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The IL-7 Signaling Pathway Regulates Lymph Node Development Independent of Peripheral Lymphocytes

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Lymph node (LN) organogenesis is initiated by the interaction between hematopoietic lymphoid tissue inducer (LTi) cells and the mesenchymal organizer cells. Mice in which the IL-7 signaling pathway has been disrupted have a severe defect in LN development; however, the reasons underlying this defect are as yet unknown. In this study, we show that the overexpression of thymic stromal lymphopoietin (TSLP) increased LTi cell numbers and restored LN development in IL-7−/− and RAG2−/− γc−/− mice. The TSLP-mediated LN restoration was strictly dependent on LTi cells and independent of lymphocyte colonization. Increased LTi cell numbers in the LN anlagen of RAG2−/− γc−/− TSLP transgenic mice were associated with the restoration of organizer cells, suggesting that LTi cell number is a critical parameter for LN organogenesis. Our results shed light on the minimal cellular requirement for LN development during ontogeny. We show that the presence of LTi and organizer cells, but not of peripheral lymphocytes, is critical for LN development and persistence and further suggest that the IL-7 signaling pathway regulates LN organogenesis by controlling the size of the LTi cell pool. The Journal of Immunology, 2010, 184: 000–000.
Flow cytometry
FITC-, PE-, PE/Cy7-, allophyocyanin-, or biotin-conjugated αCD4 (GK1.5), α-CD8a (53-6.7), α-CD19 (ID3), α-NK1.1 (PK136), α-CXCR4 (2B11), α-CXCR5 (2G8), α-e487 (DATK32), and α-ICAM-1 (3E2) Abs were purchased from BD Biosciences. α-CD3 (145-2C11), α-CD11a (M17/4), α-CD11b (M170), α-CD44 (IM7), α-CD62L (MEL14), α-CD69 (H1.2F3), α-B220 (RA3-6B2), α-Ter119 (Ter-119), α-4-integrin (R1-2), α-β1 integrin (HMb1-1), and α-β2 integrin (M18/2) Abs were purchased from Biolegend. α-CD11c (N418), α-CD45 (30-F11), α-CD45.1 (A20), α-κlt (2B8), α-CD122 (TM-b1), α-IL-7R (A7R34), α-VCAM-1 (429), α-Gr1 (RB6-8C5), and α-Flt3 (A2F10) Abs were purchased from eBioscience (San Diego, CA). As secondary reagent, streptavidin-PE and streptavidin–PE/Cy7 (Biolegend) were used. Flow cytometry acquisition was performed with a FACScalibur (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

For LTi cell number quantification, mesenteric LN (mLN) and spleen absolute cell numbers were obtained from analysis.

LT staining was performed on cell suspensions from individual organs as described previously (15). Briefly, cells were treated with α-Fc 2.4G2 Ab and 0.5% mouse and rat serum. LTBr−Fc (29) was added and detected using Biot–goat α-human IgG (Jackson Research Laboratories), pretreated for 30 min with 4% rat and mouse serum. Finally, streptavidin and surface Abs were added. Cell suspensions from individual organ genotype were pooled and analyzed.

For organ cell staining, inguinal LNs (iLNs) of 2.5-d-old mice were digested with 1 mg/ml dispase (Life Technologies) together with 100 µg/ml DNase I (AppliChem) in PBS at 37°C for 20–30 min under mild agitation. Cell suspensions were then filtered and stained on ice.

Adoptive transfer experiments
For fetal liver (FL) chimeras, IL-7−/− K14-TSLP Tg mice, IL-7−/− littermates, and C57BL/6 wild-type (WT) mice were lethally γ-irradiated (9 Gy) and i.v. injected with 10^6 total FL cells from 12.5 dpc embryos and 0.5-d-old mice. Each symbol represents LTi cell number from an individual animal.

