Ontogeny of Stromal Organizer Cells during Lymph Node Development

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The development of secondary lymphoid organs, such as lymph nodes (LNs), in the embryo results from the reciprocal action between lymphoid tissue inducer (LTi) cells and stromal cells. However, the initial events inducing LN anlage formation before the LTi stromal cells cross-talk interactions take place are not fully elucidated. In this study, we show that the inguinal LN anlage in mouse embryos developed from mesenchymal cells surrounding the lymph sacs, spherical structures of endothelial cells that bud from veins. Using inguinal and mesenteric LNs (mLNs), we provide evidence supporting a two-step maturation model for stromal cells: first, ICAM-1\(^{-}\)VCAM-1\(^{-}\) mesenchymal precursor cells become ICAM-1\(^{\text{int}}\)VCAM-1\(^{\text{int}}\) cells, in a process independent of LTi cells and lymphotoxin \(\beta\) receptor (LT\(\beta\)R) signaling. The second step involves the maturation of ICAM-1\(^{\text{int}}\)VCAM-1\(^{\text{int}}\) cells to ICAM-1\(^{\text{high}}\)VCAM-1\(^{\text{high}}\) mucosal addressin cell adhesion molecule-1\(^{-}\) organizer cells and depends on both LTi cells and LT\(\beta\)R. Addition of \(\alpha\)LT\(\beta\)R agonist to LN organ cultures was sufficient to induce ICAM-1\(^{\text{int}}\)VCAM-1\(^{\text{int}}\) cells to mature. In \(\text{LT}^{-}\) embryos, both inguinal and mLN stromal cells showed a block at the ICAM-1\(^{\text{int}}\)VCAM-1\(^{\text{int}}\) stage, and, contrary to inguinal LNs, mLNs persist longer and contained LTi cells, which correlated with the sustained gene expression of \(\beta\)-7, Cxcl13, and, to a lesser degree, Ccl21.

Taken together, these results highlight the importance of the signals and cellular interactions that induce the maturation of stromal cells and ultimately lead to the formation of lymphoid tissues. The Journal of Immunology, 2010, 184: 000–000.
Materials and Methods

**Mice**

BALB/c (H-2d), C57BL/6 (H-2b), and C57BL/6–Rorγt−/− (H-2b) (13) (C57BL/6 background), and R;oyt−/− (H-2b) (14) (C57BL/6 background) mice were bred and maintained under specific pathogen-free conditions in the Biomedical Services Unit at the University of Birmingham (Birmingham, U.K.) according to Home Office and local ethics committee regulations. Day of vaginal plug detection was designated as E0.

**Isolation of LN stromal and CD45+ cells and mLN organ culture**

iLNs and mLNs were isolated at the indicated embryonic stages by microdissection and were disaggregated by incubation in 2.5 mg/ml collagenase/disprase (R&D Systems, Minneapolis, MN) and 100 μg/ml DNase I (Sigma-Aldrich, St. Louis, MO) in RF10 media at 37°C to obtain single-cell suspensions. In some experiments, freshly isolated mLNs were explanted in fetal organ culture prepared as described (15) and treated with the agonistic anti-LTβR Ab (clone 4H8) at 2 μg/ml for 3 d (16, 17).

**Abs and flow cytometry**

The following Abs were used for flow cytometry: CD45.2–FITC clone 104, ICAM–1–PE clone YN1/1.7,4, VCAV–1–APC clone 429, MadCAM-1–biotin clone MECA-367, VEGFR–3–biotin clone AFLA4, PDGFR–α–APC clone AP5, CD44–FITC clone IM7, receptor activator for NF-κB ligand (RANKL)–biotin clone IK22/5, MadCAM-biotin clone MECA-367 (eBioscience), collagen type I (Chemicon International, Temecula, CA), laminin α5 (rat; gift of A. Zapata, Complutense University, Madrid, Spain), perlecain (rat; gift of Z. Lokmic, Bernard O’Brien Institute of Microsurgery, Melbourne, Australia), CCL21 (R&D Systems), Lyve-1 (rabbit polyclonal; Abcam, Cambridge, MA), fibronectin clone FN–3E2 (mouse IgM; Sigma-Aldrich), ER-TR7 supernatant.

