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Presumptive Lymph Node Organizers are Differentially Represented in Developing Mesenteric and Peripheral Nodes

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During murine embryogenesis, the formation of Peyer’s patches (PPs) is initiated by CD45+CD4+CD3- lymphoid tissue inducers that trigger adhesion molecule expression and specific chemokine production from an organizing stromal cell population through ligation of the lymphotoxin-β receptor. However, the steps involved in the development of lymph nodes (LNs) are less clear than those of PPs, and the characteristics of the organizing cells within the LN anlagen have yet to be documented. In this study, we show for the first time that the early anlage is bordered by an endothelial layer that retains a mixed lymphatic and blood vascular phenotype up to embryonic day 16.5. This in turn encompasses CD45+CD4+CD3- cells interspersed with ICAM-1/VCAM-1/mucosal addressin cell adhesion molecule-1, lymphotoxin-β receptor-positive, chemokine-producing cells analogous to the organizing population previously observed in PPs. Moreover, these LN organizers also express the TNF family member, TRANCE. Lastly, we show that the ICAM-1/VCAM-1/mucosal addressin cell adhesion molecule-1 cells present in peripheral and mesenteric LN form two discrete populations expressing either intermediate or high levels of these adhesion molecules but that the former population is specifically reduced in PLN. These findings provide a possible explanation for the well-known differences in developmental requirements for nodes at peripheral or mesenteric locations. The Journal of Immunology, 2004, 173: 2968–2975.

The correct generation of secondary lymphoid organs requires a high degree of cross-talk between local mesenchymal cells and circulating hemopoietic cells (1–7). Formation of the tissue lymphatic vasculature during murine embryogenesis is initiated at embryonic day 10.5 (E10.5) by budding from the larger veins, establishing a primordial lymph sac (8–11). Among the first markers to be expressed on the developing lymphatic endothelium are the homeobox gene Prox-1 (11, 12) and the lymphatic endothelium-restricted hyaluronan receptor Lyve-1, both of which become restricted to the lymphatics by E15.5.

Formation of the various lymphoid organs occurs after development of the tissue lymphatics by the action of CD45+CD4+CD3- cells (13, 14) that express surface bound lymphotoxin-α1β2 (LT), which engages the lymphotoxin-β receptor (LTβ-R) on stromal cells and provides the inductive signal for tissue remodeling (3, 15, 16).

These CD45+CD4+CD3- cells, which arise from a multipotent precursor in the fetal liver (17, 18), have been shown to induce Peyer’s patches (PPs) (7) and nasal-associated lymphoid tissue (19, 20), and therefore they have been assumed to play a similar role in the generation of lymph nodes (LNs). The cross talk between CD45+CD4+CD3- cells and local mesenchymal cells is considered to be the driving force behind PP formation. The understanding of the earliest developmental stages during PP genesis has been significantly increased by whole mount in situ hybridization studies (1–5). VCAM-1- and ICAM-1-positive cells have been shown to cluster on the intestinal wall as early as E15.5. These adhesion molecules are expressed on mesenchymal cells, and are induced in a LT-dependent manner by IL-7Rα+CD45+CD4++CD3- cells accumulating in the PP anlagen (1–5). From E18.5, mature lymphocytes start to colonize the PPs. The VCAM-1/ICAM-1 double-positive cells, which express the LTβ-R and produce both CCL19 (ELC) and CXCL13 (BLC; Ref. 3), have been denoted PP organizers, while the IL-7Rα+LTβ+CD4++CD3- cells have been termed PP inducers.

In contrast to PPs, the level of understanding of LN development is sketchy. The relative inaccessibility of developing LN anlagen has hampered investigation into the earliest stages of development, although recently, whole-mount in situ hybridization studies tracing expression of the LTβ-R have provided a first indication of the events underlying the initiation of LN formation during embryogenesis (21). In addition, whole-mount immunohistochemical analyses have allowed these events to be visualized in more detail (6). More recent, the first immunohistochemical visualizations of embryonic LN anlagen were described (22). As a result, it has become clear that in LN, as in PP, IL-7Rα+ cells accumulate in the LN anlagen leading to local, LT-dependent expression of VCAM-1 (6), while also ICAM-1-expressing cells were present in the anlagen (22).