FIGURE 1. Increased TSLP availability restores LN development in IL-7−/− mice. A. Quantification of LN in IL-7−/− and IL-7−/− K14-TSLP Tg littermates. Eight to 11 mice were analyzed per group. B. Aortic region of IL-7−/− (i) and IL-7−/− K14-TSLP Tg (ii) mice. ILNs are indicated by arrow heads. C. Absolute cell number of B220+ CD19+ B cell, NK1.1+ NK cell, and CD4+ and CD8+ T cells in the spleen of 4.5-d-old IL-7−/−, IL-7−/− K14-TSLP Tg, and WT mice. Shown are the mean values and SD from analyzing five animals per group. D. Representative FACS plots of IL-7−/−, IL-7−/− K14-TSLP Tg, and WT newborn spleens (0.5 d old). Gates indicate CD4+ IL-7Rα+ LTi cells. Numbers are mean and SD of percentages. Absolute CD4+ CD3− IL-7Rα+ LTi cell number in spleen (E) and mLN (F) of IL-7−/− (circles, n = 44), IL-7−/− K14-TSLP Tg (triangles, n = 17), and WT (diamonds, n = 40) 0.5-d-old mice. Each symbol represents LTi cell number from an individual animal. The mean values are indicated by a bar. G, LTIαβ2 level expression of WT CD19+ B cells (shaded), LTi cells from newborn IL−/− (plain line), and IL-7−/− K14-TSLP Tg (dotted line) mice in spleen and mLN.

Immunoﬂuorescence microscopy
Eight-micrometer acetone-fixed LN sections were incubated with combinations of α-ER−T7 (ER-T7; AbD Serotec), α-PNA (MECA-79; Pharmingen), α-Ly-1 (RELIXtech), α-CXCL13 (R&D Systems, Abingdon, U.K.), α-CCL19 (R&D Systems), biot-CDR1 (8C12, Pharmingen), and biot-CD31 (390; ebioscience) Abs. α-ER−T7 and α-PNA were detected with goat α-rat-Cy3 (Jackson Immunoresearch Laboratories), α-CXCL13, and α-CCL19 with a donkey α-goat Alexa Fluor 488 (Molecular Probes, Leiden, The Netherlands). Ly-1 with a goat α-rabbit Alexa Fluor 488 (Molecular Probes), and CR1 and CD31 with streptavidin–Alexa Fluor 488 (Molecular Probes).

Images were captured on a LSM 510 Meta laser scanning confocal microscope system (Carl-Zeiss, Fedbach, Switzerland). When required, images were assembled using Adobe Illustrator CS or Adobe Photoshop CS (Adobe).

Results
TSLP Tg expression rescues LN development in IL-7−/− mice
In IL-7−/− mice, LN development is severely impaired (16), resulting in substantial reduction or absence in LNs at multiple locations. To address whether increased TSLP availability could restore LNs in IL-7−/− mice, adult IL-7−/− K14-TSLP Tg mice and IL-7−/− littermates were i.p. injected with Chicago blue, and LNs were enumerated 7 d later. Although mLN were found in all of the animals analyzed regardless of TSLP Tg expression, the number of LNs in mice overexpressing TSLP was substantially increased compared with that of non-Tg littermates, except for sacral and deep cervical (Fig. 1A, 1B). These results show that increased TSLP availability was sufficient to restore LN development in IL-7−/− mice.
Because IL-7Rα is almost exclusively expressed by hematopoietic cells (30), LN restoration in IL-7−/− K14-TSLP Tg animals was likely mediated through the effect of TSLP on hematopoietic cells. Hence, to gain insight into the mechanism underlying the restoration of LN development in IL-7−/− K14-TSLP mice, we investigated in neonatal mice which hematopoietic cells were affected by TSLP Tg expression. TSLP overexpression increased the B cell compartment ~100-fold in the spleen of 4.5-d-old IL-7−/− mice when compared with that of littermate controls (Fig. 1C). CD4 and CD8 T cell numbers were increased 18- and 20-fold, respectively, whereas NK cells were less affected (Fig. 1C). TSLP Tg expression in IL-7−/− newborn mice restored T cell numbers to WT levels and increased B cell numbers 3-fold when compared with those of WT controls (Fig. 1C). These results show that TSLP overexpression increased the size of B, T, and NK cell compartments in the periphery of newborn IL-7−/− mice. In the spleen of 0.5-d-old IL-7−/− K14-TSLP Tg mice, the percentage of CD4+ IL-7Rα+ LTi cells was 3-fold increased when compared with that of non-Tg littermates (Fig. 1D). LTi absolute cell numbers were 3.8- and 2-fold increased in the spleen and the mLN, respectively (Fig. 1E, 1F). Hence, TSLP Tg expression restored LTi cell number in the mLN of newborn IL-7−/− mice to WT values (Fig. 1F). LTi cell number in IL-7−/− spleen was 2-fold increased when compared with that of WT controls (Fig. 1E), suggesting that the failure of formation of LN anlage in IL-7−/− animals leads to the accumulation of LTi cells in the spleen.

