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Ontogeny of Stromal Organizer Cells during Lymph Node Development

Cécile Bénézech,* Andrea White,* Emma Mader,* Karine Serre,* Sonia Parnell,* Klaus Pfeffer,† Carl F. Ware,‡ Graham Anderson,* and Jorge H. Caamaño*

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 anlagen formation before the LTi stromal cells cross-talk interactions take place are not fully elucidated. In this study, we show that the inguinal LN anlagen 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-1VCAM-1 mesenchymal precursor cells become ICAM-1VCAM-1 cells, in a process independent of LTi cells and lymphoxygen b receptor (LTbR) signaling. The second step involves the maturation of ICAM-1VCAM-1 cells to ICAM-1VCAM-1 mucosal addressin cell adhesion molecule-1 (MAdCAM-1) organizer cells and depends on both LTi cells and LTbR. Addition of αLTbR agonist to LN organ cultures was sufficient to induce ICAM-1VCAM-1 cells to mature. In LtβR−/− embryos, both inguinal and mLN stromal cells showed a block at the ICAM-1intVCAM-1int stage, and, contrary to inguinal LNs, mLNs persist longer and contained LTi cells, which correlated with the sustained gene expression of IL-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: 4521–4530.

Lymph nodes (LNs) are specialized structures situated at strategic sites in the body along the lymphatic vasculature network. They enable filtration of Ags and pathogens and provide a site for Ag presentation to lymphocytes and induction of adaptive immune responses. LN development is not fully elucidated, although several studies have reported on the role of lymphoid tissue inducer (LTi) cells at late stages of LN formation. Recruitment of LTi cells that express the TNF family molecule lymphoxygen b receptor (LTbR)-expressing stromal cells. Subsequently, activation of two different pathways of the NF-kB transcription factor results in expression of high levels of ICAM-1,

VCAM-1, chemokines, and cytokines by the stromal cells (1–3). However, the early events regulating LN anlagen formation remain unknown.

LN formation is tightly linked to the development of the lymphatic system and in particular to the formation of lymph sacs. Although these events have been described a century ago, our understanding is still limited. In 1902, Sabin (4, 5) proposed that lymph sacs form from the budding and sprouting of endothelial cells from veins. In contrast, Huntington and McClure (6) suggested that lymph sacs develop from mesenchymal cells first, before venous endothelial sprouting occurs from the newly formed lymph sacs. More recently, the use of markers specific for lymphatic endothelial cells, such as VEGFR3 (7), Prox-1 (8), and the hyaluronic acid receptor Lyve-1, showed that Lyve-1−Prox-1− endothelial cells located in the anterior cardinal vein bud around embryonic day (E) 10.5–11.5 to form a lymph sac under the guidance of VEGF signals (9). These data are in agreement with Sabin’s model (10), which suggested that stromal cells derive from the lymph sac, which itself has an endothelial origin. Interestingly, a recent report showed that Prox-1−/− mouse embryos failed to develop lymph sacs, although they were still able to form primitive LN anlagen, indicating that endothelial cells are not required to form the LN primordium and that the LN anlage derives from distinct cell types (11).

We have previously reported on the isolation of inguinal LN (iLN) anlagen prior to the recruitment of LTi cells (12). In this study, we report on the sequence of events leading to the formation of iLN and mesenteric LN (mLN) anlagen in the mouse embryo. Our observations indicate that iLN anlage develops at the site of interactions between the budding endothelium and surrounding layers of mesenchymal cells that appear to invade the former. Combining confocal microscopy and FACS analysis of E15 iLNs, we show the appearance of the very first stromal cells coexpressing mesenchymal markers, like platelet-derived growth factor receptor (PDGFR)α, and the stromal markers ICAM-1 or
gp38/podoplanin. These cells, characterized by their intermediate levels of expression of ICAM-1 and VCAM-1 (I<sub>int</sub>V<sub>int</sub>), seem to give rise to ICAM-1<sup>high</sup>VCAM-1<sup>high</sup> (I<sub>high</sub>V<sub>high</sub>) mucosal addressin cell adhesion molecule-1 (MadCAM-1)<sup>T</sup> stromal organizer cells. This is supported by our findings using an in vitro organ culture system with an agonistic anti-LTβR Ab. The stromal cell maturation correlates with a gradual increase in mRNA levels of cytokine and chemokine genes. We also show that LTβR and LTI cells are required for the I<sub>int</sub>V<sub>int</sub> cells to become I<sub>high</sub>V<sub>high</sub> organizer cells. Finally, our results demonstrate that in E17 LTB/−/− mLNs, LTI cells are present and that LTβR is not fully necessary for the initial recruitment of LTI cells to the mLNs.

