Chapter 1

The origin of lymph node stromal cells

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Summary

During embryogenesis, lymph nodes form through intimate interaction between lymphoid tissue inducer (LTI) and lymphoid tissue organizer (LTO) cells. Shortly after birth in mice, these LTO cells differentiate into specialized stromal cell subsets which organize microenvironments within the lymph nodes. Although stromal cells are now identified as crucial players for proper immune function, their direct precursors have not been identified yet. Using a fluorescent reporter, we describe a population of nestin-expressing mesenchymal cells which is present during early lymph node development. They are contained within the LTO population at day of birth, and only this population shows stem cell activity. The reporter-marked cells remain present in adult lymph nodes. Furthermore, genetic cell-lineage tracing experiments show relationship between this precursor population and different subpopulations of mature lymph node stromal cells. Our findings provide tools for delineating exact molecular mechanisms by which mesenchymal precursors can differentiate into different stromal cell lineages.
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Introduction

Lymph nodes are situated such that incoming antigens are efficiently presented to immune cells allowing rapid responses to infectious agents. Their formation starts during embryogenesis with the attraction of lymphoid tissue inducer (LTi) cells, which are of hematopoietic origin and belong to the family of innate lymphoid cells, to the presumptive lymph node site. This attraction is mediated by triggering of mesenchymal precursors with retinoic acid leading to CXCL13 expression. Accumulating LTi cells start to express lymphotoxin-αβ, that allows for the interaction with mesenchymal precursors cells that express the lymphotoxin-β-receptor. These cells then differentiate into lymphoid tissue organizer (LTo) cells and start to produce chemokines, cytokines and adhesion molecules that results in the attraction, survival and retention of more LTi cells eventually leading to a lymph node aggregate. Shortly after birth when lymph nodes are being populated with lymphocytes, lymph node increase in size and in addition, microdomains for T and B cells are being established by various stromal populations.

The lymph node stromal compartment comprises several cell types thought to be of endothelial and mesenchymal origin and they serve crucial functions for proper immune responses. Specialized blood endothelial cells (BEC) line the high endothelial venules (HEV) located in the paracortex, which allow entry of (naïve) lymphocytes, whereas lymphatic endothelial cells (LEC) form lymphatic vessels, which allow the entry of antigen, either freely floating in lymph fluid or captured by antigen presenting cells (APC). The non-endothelial stromal cells can be divided into cells that reside in the T cell area, the fibroblastic reticular cells (FRC); cells that are present in the B cell area, the follicular dendritic cells (FDC); and cells that associate with the subcapsular sinus, the marginal reticular cells (MRC). The FRC subset has been shown to not only provide a structural backbone for the migration of T cells searching for their cognate antigen but they are in fact actively guiding T cells, while providing them with survival signals. Furthermore, they regulate the pool of activated T cells, have the ability to present peripheral tissue antigens to induce antigen specific T cell tolerance and can induce tissue specific homing molecules on T cells.

The direct precursors for the different lymph node stromal subsets have not been identified, although the expression of the mesenchymal lineage markers PDGFR-α and PDGFR-β supports the idea that they are of mesenchymal origin. Therefore, mesenchymal stem cells (MSC) serve as good precursor candidates. The discovery that MSCs in the bone marrow are confined to a population of cells that are marked by transgenic expression of nestin led us to investigate whether these nestin-expressing precursors could give rise to various populations of lymph node stromal cells. Our data show that these mesenchymal precursors were present in primitive lymph nodes during the early stages of development and remained present during definitive organ formation. These precursors had stem cell activity, as measured by colony forming units for fibroblasts, were restricted to lymphoid tissue organizer cells, and gave rise to mature lymph node stromal cells.
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Results

Nestin expressing cells are present during initiation of lymph node development

We started off by assessing the presence of nestin\textsuperscript{pos} mesenchymal precursor cells in early presumptive lymph nodes of E14.5 embryo’s from mice that express GFP under the nestin-promotor (nestin-GFP mice) \textsuperscript{22}. These early lymph node structures can be identified by the combined analysis of CD4 on LTi cells and MAdCAM-1 on both LTo cells and (lymphatic) endothelial cells \textsuperscript{3,5,23,24}. Analysis of E14.5 nestin-GFP embryos revealed normal clusters