Because LTi cell function directly relies on the expression of LTα1β2, we analyzed LTi cells from newborn IL-7−/− K14-TSLP Tg and non-Tg littermates and found that they expressed similar levels of LTα1β2 (Fig. 1G). These results show that TSLP Tg expression increases LTi cell numbers in vivo, without altering LTα1β2 expression levels.

**TSLP promotes LTi cell generation from FL precursors**

LTi cells arise from FL progenitors (31, 32). To understand the mechanism underlying the increase in LTi cell number in IL-7−/− K14-TSLP Tg mice, we investigated whether FL progenitors could give rise to LTi cells in response to TSLP. Although IL-7 favored the generation of LTi cells from FL progenitors in vitro, we failed to detect a similar activity of TSLP in various in vitro settings (data not shown). We therefore reconstituted lethally irradiated adult Ly5.2− IL-7−/− K14-TSLP Tg, IL-7−/− littermates, and WT controls with 105 WT FL Ly5.1+ cells from 12.5 dpc embryos. Ten days after reconstitution, the percentage of LTi cells from donor origin was substantially higher in TSLP Tg recipients than that in non-Tg littermates (Fig. 2A). LTi cell numbers were increased ~5-fold in the spleen of IL-7−/− K14-TSLP Tg recipients compared with those of non-Tg littermates, reaching values equivalent to those of WT recipients (Fig. 2B). These results showed that FL progenitors efficiently generated LTi cells in response to TSLP in vivo.

**TSLP-mediated LN restoration in IL-7−/− mice is LTi cell-dependent**

LN development crucially relies on the cross talk between LTi cells and LN mesenchymal cells (5, 28). To address whether LTi cells were instrumental for the restoration of LNs in IL-7−/− K14-TSLP Tg mice, IL-7−/− K14-TSLP Tg mice were backcrossed to RORγt−/− mice. Newborn IL-7−/− RORγt−/− K14-TSLP Tg mice were devoid of LTi cells (Fig. 3), and LNs were missing in adult mice (Table I). Thus, TSLP-mediated LN restoration in IL-7−/− mice was strictly dependent on LTi cells.