**Immunofluorescence stainings**

For tissue-section stainings, iLNs and mLNs were isolated and embedded in OCT compound (Tissue Tek, Torrance, CA), then frozen in liquid nitrogen. Six-micrometer sections of tissue were cut and fixed in acetone. Abs used were gp38/podoplanin 8.1.1, ICAM–1–FITC clone YN1/1.7,4, PDGFRα clone AP5, CD44–FITC clone IM7, receptor activator for NF-κB ligand (RANKL)–biotin clone IK22/5, MadCAM-biotin clone MECA-367 (eBioscience), collagen type I (Chemicon International, Temecula, CA), laminin α5 (rat; gift of A. Zapata, Complutense University, Madrid, Spain), perlecain (rat; gift of Z. Lokmic, Bernard O’Brien Institute of Microsurgery, Melbourne, Australia), CCL21 (R&D Systems), Lyve-1 (rabbit polyclonal; Abcam, Cambridge, MA), fibronectin clone FN–3E2 (mouse IgM; Sigma-Aldrich), ER-TR7 supernatant.

**Cell sorting, RT-PCR, real-time RT-PCR**

Isolation of the different CD45+ ICAM–1/VCAV–1 mLN stromal cell populations from wild-type (WT) and LTβR−/− embryos of the indicated ages was performed by MoFlow (DakoCytomation, Carpinteria, CA) cell sorting from disaggregated LN cell suspensions and then snap frozen for RT-PCR.

High-purity cDNA was generated from purified mRNA using μMacs One-Step cDNA synthesis kit, according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) with primers specific for β-actin, Ccl21, Ccl19, Cxcl13, Il-7, IlβR, RankL, TnfαL, and Mmp9. PCRs were conducted in triplicate in 10 μl volumes containing 200 nM primers. After an initial denaturation step (95°C for 10 min), cycling was performed at 95°C for 15 s, 60°C for 20 s, and 72°C for 5 s (45 cycles). Specific amplification was verified by melt curve analysis and also by fractionation of PCR products on a 2% agarose gel that were identified by fragment size (data not shown). mRNA transcript levels of each gene were analyzed using Applied Biosytem’s SDS software (Applied Biosystems, Foster City, CA) by setting thresholds determining the cycle number at which the threshold was reached (Ct). The Ct of the β-actin was subtracted from the Ct of the target gene, and the relative amount was calculated as 2−ΔΔCt. Means of triplicate reactions (multiply 1000-fold) were represented, and data shown are representative of at least two separate cell-sorting experiments. Primer sequences are shown in Supplemental Fig. 1.

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**FIGURE 1.** The LN anlagen developed at the site of contact between mesenchymal and endothelial cell populations. A. Structure of an E13 iLN primordium. At this stage, the iLN primordium consists of a bud of endothelial cells stained with gp38/podoplanin (green) surrounded by layers of mesenchymal cells stained with ER-TR7 (red) (×40X1.4 numerical aperture [NA] water lens). B. Immunofluorescence staining of E15 iLN sections (×40X1.4 NA water lens) showing the central endothelium stained with gp38/podoplanin, ICAM-1, CCL21 (green), collagen type I (red), laminin α5 (green), and perlecain (red) and the surrounding mesenchyme stained with PDGFRα, fibronectin, ER-TR7 (red), and CD44 (green). The left panels of all sections show the overlay with DAPI (white) staining the cell nuclei.
(Biogenesis, Poole, U.K.). CD4 was directly conjugated using the Alexa Fluor 647 mAb Labeling Kit (Invitrogen, Carlsbad, CA). ICAM-1–FITC–conjugated Ab was detected using rabbit anti-FITC (Sigma-Aldrich), then goat anti-rabbit IgG–FITC (Jackson ImmunoResearch Laboratories, West Grove, PA). Anti–PDGFRα was detected using anti-rat IgG–Alexa 594 (Molecular Probes, Eugene, OR). Lyve-1 Ab was detected using goat anti-rabbit IgG–FITC (Jackson ImmunoResearch Laboratories). Collagen type I Ab was detected with goat anti-rabbit IgG–biotin (DakoCytomation). CCL21 Ab was detected using donkey anti-goat FITC (Jackson ImmunoResearch Laboratories). gp38/podoplanin and fibronectin Abs were detected using goat anti-hamster biotin (Cambridge Bioscience, Cambridge, U.K.). Biotinylated Abs were detected using streptavidin–Alexa Fluor 555 or 488 (Molecular Probes). Sections were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

