Presumptive lymph node organizers are differentially represented in developing mesenteric and peripheral nodes


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Chapter 3

Abstract

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. Here, 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/MAdCAM-1 expressing, 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/MAdCAM-1 cells present in peripheral and mesenteric lymph nodes 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.
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Introduction

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

Formation of the various lymphatic organs occurs after development of the tissue lymphatics by the action of CD45⁺CD4⁺CD3⁻ cells that express surface bound Lymphotoxin-αβ2 (LT), which engages the Lymphotoxin-β Receptor (LTβR) on stromal cells and provides the inductive signal for tissue remodeling. These CD45⁺CD4⁺CD3⁻ cells, which arise from a multipotent precursor in the fetal liver, have been shown to induce Peyer’s patches (PPs) and nasal associated lymphoid tissue (NALT), and they have therefore 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. These adhesion molecules are expressed on mesenchymal cells, and are induced in a LT dependent manner by Il7r⁺LTβ⁺CD4⁺CD3⁻ cells accumulating in the PP-anlagen. From E18.5, mature lymphocytes start to colonize the PPs. The VCAM-1/ICAM-1 double positive cells, which express the LTβR and produce both CCL19 (ELC) and CXCL13 (BLC), have been denoted PP-organizers, while the Il7r⁺LT⁺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 LTβR have provided a first indication of the events underlying the initiation of LN formation during embryogenesis. In addition, whole mount immunohistochemical analyses have allowed these events to be visualized in more detail. More recent, the first immunohistochemical visualizations of embryonic LN-anlagen were described. As a result, it has become clear that in LN as in PP, Il7r⁺ cells accumulate in the LN-anlagen leading to local, LT-dependent expression of VCAM-1, while also ICAM-1 expressing cells were present in the anlagen.

In spite of the apparent similarities in development of LNs and PPs conclusive data on the cellular make-up of the LN anlage is limited. It is also not clear whether LN-anlagen contain 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 CXCR5⁻⁻ mice, different requirements were observed for the presence of the
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mesenteric LNs (MLNs) versus the majority of peripheral LNs (PLNs) 3, 24, 25.

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 sub-populations of these cells in PLNs and MLNs may explain the observed dichotomy in development of these two lymph node systems.

Materials and Methods

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

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

Immuno-fluorescence microscopy
6 µm cryo-sections were fixed in dehydrated acetone for 2 minutes and air-dried for an additional 15 minutes. Endogenous avidin was blocked with an avidin-biotin block (Vector Labs) supplemented with 10% (v/v) mouse serum and 10% (v/v) goat serum. Sections were incubated with primary antibody for 1 hour 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 Inc., Aurora, OH) and analyzed on a Nikon Eclipse E800 microscope (Nikon Europe BV, 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, St. Louis, MO) in PBS/2%FBS for 30 min at 37° C, with constant stirring. Flow cytometric analysis was performed on a Facs Calibur (Becton Dickinson, San Jose, CA), and cell sorting on a MoFlo (Dako Cytomation, Glostrup, Denmark)

Antibodies
For immunohistology and flow cytometry the following antibodies were used: GK1.5 (anti CD4); MECA-367 (anti MAdCAM-1); 6B2 (anti B220); MP33 (anti CD45); and anti ICAM-1 (Pharmingen). All these were affinity purified from hybridoma cell
Presumptive lymph node organizers are differentially represented in developing mesenteric and peripheral nodes. Culture supernatants with protein G-Sepharose (Pharmacia, Uppsala, Sweden) and labeled with Alexa-Fluor 488 or Alexa-Fluor-594 (Molecular Probes). The mAbs A7R34 (anti Il7r, eBioscience) and 429 (anti VCAM-1, Pharmingen) were labeled with biotin, whereas MECA32, 11D4.1 (anti VE-Cadherin and CD144) (Pharmingen), 4H8Wh2 (anti LTβR, Alexis biochemicals) and IK22/5 (anti-TRANCE, e-Bioscience) were used without conjugation. The rabbit polyclonal antibody to human Lyve-1 has been described previously. For fluorescence microscopy, primary antibodies were visualized with Alexa-Fluor-594 labeled Avidin, Alexa Fluor-594 labeled anti-rat IgG, or Texas red or FITC-labeled anti rabbit IgG as appropriate. To assure specificity of the used antibodies, isotype control primary antibodies, as well as conjugate-alone controls were used. In the case of Lyve-1, total rabbit serum was used as control (data not shown).