**TSLP restores organizer cells and LNs in RAG2−/− γc−/− mice independent of peripheral lymphocytes**

Peripheral lymphocytes were proposed to play a role in the maintenance of the LN anlage during postnatal life (23). Because we could not rule out that restoration of peripheral lymphocytes partially contributed to the LN restoration in IL-7−/− K14-TSLP Tg mice, we generated RAG2−/− γc−/− K14-TSLP Tg mice. Consistent with our previous findings, the percentage of CD4+ IL-7Rα+ LTi cells in the spleen was 2.5-fold increased in RAG2−/− γc−/− K14-TSLP Tg newborn mice compared with that in non-Tg littermates (Fig. 4A). Absolute LTi cell numbers were 2.5-fold increased in the spleen and mLN of TSLP Tg newborn mice compared with those of non-Tg littermates (Fig. 4B, 4C). LTα1β2, cytokine receptors, chemokine receptors, integrins, and adhesion molecules were expressed at similar levels by LTi cells from TSLP Tg and non-Tg littermates (Fig. 4D–G), suggesting that LTi cell activity and homing were not altered by TSLP Tg expression. In 6.5-d-old WT mice, mLN were colonized by CD45+ hematopoietic cells, of which the vast majority were CD4+ and CD8+ T cells together with CD19+ B220+ B cells (Fig. 5A). WT mLN contained a mean of 3 × 104 CD4+ T cells, 9.5 × 105 CD8+ T cells, 2.2 × 107 B cells, and 6 × 107 NK cells (Fig. 5B). In contrast, mLN from RAG2−/− γc−/− K14-TSLP Tg and RAG2−/− γc−/− mice were devoid of T cells, and B and NK cell numbers were ≤100 (Fig. 5A, 5B). These results show that LN anlagen of RAG2−/− γc−/− K14-TSLP Tg mice contained higher LTi cell numbers than those of non-Tg littermates, while being almost devoid of lymphocytes.
Adult RAG2−/− γc−/− K14-TSLP Tg and non-Tg littermates were analyzed for the presence of LNs. As expected, RAG2−/− γc−/− mice had a severe defect in LN development with the frequency of inguinal, popliteal, periaortic, axillary, pancreatic, and hepatic LNs <20% of that of WT mice (Fig. 5C, 5D). TSLP overexpression fully restored inguinal, popliteal, axillary, and hepatic LN development and substantially rescued the organogenesis of periaortic LNs (Fig. 5C, 5D). The formation of brachial, superficial cervical, and deep cervical LNs was also clearly enhanced by TSLP Tg expression. Hence, RAG2−/− γc−/− K14-TSLP Tg animals had almost normal LN numbers. Furthermore, the depletion of NK cells with NK1.1 Ab for the first 2 wk of life of RAG2−/− mice did not prevent the normal formation of LNs (data not shown). These results show that peripheral B, T, and NK lymphocyte compartments are not required for the maintenance of LN anlagen.

TSLP overexpression increased LTi cell numbers and restored LN development in RAG2−/− γc−/− mice. Because LTi cells are mandatory for the formation of organizer cell clusters (5), the increase in LTi cell number may have a direct effect on the organizer compartment. To test this hypothesis, we analyzed iLN anlagen from RAG2−/− γc−/− K14-TSLP Tg and non-Tg newborn mice. Consistent with the absence of peripheral lymphocytes, iLNs from both RAG2−/− γc−/− and RAG2−/− γc−/− K14-TSLP Tg animals contained few CD45+ cells (Fig. 5E). Within the CD45+ fraction, a homogenous VCAM-1+ ICAM-1+ organizer population, which was absent from non-Tg littermates, was present in TSLP Tg animals (Fig. 5E). These results suggest that the TSLP-mediated increase in LTi cell number is instrumental for the restoration of organizer cells in newborn RAG2−/− γc−/− mice and later for the restoration of LNs in adult mice.

Absence of lymphocytes compromises LN architecture but not the presence of chemokine-producing stromal compartments and high endothelial venules

The LN microenvironment is composed of different stromal populations, which constitute the LN framework, regulate lymphocyte segregation, and actively participate in adaptive immunity (33–35). The contribution of lymphocytes to LN architecture and microenvironment remains elusive. To address these issues, we compared the iLNs from WT and RAG2−/− γc−/− K14-TSLP Tg mice.