Image acquisition and analysis of confocal images

For tissue-section stainings, confocal images were acquired using a Zeiss LSM 510 laser scanning confocal head with a Zeiss Axio Imager Z1 microscope and a 63×/1.4 NA water lens (Zeiss, Oberkochen, Germany). Digital images were recorded in four separately scanned channels with no overlap in detection of emissions from the respective fluorochromes. Confocal micrographs were stored as digital arrays of 2048 × 2048 pixels with 8-bit sensitivity; detectors were routinely set so that intensities in each channel spanned the 0–255 scale optimally.

Results

Inguinal LN anlagen formed by budding endothelial cells surrounded by mesenchymal cells

Over the past few years, the structure of the developing LN anlagen in the mouse embryo had been studied by assessing the recruitment and presence of LTi cells in sections of whole mouse embryos (11, 18–20). However, the iLN anlage forms a very distinct structure that can be dissected as early as E13. Our previous studies have shown that E15 iLNs contain very few CD45+ cells and no PDGFRα+ LTi cells (12). Therefore, experimental approaches relying on the presence of LTi cells for the identification of LN anlagen do not allow for early detection and analysis of LN primordium. For an in-depth analysis of LN anlage development prior to LTi cell colonization, we examined sections of dissected E13 and E15 iLNs.

At E13, the primitive iLN anlage had a spherical shape formed by a bud of tightly packed gp38/podoplanin+ cells surrounded by layers of fibroblast-like cells that expressed the component of the reticular network ER-TR7 (Fig. 1A). We further characterized these two cellular compartments for the expression of endothelial and mesenchymal markers on E15 iLNs by immunofluorescence staining and confocal imaging.

The bud of gp38/podoplanin+ cells expressed the endothelial markers ICAM-1 and CCL21 and the extracellular matrix proteins collagen type I, and laminin α5 (Fig. 1B, left columns). Perlecan staining was restricted to a thin layer at the interface between the endothelial and the mesenchymal cells, indicating the presence of an intact basement membrane produced by the former. The fibroblast-like cells that formed the outer layers of the LN primordium expressed several molecules characteristic of mesenchymal cells, such as PDGFRα, the extracellular matrix protein fibronectin, ER-TR7, and the hyaluronic acid receptor CD44 (Fig. 1B, right columns).

Staining of iLN sections demonstrated that ER-TR7+ stromal cells and the gp38/podoplanin+ lymphatic endothelial cell compartment remained separated up to E16, and then ER-TR7+ cells appeared to invade the endothelial core to form the proper internal compartments of the anlage (Fig. 2, Supplemental Fig. 2). Following that process, a large number of mesenchymal cells at E17 expressed both ER-TR7 and gp38/podoplanin and could be distinguished from the single-positive cells (Supplemental Fig. 2).

Based on the above observations, we envision the structure of the E13–15 iLN anlage comprising two distinct cells types, a bud of lymphatic endothelial cells forming the lymph sacs, surrounded by a basement membrane and layers of mesenchymal cells.

Ongoing remodeling of the iLN anlage structure between E15 and E17

To further define the organ remodeling taking place between E15 and E17 in iLNs, we tracked the changes occurring in the mesenchymal and endothelial compartments by analyzing E17 iLNs, when LTi cells arrived to the anlagen. Immunofluorescence staining of molecules expressed in endothelial and mesenchymal cells were performed in E17 iLN sections and compared with the stainings of these organs at E15.

The endothelial cells that formed a central bud at E15 still form the core of the anlagen at E17 (Fig 2, circled by a white line), but...
now contained perlecan+ laminin α5+ structures that form the LN vasculature (Fig. 2A, 2D).