Figure 1 Nestin-expressing stromal cells are present in E14.5 developing lymph nodes

(A-D) Immunofluorescence microscopy of primordial lymph node sites of E14.5 nestin-GFP mice. (A-C) Primitive lymph nodes were identified by CD4 (in red) and MAdCAM-1 (in blue) and contained nestin-GFP\textsuperscript{dim} cells. (D) All primordial lymph nodes are in close vicinity to bright GFP-expressing structures that co-expressed βIII-tubulin (in blue). Scale bars; 25 μm. Data are representative for 3 individual nestin-GFP embryo’s.
of LTI cells together with MAdCAM-1\textsuperscript{pos} cells (Fig. 1 A–C). The preponderance of clusters that we could identify at this stage developed in the vicinity of bright GFP-expressing structures that uniformly co-expressed the neuron-specific marker βIII-tubulin, and therefore resembled nerve bundles (Fig. 1 D). Interestingly, within the LTI clusters, we observed cells that substantially expressed GFP although at a lower level when compared to the neuronal βIII-tubulin\textsuperscript{pos} structures. These cells lacked βIII-tubulin expression (Fig. 1 D), while the majority expressed intermediate levels of MAdCAM-1 (Fig. 1 A–C) which was expressed at lower levels when compared to surrounding MAdCAM-1\textsuperscript{pos} endothelial cells.

Mesenchymal precursors are restricted to the ICAM-1/VCAM-1 expressing LTo cells

During subsequent stages of embryonic lymph node development, mesenchymal precursor cells continue to differentiate into LTo cells upon lymphotoxin mediated signaling provided by LTI cells. This results in the additional induction of adhesion molecules (ICAM-1, VCAM-1), chemokines (CXCL13, CCL21, CCL19), as well as TRANCE and IL-7\textsuperscript{5,20,23}. In mice, LTo cells are abundantly present at day of birth in mesenteric lymph nodes (MLN)\textsuperscript{23}. To...
assess if MSCs could potentially be the precursors of LTo cells, we determined by means of the colony-forming units-fibroblast (CFU-F) assay, whether LTo populations contained MSC-activity.

Separating LTo subsets based on the combined expression of ICAM-1 and VCAM-1 revealed that the MSC-activity and clonogenicity was restricted to the subsets defined as LTo cells and absent in the population of stromal cells that lacked ICAM-1 and VCAM-1 (IV\textsuperscript{neg} cells) (Fig. 2 A). Accordingly, FACS-analysis of LTo cells derived from nestin-GFP day 0 MLN showed that all ICAM-1/VCAM-1 expressing cells (IV\textsuperscript{int} and IV\textsuperscript{high}) were positive for GFP, while only a small fraction of IV\textsuperscript{neg} cells expressed GFP (Fig. 2 B-C).

**LTo cells share phenotypic properties with adult lymph node stromal cells**

We subsequently addressed to which stromal cell subset, as defined previously in adult lymph nodes by combined staining for podoplanin (GP38) and CD31, the IV\textsuperscript{int} and IV\textsuperscript{high} cells were contained \textsuperscript{15}. In both day 0 mesenteric and peripheral lymph nodes, all IV\textsuperscript{int} and IV\textsuperscript{high} cells expressed GP38, while they uniformly lacked the expression of endothelial markers CD31 and VEGFR2 (Fig. 2 D-E). Therefore, in the neonatal lymph node the MSC-activity is restricted to the non-endothelial nestin-GFP\textsuperscript{pos}IV\textsuperscript{pos}GP38\textsuperscript{pos}CD31\textsuperscript{neg} stromal cell subset.