Despite the apparent similarities in the development of LNs and PPs, conclusive data on the cellular make-up of the LN anlage is limited (22). It is also not clear whether LN anlagen contains a cell population functionally homologous to the mesenchymal PP organizers. Moreover, data from different gene-targeted mice suggest a previously unappreciated complexity within the developmental
pathway of individual LNs. In several mouse models, like the LTβ−/− and the CXCL13−/− or CCR5−/− mice, different requirements were observed for the mesenteric LNs (MLNs) vs the majority of peripheral LNs (PLNs; Refs. 3, 23, and 24).

In this study, we describe in detail the cellular make-up of LN anlagen in murine embryos. We demonstrate for the first time that phenotypic and functional homologues to the PP organizers are indeed present in LNs, and present new evidence that differences in subpopulations of these cells in PLNs and MLNs may explain the observed dichotomy in development of these two LN systems.

Materials and Methods

Mice

C57BL/6 mice were purchased from Harlan (Horst, The Netherlands) and kept under routine laboratory conditions.

Time-pregnancies

Mice were mated overnight, and the day of vaginal-plug detection was marked as E0.5. Pregnant females were sacrificed at different time points, and embryos were harvested and either frozen directly in OCT compound for TRANCE visualization, or fixed in 4% formaldehyde for 3 h and transferred to a 20% (w/v) sucrose solution in PBS. The following day, animals were frozen in OCT compound.

Immunofluorescence microscopy

Six-micrometer cryosections were fixed in dehydrated acetone for 2 min and air-dried for an additional 15 min. Endogenous avidin was blocked with an avidin-biotin block (Vector Laboratories, Burlingame, CA) supplemented with 10% (v/v) mouse serum and 10% (v/v) goat serum. Sections were incubated with primary Ab for 1 h at room temperature followed by a 30 min incubation with Fluor-Alexa-labeled conjugate (Molecular Probes, Eugene, OR) when needed. Sections were embedded in Fluorstab (ICN Biomedicals, Aurora, OH) and analyzed on a Nikon Eclipse E800 microscope (Nikon Europe, Haarlem, The Netherlands).

Flow cytometry and cell sorting

LN rudiments were dissected using a stereomicroscope, and single cell suspensions were made by digestion with 0.5 mg/ml collagenase type IV (Sigma-Aldrich, St. Louis, MO) in PBS, 2% FBS for 30 min at 37°C with constant stirring. Flow cytometric analysis was performed on a FACSCalibur (BD Biosciences, San Jose, CA) and cell sorting was performed on a MoFlo (DakoCytomation, Glostrup, Denmark).

Antibodies

For immunohistology and flow cytometry, the following Abs were used: GK1.5 (anti-CD4); MEC-367 (anti-mucosal addressin cell adhesion molecule-1 (MAdCAM-1)); 6B2 (anti-B220); MP33 (anti-CD45); and GK1.5 (anti-CD4); MECA-367 (anti-mucosal addressin cell adhesion molecule-1 (MAdCAM-1)); 6B2 (anti-B220); MP33 (anti-CD45); and anti-CD3

Real-time quantitative PCR

RNA was extracted from sorted MLN populations using TRizol (Invitrogen Life Technologies, Gaithersburg, MD), and reverse transcribed with oligo(dt)12–18 (Life Technologies) and random hexamer primers (Invitrogen Life Technologies) using standard protocols. Quantitative real-time PCR was performed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). The reaction mixture was composed of SYBR Green Mastermix, 300 nM of each primer, and cDNA in a total volume of 20 μl according to the manufacturer’s instructions. Primers were designed using Primer Express software and guidelines (Applied Biosystems). The following sequences were used: CXCL13 forward, CATAGATCGGATCTCAAGTTACGCC, and reverse, TCTTGTCGAGAGACCACTTCA; CCL21 forward, GCTGCAAGAGAACGACGACA, and reverse, CTGTAACCCACCTGTTGA; CCL19 forward, ATGCCGAAGACTGCGCTTACGAT.

