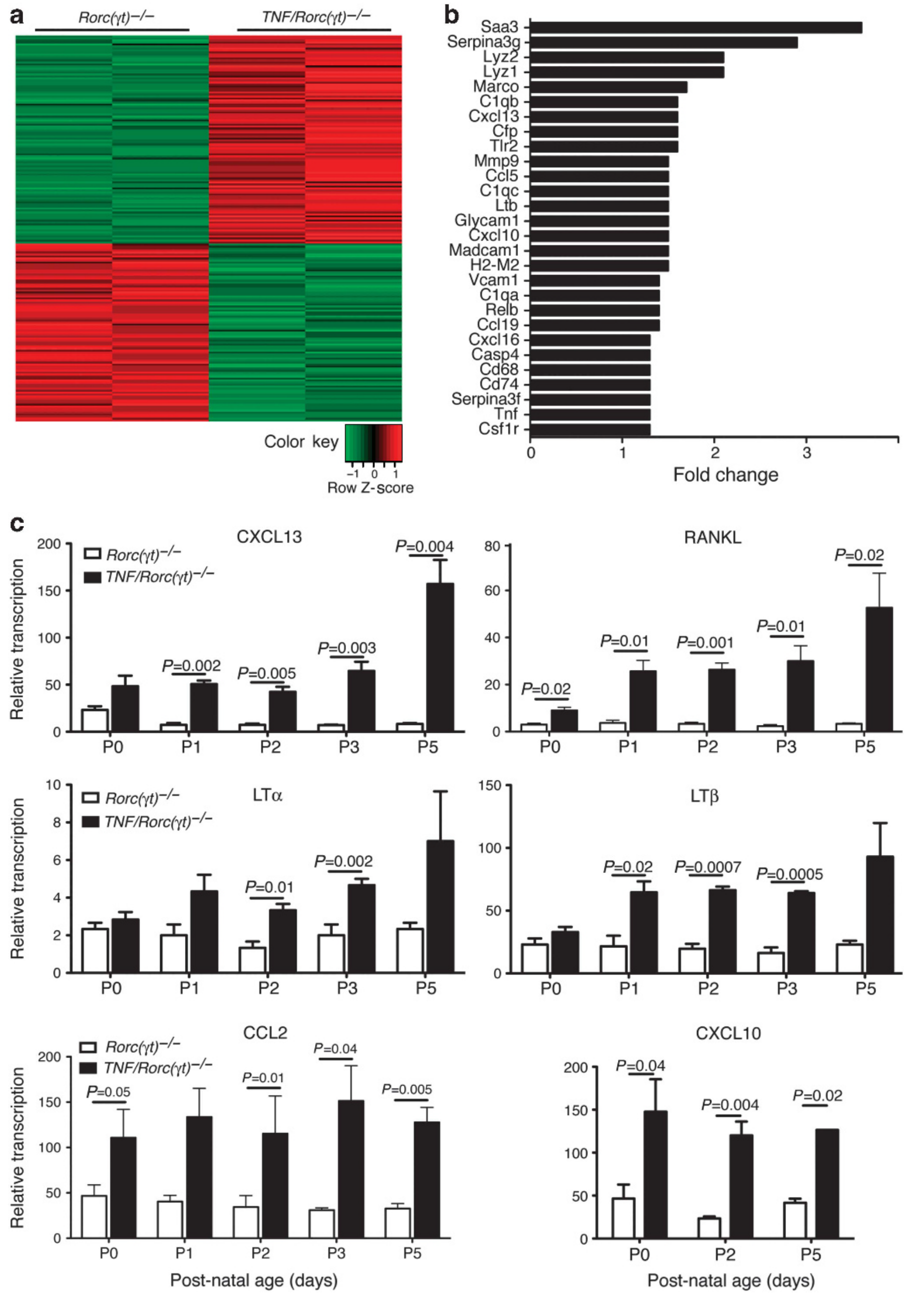

Figure 6 Increased expression of genes involved in lymph node organogenesis in the mesenteric lymph nodes (mLNs) of *TNF/Rorc(γt)*$^{-/-}$ mice. (**a**, **b**) Transcriptional profiles of mLN anlagen of *Rorc(γt)*$^{-/-}$ and *TNF/Rorc(γt)*$^{-/-}$ mice at P1. Samples (each column corresponds to a pool of 2–3 anlagen) were compared using MouseRef–8 v2.0 Expression BeadChip. Quantile-normalized expression values were filtered for $P<0.01$ and log fold change (logFC) > 1.25 (= fold 2.38). (**a**) Heatmap analysis sorted by logFC of the 193 resulting probe sets were Z-score normalized and subjected to hierarchical clustering; increased (red) and decreased (green) expression in *TNF/Rorc(γt)*$^{-/-}$ compared with *Rorc(γt)*$^{-/-}$ mice. (**b**) Fold change of selected upregulated genes. (**c**) Quantitative PCR analysis of the selected genes in the mLN region of *Rorc(γt)*$^{-/-}$ and *TNF/Rorc(γt)*$^{-/-}$ mice at different stages ($n=3$/group). CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; LT, lymphotoxin; RANKL, receptor activator of nuclear factor-κB ligand.