**Mesenchymal precursors accumulate in postnatal developing lymph nodes**

The colonization of lymphocytes shortly after birth leads to a considerable increase in size of the lymph nodes and therefore requires an expansion of the stromal compartment. This expansion occurs potentially through stromal cell proliferation, which will be followed by

*Figure 3 Mesenchymal precursors accumulate in postnatal developing lymph nodes and localize throughout the adult lymph node*

Absolute number (A) of cells with CFU-F activity within single cell suspensions derived from post-natal peripheral and mesenteric lymph nodes. Each dot represents a set of LN obtained from a single mouse (n=3 or more; ANOVA: **, P < 0.01, ***, P < 0.001, data are represented as mean). (B) Distribution of nestin-GFP\textsuperscript{pos} mesenchymal precursors (green) in adult lymph nodes taken from 12 week nestin-GFP mouse, stained for B cells (B220; red) and T cells (CD3; blue) (C-E). Higher magnification of a T cell area in which GP38\textsuperscript{pos} (blue) stromal cells and nestin-GFP\textsuperscript{pos} cells (green) are present (C). Nestin-GFP\textsuperscript{pos} cells (green) in B cell area stained for FDCs (CD35; red) and B cells (B220; blue) (D) and nestin-GFP\textsuperscript{pos} (green) cells in subcapsular sinus area together with TRANCE (red) as well as MAdCAM-1 (blue) (E). (F) Immunofluorescence images of the T cell area of lymph nodes where nestin-GFP\textsuperscript{pos} cells (green) express desmin (blue, left panel) and α-SMA (blue, right panel). (G) Nestin-GFP\textsuperscript{pos} cells (green) around CD31\textsuperscript{pos} HEVs (red). (H) Nestin-GFP\textsuperscript{pos} cells surrounding CD31\textsuperscript{pos} HEVs (red) stained for desmin (blue, left panel) and α-SMA (blue, right panel). (I) Nestin-GFP\textsuperscript{pos} cells (green) around CD31\textsuperscript{pos} HEVs (blue) stained for PDGFR-β (red). Images are representative for both peripheral and mesenteric lymph nodes of 3 individual animals. Scale bars; 250 μm for low magnification (B), 50 μm (D-E) and 10 μm (C, F-I) for high magnification. axLN; axillary lymph node, cerLN; cervical lymph node, iLN; inguinal lymph node, plLN; peripheral lymph node, mlLN; mesenteric lymph node. (J) Frequency of CFU-F and frequency of GFP\textsuperscript{pos} cells within the CD45\textsuperscript{neg} fraction of peripheral or mesenteric lymph node single cell suspensions (n=5; unpaired t-test: *, p<0.05, ***, p<0.001, n.s., data are represented as mean ± SEM). (M) Representative FACS-analysis sample of single cell suspensions of both peripheral and mesenteric lymph nodes where nestin-GFP\textsuperscript{pos} cells (green) express desmin (blue, left panel) and α-SMA (blue, right panel). (K-O) Quantification of the percentage nestin-GFP\textsuperscript{pos} cells within the CD45\textsuperscript{neg}GP38\textsuperscript{neg}CD31\textsuperscript{neg} (K) or the CD45\textsuperscript{neg}GP38\textsuperscript{pos}CD31\textsuperscript{pos} (N) stromal population of nestin-GFP peripheral and mesenteric lymph nodes. (L, O) Quantification of the percentage nestin-GFP\textsuperscript{pos} cells within the CD45\textsuperscript{neg}GP38\textsuperscript{pos}CD31\textsuperscript{neg} (L) or the CD45\textsuperscript{neg}GP38\textsuperscript{pos}CD31\textsuperscript{pos} (N) fraction of peripheral and mesenteric lymph nodes (K-O, n=6; paired t-test: ***, p < 0.01, n.s., not significant, data are represented as mean ± SEM).
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differentiation and may consequently result in a decrease of mesenchymal precursors. Indeed, based on CFU-F assay, the frequency of mesenchymal precursors within lymph nodes decreased over time (data not shown) although the absolute numbers of CFU-F increased during the first 2 weeks (Fig. 3 A). Remarkably, substantial MSC-activity could still be measured at 8 weeks of age.