Results

Identification of LN anlagen

To carry out a detailed immunohistochemical analysis of the developing murine LN anlagen and its constituent cell populations, we prepared serial transverse cryo-sections of whole embryos at E16.5, the stage at which development of most LNs is already initiated (15, 16, 22). At putative sites of LN development, cell clusters consisting of mainly CD4+ cells could be detected (Fig. 1A). These CD4+ cells were CD45+ and CD3− (data not shown), consistent with the absence of mature αβTCR+ T cells in the prenatal murine immune system, and were found in close association with cells expressing
the mucosal addressin, MadCAM-1 (Fig. 1B). In Fig. 1A, CD45^+CD4^-CD3^- cells can be seen clustering near the aortic wall (visible on the left), a site of presumed sacral/iliac LN development. Fig. 1B shows a high power magnification of a LN anlage at a brachial LN location. Although abundant MadCAM-1 staining was detected, careful morphological analysis failed to identify high endothelial venules at E16.5, suggesting that the initial migration of hemopoietic cells to the LN anlagen precedes the formation of these specialized structures.

The earliest cells to enter the LN or PP anlagen are expected to be IL-7Ra^+ cells emigrating from the fetal liver, which differentiate into CD45^+CD4^-CD3^- cells (6, 17, 22). To study the spectrum of hemopoietic cells present within the developing LN, E16.5 LN anlagen were analyzed for expression of the IL-7Ra chain, in combination with various lineage markers (Fig. 1, C–E, shows an inguinal LN anlage, while Fig. 1F depicts a sacral/iliac LN anlage). As expected, the majority of IL-7Ra^+ cells in the early LN anlagen expressed the hemopoietic marker CD45 (Fig. 1C; Ref. 22). CD45^+CD4^-CD3^- cells would be predicted to form a large proportion of the hemopoietic cells at this time point, and indeed most of the IL-7Ra^+ cells coexpressed CD4, although an IL-7Ra^-CD4^- population was also present (Fig. 1D). This remaining CD4^-IL-7Ra^- population likely contains IL-7Ra^- fetal liver-derived precursors to CD45^-CD4^-CD3^- cells. In addition, B220 expressing IL-7Ra^- cells were also present in the LN anlagen, albeit in very low numbers (Fig. 1E). It remains to be determined whether these B220^- cells are B cells, or an additional precursor population.

Finally, groups of both CD4^+ and CD4^- cells among the CD45^-CD3^- cell population also expressed the Ig superfamily adhesion molecule, ICAM-1 (Fig. 1F). As the latter may well be the immediate precursors to the CD45^-CD4^-CD3^- inducer cell population, it is possible that the expression of ICAM-1 is involved in recruitment or retention of these precursors within the LN anlagen.

The LN anlagen are bordered by differentiating lymphatic endothelium

The lymphatic endothelium of embryonic LNs is generated from local blood vessel-endothelial cells that are induced to differentiate toward a lymphatic phenotype. In the E16.5 LN anlagen, both at mesenteric and peripheral locations (Figs. 2A–H, and I–J, respectively), the most distal cell layer consists of cells expressing the lymphatic endothelium specific hyaluronan receptor Lyve-1 (Fig. 2, B and I; Refs. 25 and 26). A large portion of this lymphatic endothelium coexpresses MadCAM-1 (Fig. 2, B and J), while several cells also stain positive for the junctional adhesion molecule,
FIGURE 4. Two populations of LN organizers in the LN anlagen. A and D, In both E16.5 and day 0, MLNs, ICAM-1/VCAM-1\textsuperscript{high} (R3), and ICAM-1/VCAM-1\textsuperscript{int} (R2) populations are present. B and E. The IV\textsuperscript{high} population also expressed high levels of MAdCAM-1 (IVM\textsuperscript{high}), while the IV\textsuperscript{int} population expressed MAdCAM-1 at low to intermediate levels (IVM\textsuperscript{int}), both at E16.5 and day of birth. C and F. Both the IVM\textsuperscript{high} (light line) and IVM\textsuperscript{int} (dark line) population express LT\(\beta\)-R. Highest level of LT\(\beta\)-R was expressed by the IVM\textsuperscript{int} population. As a negative control the CD45\textsuperscript{+} cells within R1 are shown (filled histogram; representative results of at least four different flow cytometric analyses).
VE-cadherin (Fig. 2, C–E), which is expressed on both blood vessel- and lymphatic endothelium (27). However, in contrast to the situation in adult animals, lymphatic endothelium at E16.5 also shows expression of the blood vessel endothelial restricted marker MECA-32 (Figs. 2, F–H and J; Ref. 28). These data demonstrate that at E16.5, the endothelial cells which line the PLN as well as MLN anlagen, are undergoing phenotypic changes, converting from blood vessel endothelium to lymphatic endothelial cells.