Our results indicate that lymphoid tissue stromal cells undergo a gradual maturation program to create the proper milieu for recruitment of LTI cells followed by the generation of the specific T and B cell areas in the mature organ.

**Materials and Methods**

**Mice**

BALB/c (H-2<sup>b</sup>), C57BL/6 (H-2<sup>d</sup>), Ly5<sup>b</sup>−/− (H-2<sup>d</sup>) (13) (C57BL/6 background), and R or y<sup>b</sup>−/− (H-2<sup>b</sup>) (14) 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<sup>+</sup> 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/dispase (R&amp;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, VCAM-1–APC clone 429, MadCAM-1–biotin clone MECA-367, VEGFR–biotin clone AFLA4, PDGFR–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), perlecian (rat; gift of Z. Lokmic, Bernard O’Brien Institute of Microsurgery, Melbourne, Australia), CCL21 (R&amp;D Systems), Lyve-1 (rabbit polyclonal; Abcam, Cambridge, MA), fibronectin clone FN–3E2 (mouse IgM; Sigma-Aldrich), ER–TR7 supernatant containing 200 nM 5-gp38/podoplanin.

**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), perlecian (rat; gift of Z. Lokmic, Bernard O’Brien Institute of Microsurgery, Melbourne, Australia), CCL21 (R&amp;D Systems), Lyve-1 (rabbit polyclonal; Abcam, Cambridge, MA), fibronectin clone FN–3E2 (mouse IgM; Sigma-Aldrich), ER–TR7 supernatant containing 200 nM 5-gp38/podoplanin.
(Biogenesis, Poole, U. K.). CD4 was directly conjugated using the Alexa Fluor 647 Ab 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 Axios Imager Z1 microscope (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 CD4+IL7Rα+ 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 sections 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 cell 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

![FIGURE 2. Mesenchymal cells invade the endothelial bud around E17 in iLNs. Immunofluorescence stainings of E17 iLN sections (×25/1.4 NA water lens) showing the endothelial markers perlecan in green (A), collagen type I in red (B, F, H, and J), and laminin α5 in red (D). The following molecules expressed by mesenchymal cells have been stained in green: PDGFRα (G), fibronectin (C and E), and CD44 (I). Note that fibronectin also stained some cells of the vasculature and CD44 also stained hematopoietic cells. A white line has been drawn encircling the endothelial cell compartment. The bottom row shows the DAPI (white) staining the cell nuclei. Scale bar, 50 µm (A).](http://www.jimmunol.org/)
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 anlage 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 (I2V2) and PDGFRα+ that correlated with the fibroblast-like cells of the surrounding mesenchyme in Fig. 1B; and 2) the ICAM-1 single-positive (I+V2) 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 (IintVint) that were PDGFRα+ and gp38/podoplanin+, suggesting that they derived from the I−V−