Nestin expressing cells can be identified throughout the adult lymph node
In order to determine where these mesenchymal precursors are located in fully developed lymph nodes, we analyzed lymph nodes from adult nestin-GFP mice. Nestin-GFP\textsuperscript{pos} cells could be observed in both peripheral as well as mesenteric lymph nodes and they were present within the T cell area, the B cell area, as well as the subcapsular sinus (Fig. 3 B-E). Within the T cell area, nestin-GFP cells expressed GP38 (Fig. 3 C) as well as α-smooth muscle actin (α-SMA) and desmin (known markers for FRCs as well as pericytes \textsuperscript{15}, Fig. 3 F) They were often found in close association with HEVs (Fig. 3 G), while they were devoid of endothelial markers (Fig. 3 H). Nestin-GFP cells never expressed neuronal markers βIII-tubulin or neurofilament (data not shown). Recently, splenic FDCs precursors were described as perivascular cells that expressed PDGFR-β and NG2 \textsuperscript{25}. In lymph nodes, we could also identify perivascular nestin-GFP\textsuperscript{pos} cells that expressed PDGFR-β (Fig. 3 I) but not NG2 (data not shown).

Further phenotyping of these cells using FACS-analysis (Fig. 3 M) confirmed that nestin-GFP\textsuperscript{pos} cells were contained within the FRC-subset (CD45\textsuperscript{neg}GP38\textsuperscript{pos}CD31\textsuperscript{neg}, Fig. 3 K-L). Within the blood endothelial cell (BEC) population (CD45\textsuperscript{neg}GP38\textsuperscript{neg}CD31\textsuperscript{pos}) we also observed nestin-GFP\textsuperscript{pos} cells (Fig. 3 N-O). Careful immunofluorescence analysis however showed that nestin-GFP\textsuperscript{pos} cells associated with small blood vessels, rather than being blood endothelial cells (Fig 3 F-H and data not shown) indicating that this fraction contained doublets consisting of nestin-GFP\textsuperscript{pos} cells that strongly adhered to endothelial cells. Neither within the lymphatic endothelial cell (LEC) population (CD45\textsuperscript{neg}GP38\textsuperscript{pos}CD31\textsuperscript{pos}) nor the double negative population we observed nestin-GFP\textsuperscript{pos} cells (data not shown).

In the B cell area, nestin-GFP\textsuperscript{pos} cells localized close to FDCs but never expressed FDC specific markers (Fig. 3 D). In the subcapsular sinus, nestin-GFP\textsuperscript{pos} cells occasionally expressed TRANCE although never in combination with MAdCAM-1 (Fig. 3 E), indicating these nestin-GFP\textsuperscript{pos} cells were distinct from MRCs \textsuperscript{12}.

Although the frequency of nestin-GFP\textsuperscript{pos} cells within the CD45\textsuperscript{neg} population reached 4.0% ± 0.53% in PLN and 2.13% ± 0.82% in MLN, a smaller fraction of the CD45\textsuperscript{neg} population gave rise to CFU-F in culture (0.066% ± 0.019% for PLN and 0.040% ± 0.008% for MLN, Fig. 3 I). This agrees with the observation that only a small percentage of nestin-GFP\textsuperscript{pos} cells present within the bone marrow could give rise to CFU-F \textsuperscript{21}.

Nestin precursors give rise to the major subsets of mesenchymal and endothelial derived lymph node stromal cells
Our data so far suggests that nestin-expressing progenitor cells are present at all stages in lymph nodes and as such, they may form a source of precursors for the different stromal
Figure 4 Mesenchymal lymph node stromal subsets are derived from nestin-expressing precursors