**Presence of a LN homologue to the PP organizer**

Previous studies of fetal intestines identified a population of VCAM-1/ICAM-1/MAdCAM-1-positive cells that was proposed to act as PP organizers by expressing adhesion molecules and homeostatic chemokines upon LTβ-R triggering (2–4, 6). In search of a similar population in developing LNs, we assessed the expression of adhesion molecules in E16.5 LN anlagen. Fig. 3, A and B, show a representative example of a MLN anlage, while Fig. 3, C and D, display an anlage at a peripheral location, situated adjacent to one of the large vessels (left side of picture). The MAdCAM-1+ cells within the LN anlagen showed coexpression of VCAM-1 (Fig. 3, A and C) as well as ICAM-1 (Fig. 3, B and D), thus identifying these cells as the LN equivalent of the PP organizing cells in fetal intestine (1, 3, 4). ICAM-1+VCAM-1+MAdCAM-1+ (IVM+) cells were concentrated in a polarized fashion in the outer regions of the LN anlagen (Fig. 3, A, B, D, and E). In addition, VCAM-1+ICAM-1+MAdCAM-1+ cells were present in the deeper regions of the LN anlagen. These VCAM-1+LN-organizer cells also expressed the TNF family member, TRANCE (Fig. 3, E–G). In fact, throughout the embryo, TRANCE expression was only observed in the developing LNs and bones (data not shown). TRANCE was previously implied in regulating the number of CD45+CD4+CD3+ cells in developing LN-nodes (29), and the fact that we now show abundant expression of TRANCE within the LN anlagen, would suggest a role for this molecule in local differentiation of CD45+CD4+CD3+ cells.

**Different populations of functional LN organizers**

ICAM-1/VCAM-1/MAdCAM-1 triple-positive cells in the developing PPs exert their function through the production of chemokines upon ligation of the LTβ-R (3). To determine whether similar functions could be fulfilled by the IVM+ putative LN organizers, MLN anlagen were dissected at E16.5 and at day of birth, and analyzed by flow cytometry (Fig. 4). Based upon expression of ICAM-1, VCAM-1, and MAdCAM-1, two different populations of IVM+ cells could be observed, both at E16.5 and day of birth (Fig. 4, A and D). The largest population expresses all three adhesion molecules at intermediate levels (IVMint, Fig. 4, A, B, D, and E, R2). An additional, clearly distinct, smaller population expresses high levels of IVM (IVMhigh) (Fig. 4, A, B, D, and E, R3), and this population resembles the previously described organizing population in the PPs (3, 30). Furthermore, both populations displayed expression of the LTβ-R, further supporting the likelihood that they represent LN organizers analogous to those present in developing PPs. However, surprisingly, the IVMhigh population expressed higher levels of LTβ-R than the IVMhigh cells (Fig. 4, C and F).