The mesenchymal cells formed the outer structure of the LN anlage, similar to what was observed at E15. However, at E17, mesenchymal cells expressing fibronectin and PDGFRα started to migrate to the endothelial core, as shown in the central region delineating the endothelium (Fig. 2C–H). CD44 staining (Fig. 2I) highlighted the structure of the mesenchymal compartment as well as hematopoietic cells (round cells) that have entered the LN anlage. Fibronectin also started to be expressed by cells of the vasculature (Fig. 2C, 2E, arrowheads). Taken together, these observations show the dynamic changes occurring between E15 and E17 in the iLN anlage involving both the endothelium and the mesenchyme. For the endothelium, the most dramatic change consisted of the remodeling of the whole vasculature and for the mesenchyme of the invasion of the central endothelial compartment. We further characterize the endothelial and mesenchymal cell populations to better understand their contribution to LN anlagen formation.

Concomitant emergence of stromal organizer cells during remodeling of the iLN structure

The LN organizer cells have been previously characterized by their coexpression of ICAM-1 and VCAM-1 on mLNs, and the stainings suggested a differentiation of the organizer cells from mesenchymal cells (18, 21, 22). We assessed by FACS analysis the presence of these cells at different stages of embryonic iLN development. At E15, we were able to clearly identify the mesenchymal and endothelial cell compartments described in Fig. 1: 1) the mesenchymal cell precursors that were negative for ICAM-1 and VCAM-1 (I low V low) and PDGFRα that correlated with the fibroblast-like cells of the surrounding mesenchyme in Fig. 1B; 2) the ICAM-1 single-positive (I high V low) endothelial cells that also expressed gp38/podoplanin and MAdCAM-1 and were VEGFR3−, similar to the central endothelium shown in Fig. 1B (Fig. 3A, top panels). Interestingly, we identified a third cell population that expressed intermediate levels of ICAM-1 and VCAM-1 (I mid V mid) that were PDGFRα+ and gp38/podoplanin+, suggesting that they derived from the I− V−
mesenchymal cells. At E17, the mature stromal organizer cells were identified by their high expression of ICAM-1 and VCAM-1 (I^{highV^{high}}) as well as MadCAM-1, a late differentiation marker. Mature organizer cells also expressed PDGFRα and gp38/podoplanin but not VEGFR3, suggesting that their progenitors were the I^{intV^{int}} cell population.

The I^{V^{+}} endothelial cell population was negative for both VEGFR-3 and the lymphatic marker Lyve-1 at E15 but became positive for both molecules at E17, indicating an ongoing differentiation process toward lymphatic endothelial cells (Fig. 3A, bottom panels, and data not shown). These results indicate that mesenchymal cells start to mature before the lymphatic endothelium is fully differentiated.

To summarize, vascular/lymphatic endothelial cells could be defined as I^{V^{+}} gp38/podoplanin\(^{-}\) VEGFR3\(^{-}\) PDGFRα\(^{-}\), whereas the mesenchymal cell compartment encompassed the I^{V^{-}} I^{intV^{int}}, and I^{highV^{high}} MadCAM-1\(^{+}\) gp38/podoplanin\(^{-}\) PDGFRα\(^{-}\) VEGFR3\(^{-}\) cells.

Stromal cells of the iLN and mLN followed a similar pattern of development

Blocking of LTβR in pregnant female mice has shown that mLNs are the first to develop in the embryos and are followed by formation of LNs from head to tail (1, 23, 24). Several reports have indicated the different developmental requirements for mLN and iLN formation (25–27). A recent report has also shown differences in the stromal cell subsets in these organs in newborn mice (18), and we wanted to investigate whether those differences were already present in embryos and whether mLN stromal cells expressed similar molecules than their iLN counterparts. Indeed, E15 mLN showed an increased frequency of the I^{intV^{int}} cell population, expressed the highest levels of homeostatic chemokines (Ccl21, Ccl19, and Cxcl13) (18), RankL and Il-7, confirming their potential to attract and stimulate LTI cells (Fig. 5). The I^{intV^{int}} and I^{highV^{high}} cells expressed Mmp9, which has been shown to increase cellular

![Image](http://www.jimmunol.org/)

FIGURE 4. Structures of E18 mLN and iLN. Immunofluorescence staining of E18 mLN sections (×25/1.4 NA and ×40/1.4 NA water lens) showing the Lyve-1\(^{-}\) capsule (green), the CD4\(^{+}\) LTI cells (blue), and RANKL (top row), MadCAM-1 (middle row), or IL-7Rα (bottom row) (red). Results are representative of at least three independent experiments.