1^{hi}VCAM-1^{hi} cells were present at a significantly higher frequency in the mLNs of *TNF/Rorc(γt)*$^{-/-}$ mice at E18.5 onwards when compared with the same region of *Rorc(γt)*$^{-/-}$ mice[33] (**Figure 7a,c**). One day after birth, the frequency of ICAM-1^{hi}VCAM-1^{hi} cells in mLN stroma of *TNF/Rorc(γt)*$^{-/-}$ was higher than that of *Rorc(γt)*$^{-/-}$ mice but comparable with that of WT mice (**Figure 7b**). Another parameter of stromal cell activation is the production of chemokines. To examine whether the stromal cells from *TNF/Rorc(γt)*$^{-/-}$ mice produced chemokines, we sorted CD45^{-} cells from the mLN anlagen and performed quantitative PCR analyses. Sorted stromal cells (CD45-) from the mLN anlagen of *TNF/Rorc(γt)*$^{-/-}$ mice at P1 expressed increased levels of Cxcl13, Ccl19, and Ccl21 mRNA when compared with sorted stromal cells of *Rorc(γt)*$^{-/-}$ mice

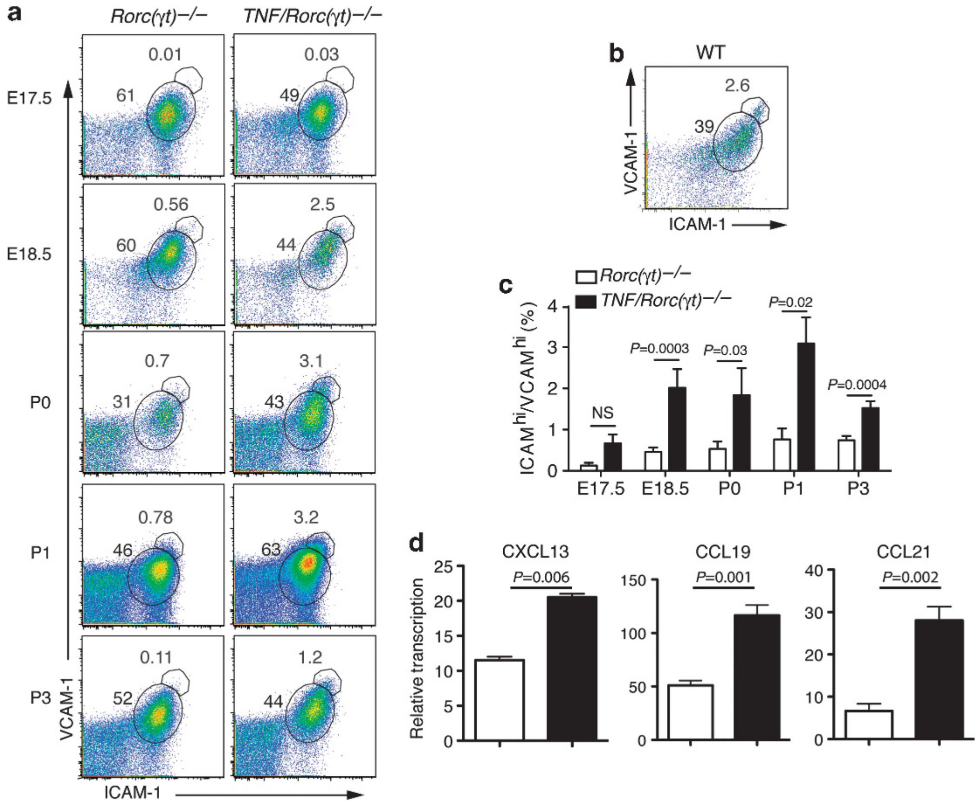

Figure 7 Tumor necrosis factor α (TNFα) induces stromal cell activation. (**a**) Fluorescence-activated cell sorting analysis of single-cell suspensions from the mesenteric lymph node (mLN) region of *Rorc(γt)*$^{-/-}$ and *TNF/Rorc(γt)*$^{-/-}$ mice at E17.5, E18.5, P0, P1, and P3 showing the increased expression of intercellular adhesion molecule 1/vascular cell adhesion molecule 1 (ICAM-1/VCAM-1) in the CD45-negative stromal cell population. (**b**) Expression of ICAM-1/VCAM-1 in stromal cell population in the mLNs of wild-type (WT) and *TNF*$^{\Delta ARE/+}$ mice at P1. (**c**) Relative number of ICAM-1^{hi}VCAM-1^{hi} cells in the CD45-negative stromal cells in the mLN region of *Rorc(γt)*$^{-/-}$ and *TNF/Rorc(γt)*$^{-/-}$ at E17.5, E18.5, and P0 ($n=4$ mice/group), P1 ($n=7$ mice/group), and P3 ($n=6$ mice/group). (**d**) Expression of C-X-C motif chemokine ligand 13 (CXCL13), CXCL19, and C-C motif chemokine ligand 21 (CCL21) in the stromal (CD45 negative) cell population sorted from the mLN region of *Rorc(γt)*$^{-/-}$ and *TNF/Rorc(γt)*$^{-/-}$ mice at P0.5-P1 ($n=9$–10 anlagen/group).

(**Figure 7d**). Taken together, these results indicate that increased levels of TNFα are sufficient to induce LN stromal cell maturation in the absence of LTi cells.