The production of homeostatic chemokines is recognized as one of the defining properties of the PP organizers. Hence, we assessed production of the chemokine mRNA in both the IVMhigh and IVMint populations sorted to purity from E18.5 MLN anlagen by real-time quantitative PCR. As shown in Fig. 5, after normalization to β-actin (or cyclophilin, data not shown) both the IVMhigh and IVMint populations in the MLN anlagen produced mRNA for the homeostatic chemokines CXCL13, CCL21, and CCL19. However, IVMhigh cells clearly contained higher levels of chemokine mRNA. As a control, the IVM−, LTβ-R-negative population did not produce any of the homeostatic chemokines (data not shown). These data identify the IVMhigh and IVMint populations as functional LN organizers.

**PLNs and MLNs contain different organizing populations**

Different requirements exist for the generation of PLNs and MLNs. Several gene-targeted mice lack one or more PLNs, while MLN formation is unaffected. Because the mechanism underlying this difference is unknown, we set out to analyze the LN organizers in both PLNs and MLNs at day of birth. Both sets of LNs were found to contain the IVMint and IVMhigh populations of LN organizers (Fig. 6A), with highest levels of MAdCAM-1 on the IVMhigh cells (Fig. 6B). Strikingly however, the IVMhigh population was severely diminished in PLNs compared with MLNs (~10-fold reduction) while the IVMhigh population showed only a slight decrease. As a result, the ratio of IVMhigh/IVMint cells, which in the MLN ranged from 0.1 to 0.4, was in the PLN completely opposite, ranging from 1 to 1.9.

To further compare the organizing populations from MLNs and PLNs, expression of the LTβ-R was analyzed. In line with MLN organizers, IVMint and IVMhigh cells from PLNs expressed the LTβ-R (Fig. 6C). Levels of LTβ-R were again highest in the IVMhigh population, indicating that comparable populations of organizers are present in the PLNs and MLNs. As the IVMint population in MLNs is by far the most prevalent in terms of absolute cell numbers (on average 10-fold more cells then the IVMhigh, data not shown), and because these express the highest levels of LTβ-R, their virtual absence from PLN may well explain why these particular nodes have different signaling requirements to MLN during embryonic development.

**Discussion**

In this study, we have identified two distinct LN-organizing populations in developing LN anlagen. These anlagen were characterized by the accumulation of CD45+CD4+CD3+ cells, which were found to be in close proximity to MAdCAM-1, VCAM-1, and ICAM-1 expressing cells. A layer of Lyve-1-expressing lymphatic endothelium, which forms the most distal part of the LN anlage, ensheathes these IVM+ cells. The coexpression of the lymphatic endothelial marker, Lyve-1, and the blood vascular marker,
FIGURE 6. Differential presence of LN organizers in PLNs and MLNs. Both PLNs (inguinal) as well as MLNs were analyzed for the presence of IVM\textsuperscript{high} and IVM\textsuperscript{int} populations at one day after birth. A, In PLNs, both populations were reduced when compared with MLNs, with the IVM\textsuperscript{int} population being severely diminished (23.9 to 2.2%) while the IVM\textsuperscript{high} was less affected (reduced from 8.9 to 4.2%). B, Similarly to MLNs, PLN IVM\textsuperscript{int} cells expressed lower levels of MAdCAM-1 when compared with the IVM\textsuperscript{high} population. C, Both populations of organizers in the PLNs express the LT\textbeta-R. Representative analysis of at least three independent experiments is shown.
MECA-32, by the endothelial cells within the LN anlagen reflects the fact that these cells have recently sprouted from the large veins, and are still undergoing transition from a vascular to a lymphatic phenotype. This “bipolar” endothelial cell layer, which has not previously been reported, eventually differentiates to form the subcapsular sinus of the LN.

Inward from the layer of VCAM-1/ICAM-1/MAdCAM-1 triple-positive cells, mostly VCAM-1 and ICAM-1 single-positive cells were found to extend into the deeper regions of the developing LN. A fraction of the single ICAM-1⁺ cells are CD45⁺CD4⁺CD3⁻ cells, and the expression of ICAM-1 on these cells may well have a function in the clustering of these cells in the LN anlage. However, because LN development is normal in mice that lack the ICAM-1 ligand, LFA-1 (31), it is likely that there is redundancy among the adhesion molecules that fulfill this function.