**Real time quantitative PCR**

RNA was extracted from sorted MLN populations using TRIZOL (Gibco BRL, Gaithersburg, MD), and reverse transcribed with oligo dT12–18 (Life Technologies) and random hexamer primers (Life Technologies) using standard protocols. Quantitative real-time PCR was performed on an ABI Prism® 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The reaction mixture was composed of SYBRgreen 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 Fw CATAGATCGGATCTAAGTACGCC Rev TCTTGGTCAGATCACACTTCA, CCL21 Fw GCTGCAAGAGGAACAGACAGAC Rev CGTGAACCACCCAGCTTGA, CCL19 Fw ATGCAGGAAGAAGTGCCTGCC Rev AGCAGGAAGGCTTCCACGAT.

**Results**

**Identification of lymph node anlagen**

To carry out a detailed immunohistochemical analysis of the developing murine LN anlagen and its constituent cell populations we prepared serial transverse cryosections of whole embryos at E16.5, the stage at which development of most LNs is already initiated. At putative sites of LN development, cell clusters consisting of mainly CD4+ cells could be detected (Figure 1A). These CD4+ cells were CD45+ and CD3- (data not shown), consistent with the absence of mature αβ TCR+ T cells in the pre-natal murine immune system, and were found in close association with cells expressing the mucosal addressin MadCAM-1 (Figure 1B). In figure 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. Figure 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 (HEVs) at E16.5, suggesting that the initial migration of hematopoietic 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 Il7r+ cells emigrating from the fetal liver, which differentiate into CD45+CD4+CD3- cells. To study the spectrum of hematopoietic cells present within the developing LN, E16.5 LN-anlagen were analyzed for expression of Il7r, in combination with various lineage markers (Figure 1C-E show an inguinal LN-anlage, while Figure 1F depicts a sacral/iliac LN-anlage). As expected, the majority of Il7r+ cells in the early LN anlagen expressed the hematopoietic marker CD45 (Figure 1C). CD45+CD4+CD3- cells would be predicted to form a large proportion of the hematopoietic cells at this time point, and indeed most of the Il7r+ cells co-expressed CD4, although an Il7r+CD4- population was also present (Figure 1D). This remaining CD4+Il7r population likely contains Il7r+ fetal liver derived precursors to CD45+CD4+CD3- cells. In addition, B220 expressing Il7r+ cells were also present in the LN-anlagen, albeit in very low numbers (Figure 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 (Figure 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 towards a lymphatic phenotype. In the E16.5 LN-anlagen, both at mesenteric and peripheral locations (Figures 2A-D and E-F respectively), the most distal cell layer consists of cells expressing the lymphatic endothelium specific hyaluronan receptor Lyve-1 (Figure 2). A large portion of this lymphatic endothelium co-expresses MAdCAM-1 (Figures 2B and E), while several cells also stain positive for the junctional adhesion molecule VE-cadherin (Figure 2C), which is expressed on both blood vessel- and lymphatic endothelium. However, in contrast to the situation in adult animals, lymphatic endothelium at E16.5 also shows expression of the blood vessel endothelial restricted marker MECA32 (Figures 2D and F). 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 LTβR triggering. In search of a similar population in developing LNs, we assessed the expression of adhesion molecules in E16.5 LN anlagen. Figures 3A-B show a representative example of a MLN-anlage, while figures 3C-D display an anlage at a peripheral location, situated...
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Figure 1: Identification of LN-anlagen in E16.5 embryos. (A) E16.5 LN anlage at a sacral/iliac location, consisting of MAdCAM-1+ stromal cells and a cluster of CD4+ cells (MAdCAM-1 in red, CD4 in green). (B) High power magnification of an E16.5 LN anlage at a brachial location showing the close interaction between MAdCAM-1+ stromal cells and CD4+ cells (MAdCAM-1 in red, CD4 in green). (C) E16.5 LN anlage at an inguinal location. The majority of hematopoietic cells in the LN anlage expressed Il7r (CD45 in green, Il7r in red). (D) CD4+ cells in the LN anlage also expressed Il7r (CD4 in green, Il7r in red), while (E) a small Il7r−B220+ population could also be detected (B220 in green, Il7r in red). (F) CD4+ cells in the embryonic anlage expressed ICAM-1 (CD4 in green ICAM-1 in red). D-F: LN anlagen at a sacral/iliac location, representative of at least 10 different LN anlagen throughout the embryo.
adjacent to one of the large vessels (left side of picture). The MAAdCAM-1+ cells within the LN-anlagen showed co-expression of VCAM-1 (Figures 3A and C) as well as ICAM-1 (Figures 3B 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+MAAdCAM-1+ (IVM+) cells were concentrated in a polarized fashion in the outer regions of the LN-anlagen (Figures 3A-B and D-E). In addition, ICAM-1+VCAM-1+MAAdCAM-1- (IV+) cells were present in the deeper regions of the LN-anlagen. These VCAM-1+ LN-organizer cells also expressed the TNF-family member TRANCE (Figures 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 30, 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/MAAdCAM-1 triple positive cells in the developing PPs exert their function through the production of chemokines upon ligation of the LTβR 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 (Figure 4). Based upon expression of ICAM-1, VCAM-1, and MAAdCAM-1, two different populations of IVM+ cells could be observed, both at E16.5 and day of birth (Figure 4A/D). The largest population expresses all three adhesion molecules at intermediate levels (IVMint) (Figures 4 A-B/D-E, R2). An additional, clearly distinct, smaller population expresses high levels of IVM (IVMhigh) (Figures 4A-B/D-E, R3), and this population resembles the previously described organizing population in the PPs 3, 31. Furthermore, both populations displayed expression of the LTβR, further supporting the likelihood that they represent LN organizers analogous to those present in developing PPs. Surprisingly however, the IVMint population expressed higher levels of LTβR than the IVMhigh cells (Figures 4C and F).