The LN reticular network can be visualized by staining with an Ab specific for the ER-TR7 Ag (34). Structures such as capsule, high endothelial venules (HEVs), cortex, and medulla were readily detectable in iLNs from both WT and RAG2−/− γc−/− K14-TSLP Tg mice and non-Tg littermates. The contribution of lymphocytes to LN architecture and microenvironment remains elusive. To address these issues, we compared the iLNs from WT and RAG2−/− γc−/− K14-TSLP Tg mice.

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Table I. LN restoration in IL-7−/− K14-TSLP Tg mice is LTi cell-dependent

<table>
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Adult IL-7−/− RORγ−/− K14-TSLP (n = 6) mice were analyzed for LN presence together with WT (n = 5) controls.

FIGURE 4. TSLP overexpression increases LTi cell numbers in RAG2−/− γc−/− mice without altering their phenotype. A. FACS profiles gated on CD3− CD19+ cells show the percentage of CD4+ IL-7+ LTi cells found in RAG2−/− γc−/− and RAG2−/− γc−/− K14-TSLP Tg newborn spleens (0.5 d old). Numbers are mean and SD of percentages. Absolute CD4+ CD3+ IL-7+ LTi cell number in spleen (B) and mLN (C) of 0.5-d-old RAG2−/− γc−/− (circles, n = 43) and RAG2−/− γc−/− K14-TSLP Tg (triangles, n = 32) mice. Each symbol represents LTi cell number for an individual animal. The mean values are indicated by a bar. D, LTTα1β2 expression level of LTi cells from LTTα1β2 (shaded), RAG2−/− γc−/− (plain line), and RAG2−/− γc−/− K14-TSLP Tg (dotted line) newborn mice in spleen (left) and mLN (right). Expression level of (E) cytokine receptors, (F) integrins and adhesion molecules, and (G) chemokine receptors on mesenteric CD4+ IL-7+ LTi cells from RAG2−/− γc−/− (plain line) and RAG2−/− γc−/− K14-TSLP Tg (dotted line) 0.5-d-old mice. Shaded histograms show background fluorescence levels of CD4+ IL-7+ LTi cells.

Tg mice (Fig. 6A, 6B), ER-TR7 staining clearly resolved B and T cell zones in WT LNs, whereas these areas could not be discriminated in LNs of RAG2−/− γc−/− K14-TSLP Tg mice (Fig. 6A, 6B). As previously shown in mice devoid of B cells (36), HEVs were localized below the subcapsular sinus of the LNs from RAG2−/− γc−/− K14-TSLP Tg mice. These results show that lymphocytes actively contribute to the LN architecture, because their presence is required for the proper reticular organization of the B and T cell areas and for the correct localization of HEVs.

Follicular dendritic cells (FDCs) are stromal cells that express high levels of the complement receptor 1 (CR1 or CD35), allowing them to capture immune complexes. They belong to the population of stromal cells that produce the B cell attractant CXCL13 (37), hence ensuring the segregation of B cells into follicles. Within WT B cell follicles, FDCs formed distinct CR1+ networks, which were undetectable in the LNs of RAG2−/− γc−/− and RAG2−/− γc−/− K14-TSLP Tg animals (Fig. 6C, 6D). As expected in WT LNs (38, 39), the B and T cell stroma were positively stained for CXCL13 and CCL19, respectively (Fig. 6E, 6G). As for WT, CCL19 staining was observed in the inner part of the RAG2−/− γc−/− K14-TSLP Tg
Discussion