FIGURE 5. Gradient of expression of stromal organizer markers during maturation of the mesenchyme. Cell sorting and gene expression analysis of E18 mLN I^{V^{+}} (black), I^{lowV^{low}} (blue), and I^{highV^{high}} (red) stromal cell populations. Real-time RT-PCR analysis of LTB, ReLB, Ccl21, Ccl19, Cxcl13, Il-7, RankL, Mmp9, and Tnfr1 genes. Ratio of gene of interest to β-actin is shown. Results are representative of at least three independent experiments.
invasiveness and mobility, indicating a role in tissue remodeling by these cell subsets. Finally, we noticed that all the markers studied showed a gradient of expression, with the \( I^{\text{int}}V^{\text{int}} \) cells showing the highest levels, followed by \( I^{\text{int}}V^{\text{high}} \) and \( I^{\text{high}}V^{\text{high}} \) that reflected the stage of maturation of the stroma (Fig. 5).

**Absence of LtβR or LTi cells resulted in a block of stromal cell maturation at the \( I^{\text{int}}V^{\text{int}} \) stage**

LN development is strictly dependent on LTi cells and LTβR signaling, as adult Rorγ2/2, LTβR2/2, and Rorγ2/2 mice lack all LNs (13, 14, 26, 28, 29). We assessed whether LTβR signaling and LTi cells were required for the initial step of stromal cell maturation or for full progression of these cells to the \( I^{\text{high}}V^{\text{high}} \) stage. FACS analysis of stromal cell populations from E15 iLN and mLN anlagen from WT, LtβR2/2, and Rorγ2/2 embryos showed the emergence of the \( I^{\text{int}}V^{\text{int}} \) cells in all strains (Fig. 6A). However, at E17, the iLNs were difficult to find in both LtβR2/2 and Rorγ2/2 embryos, and what remained had the appearance of fibrous structures (data not shown). FACS analysis of the few iLNs isolated from these mutants confirmed the absence of the \( I^{\text{high}}V^{\text{high}} \) mature stromal organizer cells in both strains (Fig. 6A).

We next investigated whether mLNs were similarly affected in their development as iLNs in these mutants. In contrast to the low numbers of iLNs found in E17 LtβR2/2 or Rorγ2/2 mice, mLNs were present in normal numbers in embryos from these strains. However, FACS analysis of stromal cells revealed a similar block on the progression from \( I^{\text{int}}V^{\text{int}} \) to \( I^{\text{high}}V^{\text{high}} \) mature organizer cells (Fig. 6A).

Recruitment of CD45+ cells to iLNs and mLNs between E15 and E17 was impaired in LtβR2/2 and Rorγ2/2 embryos. For example, in WT iLNs, we observed an 18-fold increase in the

**FIGURE 6.** LtβR signaling induce maturation of stromal cells from \( I^{\text{int}}V^{\text{int}} \) to \( I^{\text{high}}V^{\text{high}} \). A, FACS analysis of single-cell suspensions from WT, LtβR2/2, and Rorγ2/2 E15 and E17 iLNs and mLNs showing the recruitment of CD45+ cells and the concomitant phenotypic changes in the CD45\(^{\text{int}} \) stromal cells. Percentages shown in histograms correspond to CD45\(^{\text{int}} \) stromal cells and CD45+ hematopoietic cells. The different ICAM-1 and VCAM-1 CD45\(^{\text{int}} \) stromal cell populations described in Fig. 3 are shown with corresponding percentages. Note the normal development of the LtβR2/2 and Rorγ2/2 E15 iLN and the absence of the \( I^{\text{high}}V^{\text{high}} \) stromal cell populations in the LtβR2/2 and Rorγ2/2 E15 mLNs and E17 iLNs and mLNs. Results are representative of at least three independent experiments. B, LTβR stimulation induces the maturation of stromal cells from \( I^{\text{int}}V^{\text{int}} \) to \( I^{\text{high}}V^{\text{high}} \) in E14 mLNs and E15 iLNs in organ cultures. FACS analysis of single-cell suspensions of E14 mLNs (left panels) and E15 iLNs (right panels) in organ cultures for 3 d showing the phenotypic changes in the CD45\(^{\text{int}} \) stromal cells induced by agonistic αLTβR Ab (bottom panels). Percentages shown in histograms correspond to CD45\(^{\text{int}} \) stromal cells. Three different stromal cells populations were identified: \( I^{\text{int}}V^{\text{int}} \) (black), \( I^{\text{high}}V^{\text{high}} \) (blue), and \( I^{\text{high}}V^{\text{high}} \) (red). Expression levels of MAdCAM-1 for each cell population are shown in histograms. Results are representative of three independent experiments.
FIGURE 7. LTβR is not fully required for the recruitment and retention of LTi cells to mLNs