The production of homeostatic chemokines is recognized as one of the defining properties of the PP-organizers. Hence, we assessed production of 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 figure 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 contain higher levels of chemokine mRNA. As a control, the IVMnegative, LTβR 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. Since 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 (Figure 6A), with
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Figure 2: LN anlagen are ensheathed by differentiating endothelium. Endothelial cells expressing the lymphatic endothelium restricted hyaluronan receptor Lyve-1 bordered both MLNs (B) and PLNs (renal node) (I) (A: MAdCAM-1 in green, CD4 in red. B and I: Lyve-1 in green, MAdCAM-1 in red). (C-E) The Lyve-1+ endothelial cells co-expressed junctional adhesion molecule VE-cadherin (Lyve-1 in green, VE-cadherin in red). (F-H and J) A proportion of the PLN and MLN anlagen also expressed the blood vessel restricted marker MECA32, indicating that the cells were still differentiating towards their lymphatic fate (Lyve-1 in green, MECA32 in red). These stainings are representative for at least 5 different LN anlagen.
highest levels of MAdCAM-1 on the IVM\textsuperscript{high} cells (Figure 6B). Strikingly however, the IVM\textsuperscript{int} population was severely diminished in PLNs compared to MLNs (approximately 10 fold reduction) while the IVM\textsuperscript{high} population showed only a slight decrease. As a result, the ratio of IVM\textsuperscript{high}/IVM\textsuperscript{int} cells, which in the MLN ranged from 0.1-0.4, was in the PLN completely opposite, ranging from 1-1.9.

To further compare the organizing populations from MLNs and PLNs expression of the LTβR was analyzed. In line with MLN organizers, IVM\textsuperscript{int} and IVM\textsuperscript{high} cells from PLNs express the LTβR (Figure 6C). Levels of LTβR are again highest in the IVM\textsuperscript{int} population, indicating that comparable populations of organizers are present in the PLNs and MLNs.

As the IVM\textsuperscript{int} population in MLNs is by far the most prevalent in terms of absolute cell numbers (on average 10 fold more cells then the IVM\textsuperscript{high}, data not shown), and since these express the highest levels of LTβR, 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\textsuperscript{+}CD4\textsuperscript{+}CD3\textsuperscript{-} 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 ICAM-1\textsuperscript{-}VCAM-1\textsuperscript{-}MAdCAM-1\textsuperscript{-} cells. The co-expression of the lymphatic endothelial marker Lyve-1 and the blood vascular marker MECA32 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\textsuperscript{+} cells are CD45\textsuperscript{-}CD4\textsuperscript{-}CD3\textsuperscript{-} 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, since LN development is normal in mice that lack the ICAM-1 ligand LFA-1\textsuperscript{32}, it is likely that there is redundancy among the adhesion molecules that fulfill this function.