This study aimed at understanding the role of the IL-7 pathway in LN organogenesis. Our results show that LN development occurs independent of peripheral lymphocytes and suggest that the IL-7 signaling pathway controls LN development by regulating the size of the LTi cell pool. We show that TSLP Tg expression increases the size of the LTi cell pool and restores LN development in IL-7−/− mice. These results extend our previous finding on the overlapping activity of TSLP and IL-7 in adult lymphopoiesis (24) and suggest that the IL-7 signaling pathway controls LN development by regulating the size of the LTi cell pool. We show that TSLP Tg expression increases the size of the LTi cell pool and restores LN development in IL-7−/− mice. These results extend our previous finding on the overlapping activity of TSLP and IL-7 in adult lymphopoiesis (24) and suggest that the IL-7 signaling pathway controls LN development by regulating the size of the LTi cell pool. We show that TSLP Tg expression increases the size of the LTi cell pool and restores LN development in IL-7−/− mice. These results extend our previous finding on the overlapping activity of TSLP and IL-7 in adult lymphopoiesis (24) and suggest that the IL-7 signaling pathway controls LN development by regulating the size of the LTi cell pool. We show that TSLP Tg expression increases the size of the LTi cell pool and restores LN development in IL-7−/− mice. These results extend our previous finding on the overlapping activity of TSLP and IL-7 in adult lymphopoiesis (24) and suggest that the IL-7 signaling pathway controls LN development by regulating the size of the LTi cell pool. We show that TSLP Tg expression increases the size of the LTi cell pool and restores LN development in IL-7−/− mice. These results extend our previous finding on the overlapping activity of TSLP and IL-7 in adult lymphopoiesis (24) and suggest that the IL-7 signaling pathway controls LN development by regulating the size of the LTi cell pool. We show that TSLP Tg expression increases the size of the LTi cell pool and restores LN development in IL-7−/− mice. These results extend our previous finding on the overlapping activity of TSLP and IL-7 in adult lymphopoiesis (24) and suggest that the IL-7 signaling pathway controls LN development by regulating the size of the LTi cell pool.
development in RAG2−/−γc−/−mice, suggesting that peripheral lymphocytes could play a role in maintaining the LN anlage in postnatal life (23). These findings may be explained by the fact that adult lymphocytes express LTα1β2 (40) and are therefore able to restore LN anlagen.

LTi cells are central to the TSLP-mediated LN restoration in IL-7−/−mice, as demonstrated by the fact that IL-7−/−RORγc−/−K14-TSLP Tg mice lack all LNs. TSLP Tg expression increases LTi cell numbers and restores organizer cells in newborn RAG2−/−γc−/−mice, suggesting that the increase in LTi cell number is essential for the presence of organizer cells. These data are in line with the proposal (5, 41, 42) that there is a numerical threshold of LTi cells that is required to effectively “instruct” LN stroma. Indeed, there are several lines of evidence indicating that low LTi cell numbers fail to induce proper LN development. The maintenance of a fetal LN transplanted under the kidney capsule of an adult mouse is dependent on LTi cell numbers (42). A 15.5 dpc LN fails to persist unless exogenous LTi cells are added to the graft, whereas 17.5 dpc LN, containing more LTi cells, persists without LTi addition (42). Furthermore, mice in which LTi cell recruitment to the LN anlage is impaired (15, 43–45) are devoid of LNs. Collectively, these data suggest that LN development in IL-7−/− and RAG2−/−γc−/−animals is defective due to insufficient numbers of LTi cells.

Interestingly, several cellular compartments typically found in the LN microenvironment, such as chemokine-expressing stromal cells and PNAd+ HEVs, develop in the lymphocyte-free LNs of RAG2−/−γc−/−K14-TSLP Tg mice. Within the B cell follicle of WT LNs, a substantial fraction of the CXCL13-expressing cells do not display a FDC phenotype (37). Our results suggest that these CXCL13-producing cells differentiate independent of lymphocyte
Some blocks of text are not clearly visible due to low contrast or resolution, making it difficult to extract accurate content. However, the visible portion of the text appears to discuss the role of lymphocyte colonization in lymphoid organogenesis, particularly in the context of lymphatic tissue-inducer (LTi) cells and their interactions with other cell types and cytokines. The text mentions the importance of IL-7 in lymphocyte development and the requirement for proper LN organization and HEV positioning. It also references the participation of TSLP and other cytokines in the formation of lymphatic follicles and the role of stromal networks in lymphocyte migration and differentiation.