TNFα overexpression does not bypass the requirement for LTβR signaling in lymphoid organogenesis

The high expression levels of LTβR ligands in the mLNs of *TNF/Rorc(γt)*$^{-/-}$ mice after birth suggested a role for LT-LTβR signaling in the development of LN in this model. To determine whether this was indeed the case, we crossed *TNF*$^{\Delta ARE/+}$ mice with *LTβR*-deficient animals (referred to as *TNF/LTβR*$^{-/-}$ mice) (**Figure 8**). With the exception of mLNs, no LNs, PP, and TLO were found in any of the *TNF/LTβR*$^{-/-}$ mice animals examined (age 3–36 weeks, $n=19$) (**Figure 8a,b** and see **Supplementary Figure S3a**). The mLNs of *TNF/LTβR*$^{-/-}$ mice were markedly abnormal as shown by the absence of B-cell follicles and T-cell areas and the impaired recruitment of lymphocytes to these organs (**Figure 8c**). In addition, HEVs appear to be absent, and the number of lymphatic vessels is also reduced. Finally, they lacked CD35^{bright} FDC and had an aberrant ER-TR7^{+} stroma.

LTβR-deficient mice have severe splenic defects that include loss of T-/B-cell segregation and an abnormal stroma.[12] Interestingly, the spleen of *TNF/LTβR*$^{-/-}$ mice displayed normal T-/B-cell distribution, had MAdCAM-1^{+} cells, and a normal ER-TR7^{+} cell network (see **Supplementary Figure S3b**). Thus, TNFα overexpression can compensate for the absence of LTβR signaling and promote development of an organized spleen. Together, the results indicate that TNFα overexpression corrects the splenic defects but not the lack of SLO associated with abrogation of LTβR signaling.

Influx of hematopoietic cells into the anlagen of *TNF/Rorc(γt)*$^{-/-}$ mice

To gain further insights into the mechanisms of SLO formation, we compared the kinetics of hematopoietic cell recruitment with the mLN anlagen of WT, *Rorc(γt)*$^{-/-}$, and *TNF/Rorc(γt)*$^{-/-}$ mice. A significant number of CD45^{+} cells were present in the mLNs of WT mice at P0, whereas very few cells were detected in the mesenteric area of both *Rorc(γt)*$^{-/-}$ and *TNF/Rorc(γt)*$^{-/-}$ mice (**Figure 9**). In contrast, by P5 the mLN anlagen of *TNF/Rorc(γt)*$^{-/-}$ mice appeared to be populated by CD45^{+} cells, whereas almost no bone