A. Cell sorting and gene expression analysis of the WT and LtβR−− E15 mLN I− V− (black), I− V− (blue), and I− V− (gray) stromal cell populations. Real-time RT-PCR analysis of the Ccl21, Il-7, and Cxcl13 genes. Ratio of gene of interest to β-actin is shown. Results are representative of two independent experiments. B. LTβR is required for the recruitment of LTi cells to iLN but not mLNs. FACS analysis of single-cell suspensions from WT and LtβR−− E15 and E17 iLNs and mLNs showing the percentage of CD45+ cells and CD4+IL-7Rα. Ccl21 of WT and LtβR−−. Percentages shown in histograms correspond to CD45+ hematopoietic cells. CD45+ hematopoietic cell dot plot analysis of CD4 and IL-7Rα showed the percentages of LTi cells. Note the presence of LTi cells in the LtβR−− E17 mLN and their absence in iLNs. C. Immunofluorescence staining of WT and LtβR−− E16 mLN sections (×40/1.4 NA water lens) showing the Lyve-1 capsule (green), CD45+ LTI cells (blue), and in red, IL-7Ra (left panels), MadCAM-1 (middle panels), or RANKL (right panels). Note in the LtβR−− mLNs the presence of the capsule expressing Lyve-1 and MadCAM-1, the presence of LTi cells coexpressing IL-7Ra and CD4, and the absence of RANKL expression. Results are representative of two independent experiments.
embryos confirmed the presence of LTi cells in both strains and showed a significant reduction in MadCAM-1 and undetectable levels of RANKL in the latter (Fig. 7C).

These results showed that although overall colonization by CD45+ cells is reduced in the mLNs of LtbR−/− embryos, LTi cells are still being recruited, suggesting that LTβR signals are not fully required. However, MadCAM-1 and RANKL levels are severely reduced in these organs, showing their dependence on the LTβR pathway. Based on the results shown above we propose a specific role for LTβR signaling in stromal cells during maturation (Fig. 8, see Discussion).

Discussion

The majority of LN development studies have used whole mount sections of mouse embryos and relied on the accumulation of LTi cells for the identification of LN primordium (11, 18–20). Therefore, little is known about the initiating events that take place before the recruitment/arrival of LTi cells to the LN anlage. We took advantage of the well-defined structure of the early iLN and performed microdissection of intact anlage to show that the LN primordium developed where endothelial cells formed a spherical body, the lymph sac. This endothelium is surrounded by a perlecan+ basement membrane and expressed gp38/podoplanin, ICAM-1, and CCL21. Expression of Lyve-1 and VEGFR3 was not detected by immunofluorescence staining at E13 and appeared around E17 indicating an ongoing differentiation process toward lymphatic phenotype. Based on these observations, we confirmed Sabin’s findings (4, 5) using pig embryos in that LN anlagen formed at sites of endothelial cell budding from veins to form the primitive lymph sacs.

We showed that layers of mesenchymal cells surround the iLN endothelial bud and that mesenchyme and endothelium remained two distinct compartments until E17, when mesenchymal cells started to invade the former. Remodeling of the iLN anlage is concomitant with the differentiation of the lymphatic endothelium and the recruitment of LTi cells that induce the maturation of stromal cells to become proper organizer cells. Therefore, all these essential milestones of iLN organogenesis appear to take place in a short length of time. The signals that induce the mesenchymal cells to degrade the basement membrane and invade the lymph sacs and whether endothelial-mesenchymal cell cross-talk interactions are important for this process and for the differentiation of the lymphatic endothelium remain to be investigated.