FIGURE 3. Ontogeny of stromal organizer cells. Emergence of the IhighVhigh stromal organizer cells in LNs. FACS analysis of single-cell suspensions of iLN at E15 and E17 showing the recruitment of CD45+ hematopoietic cells and the concomitant phenotypic changes in the CD45− stromal cells. Percentages shown in histogram correspond to CD45− stromal cells and CD45+ hematopoietic cells. A, in iLNs, four different stromal cell populations were distinguished according to their level of expression of ICAM-1 and VCAM-1, and the levels of PDGFRα, gp38/podoplanin, MAdCAM-1, and VEGFR3 are shown for each stromal population: I−V− (black) expressed PDGFRα, IintVint (blue) expressed gp38/podoplanin and PDGFRα, I+V2 (gray) expressed gp38/podoplanin, MAdCAM-1, and VEGFR-3 (E15 and E17), and IhighVhigh (red) expressed PDGFRα, gp38/podoplanin, and MAdCAM-1 (E17). The ICAM-1/VCAM-1 expression profile of the CD45− cells from iLNs changed between E15 and E17 independently of whether cells were harvested and analyzed on the same day or in different days. The gates in A have been set up to fit according to the profile of the different ICAM and VCAM expression levels in the cell populations rather than using the same for both E15 and E17. B, in mLNs, the same four stromal cell populations were identified. The I+V− (gray) and IhighVhigh (red) expressed MAdCAM-1 (E15 and E17). Percentages shown in scatter plots correspond to CD45− stromal cells according to their expression of ICAM-1 and VCAM-1. Results are representative of at least three independent experiments.
mesenchymal cells. At E17, the mature stromal organizer cells were identified by their high expression of ICAM-1 and VCAM-1 (I\(^{\text{high}}\)V\(^{\text{high}}\)) as well as MadCAM-1, a late differentiation marker. Mature organizer cells also expressed PDGFR\(\alpha\) and gp38/podoplanin but not VEGFR3, suggesting that their progenitors were the I\(^{\text{int}}\)V\(^{\text{int}}\) cell population.

The I\(^{\text{V}}\)− 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\(^{\text{V}}\)− gp38/podoplanin− VEGFR3− PDGFR\(\alpha\)−, whereas the mesenchymal cell compartment encompassed the I\(^{\text{V}}\)−, I\(^{\text{int}}\)V\(^{\text{int}}\), and I\(^{\text{high}}\)V\(^{\text{high}}\) MadCAM-1+ gp38/podoplanin+ PDGFR\(\alpha\)+ VEGFR3− cells.

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

Blocking of LT\(\beta\)R in pregnant female mice has shown that mLNs are the first to develop in the embryos and are followed by formation of LN 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 those 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 to those in iLN counterparts. Indeed, E15 mLNs showed an increased frequency of the I\(^{\text{V}}\)−V\(^{\text{int}}\) cell population, explained by the earlier development of the mLN anlage compared with iLN. However, the same three compartments were still identifiable: 1) mesenchymal cells that were I\(^{\text{V}}\)−V\(^{\text{int}}\); 2) I\(^{\text{int}}\)V\(^{\text{int}}\) cells; and 3) I\(^{\text{V}}\)− endothelial cells that also expressed MadCAM-1 (Fig. 2B, top panel). At E17, the mature stromal organizer cells I\(^{\text{high}}\)V\(^{\text{high}}\) were present expressing MadCAM-1, as well as the I\(^{\text{V}}\)− mesenchymal and the I\(^{\text{int}}\)V\(^{\text{int}}\) cells. However, the I\(^{\text{V}}\)− endothelial cells were not clearly identifiable in the mLNs at this stage (Fig. 3B).

Cellular localization and expression of different molecules were assessed by immunofluorescence staining in E18 iLNs and mLNs. Both organs showed the presence of CD4+IL-7R+ LTi cells as well as stromal organizer cells that expressed RANKL and MadCAM-1 (Fig. 4). Some lymphatic endothelial cells in the developing subcapsular sinus surrounding the E18 mLNs and iLNs coexpressed MadCAM-1 and Lyve-1, showing no difference in the presence and structure of the lymphatic endothelium between iLN and mLN (Fig. 4, middle panels). Thus, the absence of an I\(^{\text{V}}\)− endothelial cell population in E17 mLNs compared with iLNs might be due to the stripping of the mLN capsule during dissection of the organs prior to FACS analysis (Fig. 3A, 3B).