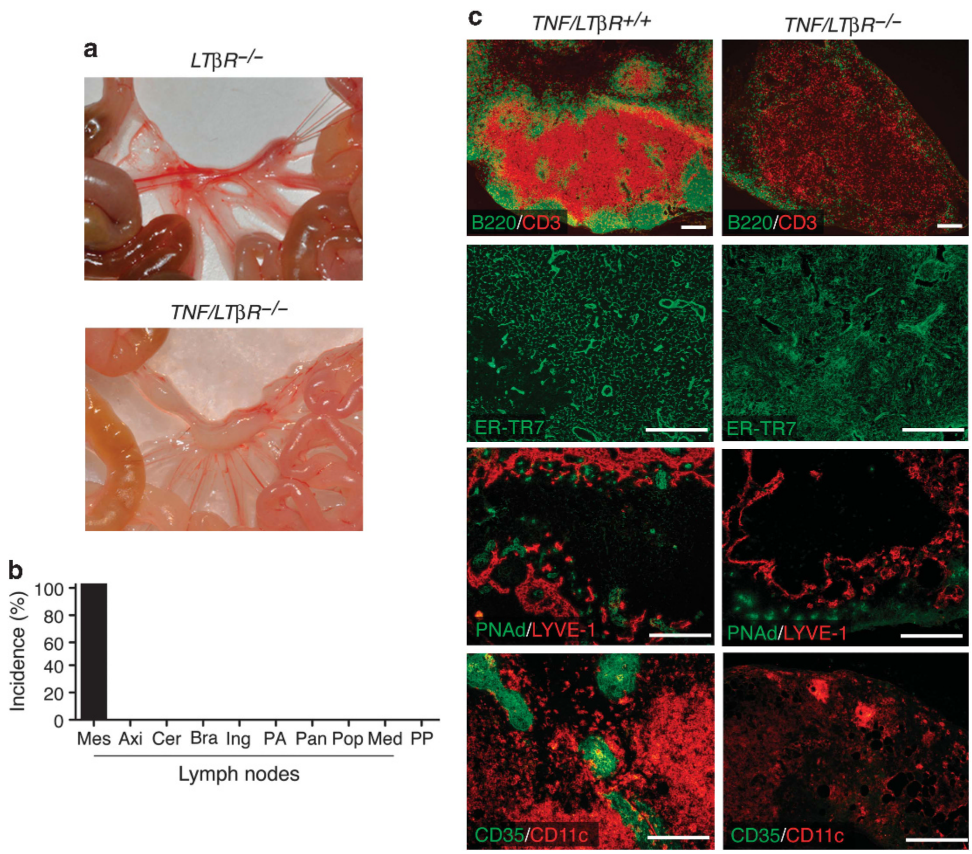

Figure 8 Tumor necrosis factor α (TNFα)-driven formation of most secondary lymphoid organs (SLO) is dependent on lymphotoxin βR (LTβR) signaling. (**a**) Mesentery of *LTβR*$^{-/-}$ and *TNF/LTβR*$^{-/-}$ mice. (**b**) With the exception of mesenteric lymph nodes (mLNs), SLO were absent in *TNF/LTβR*$^{-/-}$ mice ($n=16$). (**c**) Abnormal organogenesis of mLNs in *TNF/LTβR*$^{-/-}$ mice. mLNs of *TNF/LTβR*$^{+/+}$ and *TNF/LTβR*$^{-/-}$ mice were stained with the indicated antibodies. Notice the lack of distinct T- and B-cell areas, absence of PNAd + high endothelial venules and reduced lymphatic vasculature, lack of CD35^{+} FDC, and disorganized ER-TR7 stroma in the mLNs of *TNF/LTβR*$^{-/-}$ mice. Representative staining ($n=5$/group). Bars = 250 μm. Axi, axillary; Bra, brachial; Cer, cervical; Ing, inguinal; Med, mediastinal; Mes, mesenteric; PA, para-aortic; Pan, peripancreatic; Pop, popliteal; PP, Peyer's patches.

marrow derived cells were present in a similar area in *Rorc(γt)*$^{-/-}$ mice. Consistent with our flow cytometric data, F4/80^{+} cells were present within the anlagen of WT and *TNF/Rorc(γt)*$^{-/-}$ mLNs, and their frequency was proportionally increased in the latter strain.

Grafting of *TNF/Rorc(γt)*$^{-/-}$ anlagen under the kidney capsule induces lymphoid neogenesis

Grafting of re-aggregates of embryonic and neonatal RORγt^{+} LTi cells and LTo cells under the kidney capsule of adult mice promotes the formation of structures that resemble LNs that recruit and organize host T and B cells.[34] At P0, the mLNs of WT mice contain mostly LTi cells and myeloid cells and are not organized. Kidney grafts of WT mLNs result in organized tissues populated by host lymphocytes only 2–3 weeks after grafting. To test whether the anlagen of *TNF/Rorc(γt)*$^{-/-}$ mice could promote development of LN-like structures in adult mice, we grafted them under the kidney capsule of *Rorc(γt)*$^{-/-}$ recipient mice (**Figure 10**). Three weeks later, the kidneys were removed and processed for histological analysis. All (100%) animals transplanted with WT mLN anlagen ($n=3$) and 71% of those transplanted with *TNF/Rorc(γt)*$^{-/-}$ anlagen ($n=14$) developed lymphoid aggregates under the kidney capsule. These aggregates contained segregated T- and B-cell areas, HEV, and lymphatic vessels and were similar to host LN. We conclude that mLN anlagen of *TNF/Rorc(γt)*$^{-/-}$ mice can promote the formation of lymphoid organs at a non-predestined site in adult mice in the absence of RORγt^{+}LTi cells and that this response is not dependent on systemically increased levels of TNFα.