(A) Representative FACS-analysis example of single cell suspensions of peripheral and mesenteric lymph nodes derived from nestin-cre/ROSA26-GFP mice stained for CD45, GP38 and CD31 to identify the four stromal cell populations (FRC; GP38<sup>pos</sup>CD31<sup>neg</sup>, LEC; GP38<sup>pos</sup>CD31<sup>pos</sup>, BEC; GP38<sup>neg</sup>CD31<sup=pos</sup>, DN; GP38<sup>neg</sup>CD31<sup>neg</sup>). (B) Percentage GFP<sup>pos</sup> stromal cells within peripheral or mesenteric lymph nodes of nestin-cre/ROSA26-GFP mice (n=4, data are represented as mean ± SEM). (C) Distribution of GFP-expression identified by anti-GFP staining (in red) of a peripheral lymph node derived from adult nestin-cre/ROSA26-GFP. Scale bar; 75μm. (D, H, J) Histograms showing the expression of GFP within the FRC population (D), LEC population (H) and BEC population (J) of adult nestin-cre/ROSA26-GFP (black line) peripheral and mesenteric lymph nodes. (E-G, I, K) Immunofluorescence images of lymph nodes from nestin-cre/ROSA26-GFP stained with anti-GFP (in red) and in blue GP38 (E), 8C12 (F) identifies GFP<sup>pos</sup> stromal cells in the T cell zone and the B cell zone respectively. (G) In the subcapsular sinus, endogenous GFP expression (in green) could be identified and arrowheads indicate cells that stain positive for TRANCE (in red) and MAdCAM-1 (in blue). Subsequent sections were stained with anti-GFP (in red) and (I) Lyve-1 (in blue) to identify LECs and (K) PNAd or MAdCAM-1 (in blue) to identify HEVs. (L-N) Spleen sections of adult nestin-cre/ROSA26-GFP mice stained with anti-GFP (in red) together with (L) CD31 (in blue) to identify endothelial cells, (M) ERTR-7 (in blue) to identify stromal reticular cells and (N) 8C12 (in blue) to identify FDCs. Images are representative for both peripheral and mesenteric lymph nodes or spleens of 4 individual animals. Scale bars; 50 μm (F-G), 25μm (I, K). axLN; axillary lymph node, iLN; inguinal lymph node, mLN; mesenteric lymph node.
subsets present within the lymph nodes. To assess whether lymph node stromal cells are indeed derived from nestin-expressing precursors, we intercrossed nestin-cre transgenic mice with the ROSA26-flox-STOP-flox-GFP reporter line, which allowed lineage tracing of all cells that expressed the nestin-cre transgene. Both FACS-analysis as well as immunofluorescent staining of nestin-cre/Rosa26-GFP lymph nodes unambiguously revealed the lineage relationship between nestin-expressing precursors and mesenchymal lymph node stromal cells and. FACS-analysis of lymph node single cell suspensions confirmed that the majority of FRCs (GP38posCD31neg) were derived from nestin-expressing precursors since these cells expressed GFP (Fig. 4 A-B, D). We hardly observed GFP expression in the double negative subset (DN; GP38negCD31neg) indicating that this special subpopulation of stromal cells may originate from a different precursor (Fig. 4 A-B and data not shown). Upon immunofluorescent staining within the T cell area, we confirmed that podoplanin expressing FRCs were GFP positive (Fig. 4 C, E). Also within the B cell area, FDCs clearly co-localized with GFP (Fig. 4 D). Finally, within the subcapsular sinus, the GFP-signal also co-localized with MRCs which were identified by the combined expression of TRANCE and MAdCAM-1 (Fig. 4 G). Unforeseen, also LECs (GP38posCD31pos) expressed GFP, both by FACS as well as immunofluorescent analysis, suggesting that the majority of LECs present within lymph nodes are also derived from nestin-expressing precursor (Fig. 4 B, H-I). In addition, we also observed that the majority of BECs (GP38negCD31pos) expressed GFP (Fig. 4 B, J-K), indicating that within the lymph nodes they share this common precursor. Careful immunofluorescent analysis shows that HEVs but not other vasculature structures indeed are GFP-positive in both PLN and MLN (Fig. 4 I). To clearly demonstrate that only HEVs and not other blood endothelial cells are derived from nestin precursors we analyzed spleens from nestin-cre/Rosa26-GFP mice, since HEVs are absent from the spleen. We were unable to identify splenic CD31pos structures within the white pulp that expressed GFP (Fig. 4 L), demonstrating that HEVs within lymph nodes are unique structures with a different precursor then endothelial cells. Furthermore, within the spleen we were able to show that both MRCs (Fig. 4 M) and FDCs (Fig. 4 N) were derived from nestin precursors while stromal cells within the red pulp never stained positive GFP (data not shown).
Discussion

All together our results identify the sought after population of cells which gives rise to the mesenchymal stromal cells as well as HEVs and LECs in lymph nodes. By using a Cre-lox system which allows genetic lineage tracing, our data show for the first time a relationship between nestin-expressing precursors and adult lymph node stromal cells and demonstrates that one type of precursor cells can give rise to different stromal subpopulations. As shown through their transgenic nestin-GFP expression, these cells are present during all stages of lymph node development. These nestin-GFP<sup>pos</sup> cells have stem cell activity and this activity is restricted to lymphoid tissue organizer cells in day 0 lymph nodes. The presence of mesenchymal nestin-precursors in adult lymph nodes further implies that a reservoir of precursors remains present within the adult lymph nodes thus enabling lymph node remodelling during inflammation.