Our studies also show that CD45⁺CD4⁺CD3⁻ cells in the LN anlage express the IL-7Rα chain (32). However, not all IL-7Rα⁺ cells expressed CD4, indicative of the fact that the direct precursor to CD45⁺CD4⁺CD3⁻ cells, an IL-7Rα⁺ population derived from the fetal liver (17, 18), might also lodge in these LN anlagen. One of the factors responsible for the accumulation of CD45⁺CD4⁺ CD3⁻ cells in the LN anlage is TRANCE, because mice with deficient TRANCE-signaling have severely diminished numbers of CD45⁺CD4⁺CD3⁻ cells in these anlagen (6, 22, 29). In this study, we show that expression of TRANCE is restricted to the LN anlagen, suggesting that TRANCE acts locally to either recruit, mediate survival, or induce differentiation of CD45⁺CD4⁺CD3⁻ cells. TRANCE was previously shown to be present on CD45⁺CD4⁺CD3⁻ cells, while the TRANCE-R is expressed by both CD45⁺CD4⁺CD3⁻ and CD45⁺CD4⁻CD3⁺ cells (29), but now we show that TRANCE is also expressed on VCAM-1⁺ LNsorganizing cells within the LN anlage. This nonhemopoietic expression of TRANCE could thus further explain the inability to completely rescue the phenotype of the TRANCE⁻/⁻ mice by hemopoietic cell-specific transgenic TRANCE overexpression (29).

Dissection of PLN and MLN from newborn and embryonic animals provided us with the opportunity to study the properties of the IVM⁺ cells constituting the presumptive LN organizers. Flow cytometric analysis of both MLNs and PLNs revealed the presence of two populations of IVM cells: IVMint and IVMhigh cells. Both populations expressed the LTβR, and are, therefore, phenotypically homologous to the previously described PP organizers. The IVM⁺ and IVMhigh cells produced mRNA for CXCL13, CCL21, and CCL19, confirming that these are the functional counterparts of PP organizing cells (3).

The development of LNs at either peripheral or mesenteric sites is known to be regulated by distinct signals. For example, targeted deletion of either LTβ, components of the IL-7R signaling complex, or the CXCL13/CXCR5 pair disrupts development of PLNs but has little or no effect on the development of MLNs (3, 23, 24). The cellular basis for such differences has until now remained unclear. However, our finding in this manuscript that PLN anlagen differ markedly from MLN anlagen in the relative abundance of IVM⁺ cells may provide an explanation for this discrepancy. Because IVM⁺ cells are the organizing population that expresses the highest levels of LTβ-R, the decrease of these cells in PLNs could proof to be a limiting factor for PLN development in case of sub-optimal LTβ-R stimulation, for instance in the absence of LTβ, IL-7R triggering, or CXCR5 triggering. In LTβ⁻/⁻ mice, a role was postulated for LTβ-R triggering via LTα and homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D, for HVEM, a receptor expressed by T lymphocytes (LIGHT) (33). However, this is likely to be a less potent signal then LT, reducing the level of LTβ-R triggering. Furthermore, in the absence of IL-7R ligation, TRANCE-R signaling is still able to induce surface LT on inducer cells (6). However, if a reduction in LT-inducing signals leads to a lower number of cells that initiate expression of LT, again LTβ-R levels could become limited. The same, of course, holds true for the CXCL13/CXCR5 signaling pathway, which controls the accumulation of LT-expressing inducer cells at sites of LN development (3, 13, 32).

In summary, developing LN anlagen contain two distinct populations of organizing cells that express the LTβ-R, adhesion molecules ICAM-1, VCAM-1, and MAdCAM-1, and produce CXCL13, CCL21, and CCL19. In PLNs, the number of organizing cells is greatly diminished, with the LTβ-RhighIVM⁺ population being most severely reduced. This leads to distinct stromal micro-environments in PLN and MLN anlagen, providing an explanation for the divergent developmental requirements observed for these two LN systems.

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