DISCUSSION

RORγt is a transcription factor encoded by the *Rorc* gene whose expression is critical for the development of embryonic LTi cells and other types of group 3 innate lymphoid cells.[35] Our results show that lymphoid organogenesis can occur in the absence of *Rorc*, provided that there is increased expression of TNF. Formation of most LNs under these circumstances is dependent on LTβR signaling.

A body of work supports the notion that SLO and TLO development share common mechanisms. However, evidence first derived from the analysis of CCL21-driven transgenic

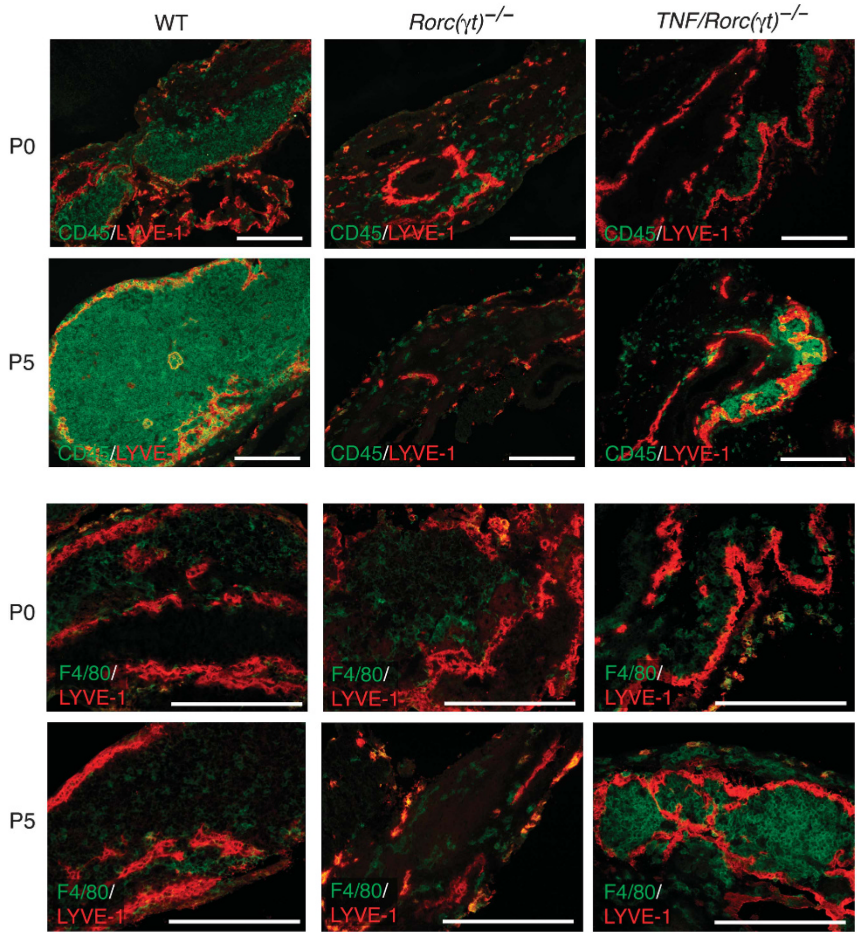

Figure 9 Hematopoietic cell influx into the mesenteric lymph node (mLN) anlagen. Sections of the mLN region of wild-type (WT), *Rorc(γt)*$^{-/-}$, and *TNF/Rorc(γt)*$^{-/-}$ (P0 and P5) mice were stained with anti-CD45, -F4/80, and -LYVE-1 antibodies. Representative staining ($n = 3$/group). Bars = 250 μm.