To understand the maturation process of the mesenchymal cell populations and their contribution to the formation of the LN anlagen stroma, we tracked the emergence of the high-high mature organizer cells by FACS analyses. E15 iLNs, that appear to lack LTi cells, do contain int-intPDGFRα+ cells suggesting that the latter are derived from the PDGFRα+ mesenchymal cell layers that surround the lymph sacs. In this regard, a recent report has shown that lymph sacs are not required for the initiation of LN anlagen development (11), suggesting that stromal cell differentiation does not depend on the endothelium and can take place in its absence. In addition, we suggest that the int^int gp38/podoplanin+ cell population will give rise to the mature high-high MadCAM-1+ stromal organizer cells. The common expression of PDGFRα by 1−V−, int-int, and high-high cells and gp38/podoplanin by int-int and high-high cells but not VEGFR3 suggested a precursor-product relationship between them. iLN and mLN anlagen develop at different times during embryogenesis, have different developmental requirement, and contain different frequencies of int-int stromal cells. As shown before in newborn mice, embryonic mLNs have a higher percentage of int-int stromal cells than iLNs but that does not reflect in a larger proportion of high-high organizer cells because the latter is lower in mLNs than iLNs (Figs. 3A, 3B, 6A) (18). However, the sequence of stromal cell maturation is similar in both LNs.

Although several reports had demonstrated that LN development is strictly dependent on LTi cells and LTβR signaling as adult Rorγ−/−, LtbR−/−, and Ltα−/− mice lack most LNs, the fate of the developing LN anlagen in those mutants had not been characterized (13, 14, 26, 28, 29). Other groups and ours (11, 12, 19) have indicated that two different signals are required for stromal organizer cell maturation. We have shown previously that in the absence of lymphotixin α and hence lymphotixin αβ2, the maturation of stromal cells was blocked at the int-int stage (12). However, a role for LTβR engagement by ligands other than lymphotxin αβ2 to induce the transition of 1−V− mesenchymal cells to the high-high stage could not be ruled out. Analysis of the LtbR−/− LNs showed that this receptor does not have a role in the first step of stromal cell maturation. Thus, the signal(s) for the initial step of stromal cell maturation remains elusive. Lymphotxin α and LTβR engagement and the presence of LTi cells were required for the second step of this process, for LtbR−/− and Rorγ−/− mouse embryos showed normal development of iLNs and mLNs up to E15 but stromal cell maturation did not progress further than the int-int stage, and iLNs quickly regressed. Stimulation of E14 mLNs through LTβR induced a strong increase in the frequency of high-high MadCAM-1+ stromal organizer cells that seems to be independent of cell proliferation. Similar results were obtained upon LTβR stimulation of E15 iLNs, although upregulation of MadCAM-1 expression was lower than in mLN stromal cells. Taken together, these results support the hypothesis of a precursor-product relationship between the int-int cells and the high-high MadCAM-1+ stromal organizer cells.

We investigated the gene expression profile of three CD45− cell populations from E18 mLNs, 1−V−, int-int, and high-high. Our