Our studies also show that CD45\textsuperscript{-}CD4\textsuperscript{-}CD3\textsuperscript{-} cells in the LN anlage express the II7r chain\textsuperscript{33}. However, not all II7r\textsuperscript{+} cells expressed CD4, indicative of the fact that the direct precursor to CD45\textsuperscript{-}CD4\textsuperscript{-}CD3\textsuperscript{-} cells, an II7r\textsuperscript{+} population derived from the fetal
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Figure 3: Putative LN organizers are present in the LN anlagen. (A and C) MAdCAM-1 and VCAM-1 double positive cells can be seen to localize in the outer edge of the anlagen, while cells expressing lower levels of MAdCAM-1 and VCAM-1 have a more central location (MAdCAM-1 in green, VCAM-1 in red). (B and D) These MAdCAM-1+ cells also expressed ICAM-1, showing the presence and location of ICAM-1+VCAM-1+MAdCAM-1+ cells (MAdCAM-1 in green, ICAM-1 in red). This staining pattern was observed in at least 10 different LN anlagen) (E-F) VCAM-1+ cells in the LN-anlagen expressed TRANCE (VCAM-1 in red, TRANCE in green) (representative results of at least 3 different LN anlagen).
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Liver 18, 19, might also lodge in these LN-anlagen. One of the factors responsible for the accumulation of CD45^+CD4^+CD3^- cells in the LN-anlagen is TRANCE, since mice with deficient TRANCE-signaling have severely diminished numbers of CD45^+CD4^+CD3^- cells in these anlagen 6, 23, 30. Here 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-Receptor is expressed by both CD45^+CD4^-CD3^- cells 30, but now we show that TRANCE is also expressed on VCAM-1^+ LN-organizing cells within the LN-anlagen. This non-hematopoietic expression of TRANCE could thus further explain the inability to completely rescue the phenotype of the TRANCE^-/- mice by hematopoietic cell-specific transgenic TRANCE overexpression 30.

Dissection of PLN and MLN from newborn and embryonic animals provided us with the opportunity to study the properties of the ICAM-1^+VCAM-1^+MAdCAM-1^+ (IVM^+) cells constituting the presumptive LN organizers. Flow cytometric analysis of both MLNs and PLNs revealed the presence of 2 populations of IVM cells: IVM^int and IVM^high cells. Both populations expressed the LTβR, and are therefore phenotypically homologous to the previously described PP organizers. The IVM^int and IVM^high cells produce mRNA for CXCL13, CCL21 and CCL19, confirming that these are the functional counterparts of PP organizing cells 3.

The development of lymph nodes at either peripheral or mesenteric sites is known to be regulated by distinct signals. For example targeted deletion of either LTβ, components of the Il7r signaling complex, or the CXCL13/CXCR5 pair disrupts development of PLNs but has little or no effect on the development of MLNs 3, 24, 25. 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^int cells may provide an explanation for this discrepancy. Since IVM^int cells are the organizing population that expresses highest levels of LTβR, the decrease of these cells in PLNs could prove to be a limiting factor for PLN development in case of sub-optimal LTβR stimulation, for instance in the absence of LTβ, Il7r triggering or CXCR5 triggering. In LTβ^-/- mice, a role was postulated for LTβR triggering via LTα and LIGHT 34. This however is likely to be a less potent signal then LTα,β2, reducing the level of LTβR triggering. Furthermore, in the absence of Il7r 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 LTβR 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, 14, 33.

In summary, developing LN anlagen contain two distinct populations of organizing cells that express the LTβR, 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 LTβR^high/IVM^int population being most severely reduced. This leads to distinct stromal microenvironments in peripheral- and mesenteric-LN anlagen, providing an explanation for the divergent developmental requirements observed for these two LN-systems.
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**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\(^\text{high}\) (R3) and ICAM-1/VCAM-1\(^\text{int}\) (R2) populations are present. (B and E) The IV\(^\text{high}\) population also expressed high levels of MAdCAM-1 (IVM\(^\text{high}\)), while the IV\(^\text{int}\) population expressed MAdCAM-1 at low to intermediate levels (IVM\(^\text{int}\)), both at E16.5 and day of birth. (C and F) Both the IVM\(^\text{high}\) (light line) and IVM\(^\text{int}\) (dark line) population express LTβR. Highest level of LTβR was expressed by the IVM\(^\text{int}\) population. As a negative control the CD45\(^+\) cells within R1 are shown (filled histogram) (representative results of at least 4 different flow cytometric analyses).
Figure 5: Production of homeostatic chemokines by LN-organizers. In order to determine the functionality of the IVM<sup>high</sup> and IVM<sup>int</sup> populations in the developing LN, both populations were sorted from MLN anlagen at E18.5 and analyzed for expression of chemokine mRNA by quantitative real-time PCR. Both populations produced mRNA for CXCL13, CCL21 and CCL19. When normalized to β-actin, IVM<sup>high</sup> cells produced higher amounts of mRNA for all three homeostatic chemokines. Representative result of 3 independent quantitative PCR reactions is shown.
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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 to 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 MA\textsuperscript{CAM-1} when compared to the IVM\textsuperscript{high} population. (C) Both populations of organizers in the PLN express the LT\textbeta R. (representative analysis of at least 3 independent experiments is shown).
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