**Gradient of gene expression of stromal organizer markers during maturation of the mesenchyme**

To confirm the sequence of maturation of the mesenchyme, we assessed the mRNA levels of several genes that are characteristic of stromal cells. The small number of cells present in embryonic iLNs prevented a more in-depth analysis. To have a significant number of stromal cells for these assays, CD45− cell populations (I\(^{\text{V}}\)−, I\(^{\text{int}}\)V\(^{\text{int}}\), and I\(^{\text{high}}\)V\(^{\text{high}}\)) were isolated from E18 mLNs, and the gene expression profile was analyzed by quantitative PCR (qPCR).

The expression of most of the genes analyzed was nil in I\(^{\text{V}}\)− cells with the exception of Tnf-R1 and Ccl21. All CD45− cells expressed Lt\(\beta\)R, but only the differentiating I\(^{\text{int}}\)V\(^{\text{int}}\) and I\(^{\text{high}}\)V\(^{\text{high}}\) cells expressed RelB. The I\(^{\text{high}}\)V\(^{\text{high}}\) stromal organizer cells expressed the highest levels of homeostatic chemokines (Ccl21, Cc119, and Cxc113) (18), RANKL and IL-7, confirming their potential to attract and stimulate LTi cells (Fig. 5). The I\(^{\text{int}}\)V\(^{\text{int}}\) and I\(^{\text{high}}\)V\(^{\text{high}}\) cells expressed Mmp9, which has been shown to increase cellular
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\textsuperscript{high}V\textsuperscript{high} cells showing the highest levels, followed by I\textsuperscript{int}V\textsuperscript{int} and I\textsuperscript{2}V\textsuperscript{2} 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\textsuperscript{int}V\textsuperscript{int} stage

LN development is strictly dependent on LTi cells and LTβR signaling, as adult Ror\textsuperscript{2/2}, LtβR\textsuperscript{2/2}, and Lta\textsuperscript{−/−} 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\textsuperscript{high}V\textsuperscript{high} stage. FACS analysis of stromal cell populations from E15 iLN and mLN anlagen from WT, LtβR\textsuperscript{−/−}, and Ror\textsuperscript{2/2} showed the emergence of the I\textsuperscript{int}V\textsuperscript{int} cells in all strains (Fig. 6A). However, at E17, the iLNs were difficult to find in both LtβR\textsuperscript{−/−} and Ror\textsuperscript{−/−} 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\textsuperscript{high}V\textsuperscript{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 LtβR\textsuperscript{−/−} or Ror\textsuperscript{−/−} 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\textsuperscript{int}V\textsuperscript{int} to I\textsuperscript{high}V\textsuperscript{high} mature organizer cells (Fig. 6A).

Recruitment of CD45\textsuperscript{+} cells to iLNs and mLNs between E15 and E17 was impaired in LtβR\textsuperscript{−/−} and Ror\textsuperscript{2/2} embryos. For example, in WT iLNs, we observed an 18-fold increase in the...
Our analyses showed that LTβR engagement and the presence of LTi cells were required for the appearance of the I\textsuperscript{high}\textsuperscript{high} mature organizer cells but not for progression of mesenchymal cells from I\textsuperscript{low}\textsuperscript{low} toward the I\textsuperscript{int}\textsuperscript{int} compartment.

**LTβR-induced maturation of stromal cells from I\textsuperscript{int}\textsuperscript{int} to I\textsuperscript{high}\textsuperscript{high}**