FIGURE 8. Model of LN stromal cell maturation. Based on our immunofluorescence and FACS analysis data (summarized in the top row in capital letters) and qPCR data (summarized in the bottom row in italics), we propose a model for the maturation of stromal cells during LN development. The 1−V− mesenchymal precursor cells (dark gray), upon stimulation with an unknown signal, will become int-int primed stromal cells (blue) that upon upregulating the expression of RelB will be able to respond to LTβR signals upon engagement by the lymphotxin αβ2 ligand expressed by LTi cells. Signals through the LTβR and other receptors induce the maturation of the int-int cells (red) to become high-high high-high stromal organizer cells (see Discussion).
results showed that although $\Gamma^{-}$ mesenchymal precursors cells expressed Lt$\beta$R mRNA, one of its downstream effectors, the transcription factor Relb, was expressed only in the maturing $\Gamma^{intV^{-}}$ and $\Gamma^{highV^{high}}$ cells. This finding suggests that the first step of stromal cell maturation enables the LN anlagen to respond to LT$\beta$R signaling by inducing the expression of the downstream proteins of this cascade. In agreement with this, Rankl was expressed at 1000-fold higher levels in the $\Gamma^{phatV^{high}}$ cell population than in the $\Gamma^{intV^{int}}$ cells. Concomitantly, mLNs from E16.5 Lt$\beta$R-deficient embryos, whose stromal cells are blocked at the $\Gamma^{intV^{int}}$ cell stage, showed no expression of RANKL. In contrast, Ccl21, Ccl13, and Il-7 were expressed in both $\Gamma^{intV^{int}}$ and $\Gamma^{highV^{high}}$ cell populations, indicating that these genes are less dependent on LT$\beta$R engagement for their full expression. In agreement with this, the mRNA levels of Ccl13 and Il-7 were not decreased in E15 mLN of Lt$\beta$R-deficient embryos. Ccl21 expression was significantly decreased in endothelial cells correlating with previous reports on the expression of CCL21 by endothelial cells of high endothelial venous venules and the role of LT$\beta$R-NF-kB2 in the latter (30–34). These results are in agreement with the findings of Moyron-Quiroz and colleagues (35) showing that CXC13 and CCL21 were expressed independently of lymphotoxin $\alpha$ during the formation of inducible bronchial-associated lymphoid tissues. A recent report has indicated that retinoic acid expressed by neurons induces the expression of Ccl13 in LN anlagen independently of LT$\beta$R signaling (36).

At E17 when iLNs from Lt$\beta$R$^{-/-}$ mice have regressed, mLNs were still present in this mutant but showed a failure to induce RANKL and MadCAM-1 in stromal cells compared with WT mLNs, highlighting the importance of LT$\beta$R signaling for the expression of these molecules (20). The absence of RANKL expression in the Lt$\beta$R$^{-/-}$ mLN may compromise the survival of LTI cells, which may be dependent on RANK signaling, as suggested by the reduced number of these cells found in the mLNs of newborn mice deficient in this pathway or the intracellular adaptor TNFR-associated factor-6 as well as in embryos treated with RANK-Ig fusion protein (19, 37, 38). These findings correlate with the fact that rudimentary mLNs have been found in newborn Lt$\beta$R$^{-/-}$ mice.

Based on the results shown in this study, we propose a two-step stromal cell maturation model (Fig. 8). First, the $\Gamma^{-}$ mesenchymal cells are primed by a yet unknown signal to give rise to a $\Gamma^{intV^{int}}$ gp38/podoplanin$^{+}$ cell population, able to recruit LTI cells by their expression of Il-7, Ccl21, and Ccl13 and to respond to LT$\beta$R engagement via acquisition of the expression of Relb. Second, the $\Gamma^{intV^{int}}$ cells give rise to the mature $\Gamma^{phatV^{high}}$ MadCAM-1$^{+}$ stromal organizer cells. Following these essential maturation steps during embryogenesis, LN stromal cells require stimulation by Il-$\gamma$R$\alpha$ cells at the time of birth to sustain LN development and organization (39).

It is clear that stromal cell function in lymphoid tissues is more than contributing a support for the exquisite architecture of these organs. Stromal cells also have a role in B and T cell homeostasis as well as in the organization of specific cell areas to facilitate the localization and interaction of cells during immune responses (40, 41). All these functions suggest the existence of different subsets of stromal cells, and further studies will be necessary to investigate the ontogeny of fibroblastic reticular cells and marginal reticular cells (42–45). We are currently investigating the origin of the $\Gamma^{-}$ mesenchymal cell precursors, the signals that induce priming on those cells to become $\Gamma^{intV^{int}}$ and the role for the recently described lymphoid tissue initiator cells have in this process (46).

The process of maturation of lymphoid tissue stromal cells might have similarities with the recently described changes in the stromal microenvironment resulting in the differentiation of lymphoid stromal cells during the formation of ectopic lymphoid tissues (47).

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Disclosures

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