models[36] and other models[37] suggested that their development differs, as canonical LTi cells, critical for SLO development, were shown not to be absolutely required for TLO development. Here we show that the generation of TLO in the ileum of *TNF*$^{\Delta ARE/+}$ animals is also independent of RORγt^{+} LTi cells. Our results complement those of Eberl and colleagues who showed that *Rorc(γt)*-deficient mice that received an inflammatory insult, such as DSS (dextran sodium sulfate)-induced colitis, develop TLO in the colon.[27] Together, these studies demonstrate that inflammatory stimuli promote development of TLO in different areas of the intestine (ileum and colon) in the absence of RORγt^{+} LTi cells and implicate TNFα as an important factor in their generation, as its expression is elevated in the ileum of *TNF*$^{\Delta ARE/+}$ mice and in the colon of DSS-treated animals.[29,38] Importantly, the development of TLO in the intestine of *TNF/Rorc(γt)*$^{-/-}$ mice also demonstrates that the formation of these organs is independent of Th17^{+} cells and other *Rorc(γt)*-dependent members of the growing family of innate lymphoid cells.

Although recent experimental evidence supports the concept that TLO formation can occur in the absence of canonical LTi cells, the bulk of the literature suggests that they are critical for the development of SLO. The current model for SLO formation suggests that Id2^{+}RORγt^{+}CD3^{-}CD4^{+}IL-7Rα^{+} LTα1*b*2^{+} RANK^{+}RANKL^{+} LTi cells are key drivers of lymphoid organogenesis based on the fact that *Id2*-, *Rorc*-, *IL-7Ra*-, and *LTa-deficient* mice lack SLO.[1,39] Exceptions to this rule include nasal associated lymphoid tissue, whose formation takes place after birth and is not dependent on LTα and *Ror(c)*,[40] and milky spots of the omentum and fat-associated lymphoid clusters are also independent of LTi cells.[41] Here we show that many SLO can form in the in absence of RORγt^{+}LTi cells, provided that the basal levels of TNFα are increased. Mesenteric, axillary, and cervical LNs were found in 60–100% of *TNF/Rorc(γt)*$^{-/-}$ mice. Other LNs such as brachial, inguinal, para-aortic, and peripancreatic were found in >10% of the mice, whereas popliteal and mediastinal LNs were not detected. The LNs detected in *TNF/Rorc(γt)*$^{-/-}$ mice were positioned in the same region as WT nodes, had similar architecture and cellularity, and could mount an efficient immune response after immunization.

How could TNFα promote organogenesis in the absence of LTi cells? Here we show that TNFα is produced at higher levels during embryogenesis in the *TNF/Rorc(γt)*$^{-/-}$ than in

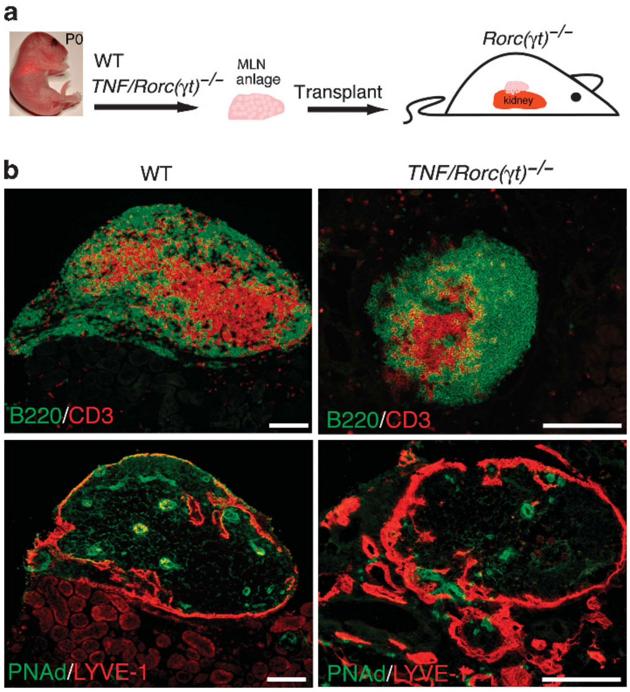

Figure 10 Mesenteric lymph node (mLN) anlagen from wild-type (WT) and *TNF/Rorc(γt)*$^{-/-}$ mice promote development of LN-like structures when grafted under the kidney capsule. (**a**) Schematic representation of the transplantation experiment. (**b**) mLN anlagen isolated from WT ($n=3$) and *TNF/Rorc(γt)*$^{-/-}$ ($n=14$) newborn mice were grafted under the kidney capsule of *Rorc(γt)*$^{-/-}$ mice. Notice normal segregation of T and B cells and development of PNAd^{+} high endothelial venules from WT and *TNF/Rorc(γt)*$^{-/-}$ grafts. Bars = 250 μm.