We next tested whether the I\textsuperscript{int}\textsuperscript{int} stromal cells were the precursors of the I\textsuperscript{high}\textsuperscript{high} organizer cells. E14 mLNs and E15 iLNs were stimulated in organ cultures with an agonistic antiLTβR Ab and the ICAM-1 and VCAM-1 expression profiles of the CD45\textsuperscript{+} cell populations were assessed by FACS analysis. As shown in Fig. 6B, a significant increase in the frequency of I\textsuperscript{high}\textsuperscript{high} cells was detected. Of note, E14 mLNs and E15 iLNs contained very few if any I\textsuperscript{high}\textsuperscript{high} cells, suggesting that LTβR stimulation induced either those few cells to proliferate or the I\textsuperscript{int}\textsuperscript{int} cells to mature, becoming I\textsuperscript{high}\textsuperscript{high} organizer cells. Cell counts prior to and after LTβR stimulation in organ cultures demonstrated no increase in cell numbers (data not shown). These results strongly suggest that the I\textsuperscript{int}\textsuperscript{int} cell population gives origin to I\textsuperscript{high}\textsuperscript{high} organizer cells and that signaling through the LTβR was necessary and sufficient to induce the maturation.

**LTβR is not fully required for the recruitment retention of LTi cells to mLNs**

The accumulation of LTi cells in the LN anlagen may result from active recruitment of these cells or proliferation of LTi cells or their precursors. A previous report showed that proliferation of hematopoietic cells was very rare in LN anlagen, indicating that recruitment of LTi cells and their precursors might be the dominant mechanism (19). IL-7Rα and CXCL13 (ligand of CXCR5 expressed by LTi cells) have been shown to play an important role in the recruitment of LTi cells, and IL-7 is thought to be an early localization signal, as late blockade of this cytokine had minor effects on LTi cell migration and LN development (19).

Cell sorting of mLN cells from E15 WT and LtβR\textsuperscript{−/−} embryos and qPCR were used to assess the effect of LTβR deficiency in stromal cell production of chemokines and survival factors for LTi cells. Cxcl13 and Il-7 were expressed at similar levels by the I\textsuperscript{int}\textsuperscript{int} cells in both WT and LtβR\textsuperscript{−/−} cells. We also noted that Ccl21 was mainly expressed by the I\textsuperscript{low}\textsuperscript{low} endothelial cells, and its expression was reduced but still present in LtβR\textsuperscript{−/−} E15 mLN cells (Fig. 7A) (2).

The presence of LTi cells was assessed in E17 iLNs and mLNs from WT and LtβR\textsuperscript{−/−} embryos. A very low percentage of both CD45\textsuperscript{+} cells and LTi cells was present in LtβR\textsuperscript{−/−} iLNs when compared with WT (Fig. 7B). Interestingly, whereas the percentage of CD45\textsuperscript{+} cells was reduced in LtβR\textsuperscript{−/−} compared with WT E17 mLNs, a similar frequency of LTi cells was found in both strains, corroborating previous findings in E16.5 Ltα\textsuperscript{−/−} embryos (19). Staining of sections of E16 mLNs from WT and LtβR\textsuperscript{−/−} and IL-7Rα showed the percentages of LTi cells. Note the presence of LTi cells in the LtβR\textsuperscript{−/−} E17 mLN and their absence in iLNs. C, Immunofluorescence staining of WT and LtβR\textsuperscript{−/−} E16 mLN sections (×40/1.4 NA water lens) showing the Lyve-1\textsuperscript{+} capsule (green), CD4\textsuperscript{+} LTi cells (blue), and in red, IL-7Rα (left panels), MadCAM-1 (middle panels), or RANKL (right panels). Note in the LtβR\textsuperscript{−/−} mLNs the presence of the capsule expressing Lyve-1 and MadCAM-1, the presence of LTi cells coexpressing IL-7Rα and CD4, and the absence of RANKL expression. Results are representative of two independent experiments.
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 spheroidal 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 IhighVhigh mature organizer cells by FACS analyses. E15 iLNs, that appear to lack LTi cells, do contain IintVintPDGFRα+ 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 IintVint gp38/podoplanin+ cell population will give rise to the mature IhighVhigh MadCAM-1+ stromal organizer cells. The common expression of PDGFRα by I−V−, IintVint, and IintVhigh cells and gp38/podoplanin by IintVint and IhighVhigh cells but not VEGFR3 suggests a precursor-product relationship between them. iLNs and mLNs anlagen develop at different times during embryogenesis, have different developmental requirement, and contain different frequencies of IintVint stromal cells. As shown before in newborn mice, embryonic mLNs have a higher percentage of IintVint stromal cells than iLNs but that does not reflect in a larger proportion of IhighVhigh organizer cells because the latter is lower in mLNs than in 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−/−, Ltβ−/−, and Lta−/− 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 cell organizer maturation. We have shown previously that in the absence of lymphotoxins α and hence lymphotoxin αβ2, the maturation of stromal cells was blocked at the IintVint stage (12). However, a role for LTβR engagement by ligands other than lymphotoxin αβ2 to induce the transition of I−V− mesenchymal cells to the IintVint stage could not be ruled out. Analysis of the Ltβ−/− 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. Lymphotoxin α and LTβR engagement and the presence of LTi cells were required for the second step of this process, for Ltβ−/− mouse embryos showed normal development of iLNs and mLNs up to E15 but stromal cell maturation did not progress further than the IintVint stage, and iLNs quickly recessed. Stimulation of E14 mLNs through LTβR induced a strong increase in the frequency of IhighVhigh 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 IintVint cells and the IhighVhigh MadCAM-1+ stromal organizer cells.