Rorc(γt)$^{-/-}$ mice and that the TNFR1 receptor is expressed by stromal cells. Stromal cell activation by LTi cells is critical for the generation of LNs. LTα1β2 expressed by LTi cells binds to LTβR expressed by stromal cells, which activates both canonical and non-canonical NF-κB signaling pathways to promote the latter cells to become mature stromal "organizer" cells that express increased levels of ICAM-1, VCAM-1, and MAdCAM-1 and the B- and T-cell chemoattractants CXCL13, CCL19, and CCL21.[1,39] We suggest that increased levels of TNFα functionally compensated for the lack of LTβR signaling during embryogenesis and contributed to the maintenance of a functional anlagen. This hypothesis is supported by our observations that stromal cells in the anlagen of *TNF/Rorc(γt)*$^{-/-}$, but not in the *Rorc(γt)*$^{-/-}$ mice, are activated. They express higher levels of ICAM-1 and VCAM-1 during embryogenesis and immediately after birth. Furthermore, cells in the *TNF/Rorc(γt)*$^{-/-}$ anlagen expressed increased levels of the TNFα-inducible chemokines CCL2 and CXCL10. These chemokines, acting in concert with TNFα, could promote recruitment of additional hematopoietic cells. At birth, influx of hematopoietic cells could further contribute to organogenesis. Interestingly, we noted that the expression of LT ligands increased after birth. This could reflect either increased expression of LT ligands by resident non-LTi cells or reflect increased influx of hematopoietic cells that express LT ligands. We favor the second hypothesis, because we have observed increased influx of hematopoietic cells in the *TNF/Rorc(γt)*$^{-/-}$ anlagen during the first 5 days of life. The increased expression of LT ligands is absolutely critical for normal LN development as shown by the analysis of the TNF/LTβR mutants. In the newborn *TNF/Rorc(γt)*$^{-/-}$ anlagen, LT ligands could potentially synergize with TNFα to activate the transcription of several molecules related to LN organogenesis, macrophage function, and inflammation. A recent report has shown the synergistic effect of TNFα signaling together with the alternative NF-κB pathway to drive high expression levels of *Ccl21*, *Cxcl13*, *Vcam1*, *Icam1*, and *Madcam1* in the spleen.[42] Notably, we have detected expression of Cxcl13, Ccl19, and Ccl21 by stromal cells located in the mLNs of *TNF/Rorc(γt)*$^{-/-}$ mice at P1. These chemokines induce lymphoid organogenesis when expressed *in vivo*,[43–45] and their expression by stromal cells in the anlagen could account for the formation of LN in *TNF/Rorc(γt)*$^{-/-}$ mice.

Myeloid cells are essential for the maintenance,[46] organization,[47] and vascularization[48] of TLO. Results presented here suggest that they may also contribute to development of SLO, working in part as LTi-like cells. We show here that TNFα is expressed by myeloid cells present in the anlagen of WT mLNs as early as E15.5. Clusters of fetal CD11b^{+} cells and LTi cells are observed at early stages of WT LN development.[5] The generation of the CD11b^{+} cells does not require RORγt, because these myeloid cells are still present in the LN anlagen of *Rorc(γt)*$^{-/-}$ mice, as shown here and in Eberl *et al.*[5] The increased number of myeloid cells that express stable *TNFa* mRNA contribute to high levels of this protein in the LN anlagen in *TNF/Rorc(γt)*$^{-/-}$ mice. Consistent with a role for myeloid cells contributing to LN development in the *TNF/Rorc(γt)*$^{-/-}$ mouse model is the observation that absence of these cells (and thus the source of TNFα necessary to activate the stromal cells) in LN anlagen of *TNF/Id2*$^{-/-}$ mice resulted in the failure to rescue the formation of these organs.

Our results indicate that the development of LNs in TNF/*Rorc(γt)*$^{-/-}$ mice appears to be delayed when compared with their WT counterparts. In addition, it is not clear why PP and some LNs do not develop in this mouse model. The development of these structures may be dependent on the local numbers or phenotype of myeloid cells in those locations. Additional research will be necessary to uncover the origin of these myeloid cells and the factors mediating their influx into different LN anlagen.