We investigated the gene expression profile of three CD45− cell populations from E18 mLNs, I−V−, IintVint, and IhighVhigh. Our
results showed that although Γ−V− mesenchymal precursors cells expressed LfβR mRNA, one of its downstream effectors, the transcription factor Relb, was expressed only in the maturing ΓintVint and ΓhighVhigh cells. This finding suggests that the first step of stromal cell maturation enables the LN anlagen to respond to LTβR 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 ΓhighVhigh cell population than in the ΓintVint cells. Concomitantly, mLN$s from E16.5 LfβR-deficient embryos, whose stromal cells are blocked at the ΓintVint cell stage, showed no expression of RANKL. In contrast, Cc121, Cc113, and Il-7 were expressed in both ΓintVint and ΓhighVhigh cell populations, indicating that these genes are less dependent on LTβR engagement for their full expression. In agreement with this, the mRNA levels of Cc113 and Il-7 were not decreased in E15 mLNs of LfβR-deficient embryos. Cc121 expression was significantly decreased in endothelial cells correlating with previous reports on the expression of CCL21 by endothelial cells of high endothelial venules and the role of LTβR-/- mice in the latter (30–34). These results are in agreement with the findings of Moyron-Quiroz and colleagues (35) showing that rudimentary mLNs have been found in newborn mice. These findings correlate with the fact that LTβR−/- mice still present in this mutant but showed a failure to induce the IintVint gp38/podoplanin+ cell population, able to recruit LTi cells by their expression of Cxcl13, and Ccl21 were expressed independently of lymphotixin α during the formation of inducible bronchial-associated lymphoid tissues. A recent report has indicated that retinoic acid expressed by neurons induces the expression of Cc113 in LN anlagen independently of LTβR signaling (36).

At E17 when iLN$s from LfβR−/- 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 LTβR signaling for the expression of these molecules (20). The absence of RANKL expression in the LfβR−/- mLNs 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 intracerebral adopter 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 LfβR−/- mice.

Based on the results shown in this study, we propose a two-step stromal cell maturation model (Fig. 8). First, the Γ−V− mesenchymal cells are primed by a yet unknown signal to give rise to an ΓintVint gp38/podoplanin+ cell population, able to recruit LTi cells by their expression of Il-7, Cc121, and Cc113 and to respond to LTβR engagement via acquisition of the expression of RelB. Second, the ΓintVint cells gave rise to the mature ΓhighVhigh MadCAM-1+ stromal organizer cells. Following these essential maturation steps during embryogenesis, LN stromal cells require stimulation by Il-7Rα+ 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 Γ−V− mesenchymal cell precursors, the signals that induce priming on those cells to become ΓintVint 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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