TNFα receptor ligation activates the NF-κB classical pathway, which involves the IκB kinase and results in the activation of RelA. LTβR ligation activates both the NF-κB classical and alternative pathways.[18] The alternative NF-κB pathway is mediated by the NIK and results in the activation of NF-κB2/Relb. Because animals genetically deficient in *LtbR*, *Nik*, and *RelB* do not form LNs,[12,17,18] it was concluded that the alternative pathway is critically important for the generation of SLO.[18] However, simultaneous deletion of TNFR1 and RelA precludes formation of all LN and PP in double knockout mice due to a stromal cell defect, even in the presence of LTi cells expressing normal levels of LT,[22] which suggests that the canonical NF-κB pathway is physiologically important for normal development. It is clear, however, that LTβR signaling has a profound effect in the generation of most LN and intestinal TLO, a role that cannot be bypassed even in the presence of increased levels of TNFα. Although increased TNFα cannot compensate for the lack of LTβR in terms of TLO and SLO development, it can partially compensate for lack of LTβR signaling in the development of mLNs. Finally, as shown here, increased TNFα expression can correct the splenic defects associated with lack of LTβR. These results are in agreement with studies that show that TNFα overexpression can correct splenic defects associated with LTα deficiency.[49,50] Taken together, the studies highlight a significant cross-talk between these receptor systems for the development and function of lymphoid structures.

In summary, our results support a model of LN development in *TNF/Rorc(γt)*$^{-/-}$ mice where increased expression of TNFα by F4/80^{+}CD11b^{+} cells is sufficient to promote the homeostasis of LN stromal cells up to early post-natal life. After birth, the recruitment of lymphoid cells and myeloid cells to the anlagen initiates a series of cross-talk interactions with stromal cells through LTα-LTβR signaling that induces the expression of chemokines and cell adhesion molecules to organize specific lymphoid areas and attract further cells to form the proper LN structure containing HEVs and lymphatic vasculature. Failure of signaling through LTβR in early post-natal life results in the collapse of the anlagen of most LNs with the exception of mesenteric LN that present with a disrupted architecture as shown in *TNF/LTβR*$^{-/-}$ mice. Thus our results show that lymphoid tissue organogenesis can occur in the absence of Rorγt^{+} LTi cells and suggest that interactions between TNFα-expressing myeloid cells and stromal cells have an important role in this process.

METHODS

Mice. *TNF*$^{\Delta ARE/+}$ and *LTβR*$^{-/-}$ mice have been described.[12,23] Id2$^{-/-}$ mice were a generous gift from Dr Y. Yokota (University of Fukui, Fukui, Japan).[6] C57BL6/J and *Rorc(γt)*$^{-/-}$ mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and bred in our facility. All mice were housed under specific-pathogen-free conditions in individually ventilated cages at the Mount Sinai School of Medicine Animal Facility. All experiments were performed following institutional guidelines. For timed pregnancies, the day of vaginal plug was considered as E0.5.

Immunostaining. Sections of frozen tissues were subjected to immunofluorescent staining as described[36] (for details, see **Supplementary Procedures**).

Cell isolation and flow cytometry. The area of the mesentery corresponding to where mLNs are found in WT mice was microdissected from *Rorc(γt)*$^{-/-}$ and *TNF/Rorc(γt)*$^{-/-}$ animals and analyzed by flow cytometry (for details, see **Supplementary Procedures**).

***In vivo* immunization.** *TNF/Rorc(γt)*$^{+/+}$ and *TNF/Rorc(γt)*$^{-/-}$ mice at 6–8 weeks were immunized with OVA (grade V; Sigma-Aldrich, St Louis, MO) by intragastric gavage of 100 μg of OVA + 20 μg cholera toxin (List Biological Laboratories, Campbell, CA) on seven occasions at 7-day intervals. One week after the last immunization, mice were killed, and the mLN was collected for cytokine analysis (for details, see **Supplementary Procedures**).

Analysis of mRNA expression. Total RNA was extracted from mesenteric region using the RNeasy mini Kit (Qiagen, Valencia, CA) as described[36] (for details, see **Supplementary Procedures**).

Microarray analysis. Microarrays were done with the Illumina TotalPrepTM RNA Amplification Kit (for details, see **Supplementary Procedures**).

Statistics. Statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA). Differences among means were evaluated by a two-tailed *t* test. $P<0.05$ was considered significant. All results shown represent mean ± s.e.m.

Transplantation of mLN anlagen. *Rorc(γt)*$^{-/-}$ mice (6–8 weeks) were anesthetized with ketamine/xylazine solution. A small incision in the skin and peritoneum was made in order to expose the kidney. A slight pressure to both sides of the incision was applied in order to exteriorize the kidney. A small nick in the kidney capsule was created using a 25-gauge needle, and the mLN anlagen was placed into the kidney capsule pocket created in the nick area. The peritoneum and skin were stitched using 5-0 silk sutures with a C-6 19-mm needle. Formation of LNs under the kidney capsule was assessed by histology 3 weeks after transplantation.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE
The authors declared no conflict of interest.

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