Using lineage tracing we were unable to show a relationship between double negative (GP38<sup>neg</sup>CD31<sup>neg</sup>) stromal cells and nestin-expressing precursors. These double negative cells were recently identified as myofibroblastic pericytes present around some vessels in lymph node cortex and medulla. Judged from their expression profile, they are most closely related to FRCs<sup>27</sup> although our data demonstrates that these cells may be derived from different precursor cells.

The challenging observation that in nestin-cre/ROSA26-GFP lymph nodes both HEVs and LECs are marked with GFP indicates that these endothelial subsets are also derived from nestin-expressing precursors. This may require readjustment of our current view on the origin of (lymphatic) endothelium. Perhaps (lymphatic) endothelial cells within lymph nodes are unique and connect to the conventional lymphatic and vascular endothelial cells of the lymph and blood vessels. This view is supported by the observation that in transplanted lymph nodes as well as in lymphoid structures induced by intradermal injection of newborn lymph node derived cells, the stromal cells, including HEVs and LECs are of donor origin<sup>19,28</sup>. Remarkably, all three stromal subsets, namely FRCs, HEVs, and LECs express CCL21, indicative of a functional similarity. Furthermore, splenic endothelial cells were not derived from nestin precursors.

Since day 0 MLN endothelial cell subsets (CD45<sup>neg</sup>ICAM<sup>high</sup>VCAM<sup>int</sup>) did not give rise to CFU-F while they still expressed nestin (data not shown), further studies are needed to elucidate when commitment of nestin-precursors to the endothelial subsets occurs during embryonic lymph node development and what the molecular requirements are.

Recently, it was reported that FDCs within the spleen are derived from perivascular progenitors that express FDC-M1 and are derived from PDGFR-β precursors<sup>25</sup>. Here we show that perivascular nestin-GFP<sup>pos</sup> cells also express PDGFR-β suggesting that these cells show similar phenotypic properties, although we never detected NG2 on nestin-GFP<sup>pos</sup> cells within lymph nodes. Furthermore, both FDCs and MRCs within splenic white pulp are derived from nestin precursors suggesting that both types of precursors must be closely related. However, we never detected GFP<sup>pos</sup> stromal cells within the red pulp of nestin-cre/ROSA26-GFP while they were abundantly labeled in spleens of Pdgfrb-
cre/ROSA26 reporter mice. These data indicate that Nestin more exclusively identifies precursors for stromal cells within lymphoid tissue. The identification of the lymph node stromal cell precursor now allows studies that specifically address the requirements for differentiation of precursors into various stromal subsets. In addition, although all stromal cells, except double negative stromal cells, share a common precursor, it remains unknown what drives certain lymph node specific expression profiles since lymph node stromal cells show site-specific characteristics. Together with the recently published transcriptome analysis of lymphoid organ stroma, our findings allow more specific research to untangle the contribution of stromal cell-derived factors in shaping immune responses by specifically deleting molecules that are expressed in stromal subsets using nestin-cre deleter mice. With this knowledge, we would then be able to modulate the function of (lymph node) stromal cells during unwanted inflammatory reactions in acute diseases (e.g. acute-GVHD, viral infections) or chronic inflammatory disorders when tertiary lymphoid structures develop as a result of continued inflammation (reviewed in), as well as in maintaining tolerance against peripheral tissue antigens.
Experimental Procedures

Mice

C57BL/6 mice were bred at our own facility and maintained under standard animal housing conditions. Nestin-GFP mouse line was generated as described\(^2\) Nestin-Cre [B6.Cg-Tg(Nestin-cre)1Kin/J] and ROSA-Isl-GFP [B6;129-Gt(Rosa)26Sor^{tm2Sho}/J] strains were obtained from Jackson Laboratory. All animal experiments were approved by the Animal Experimental Committee of the VU University Medical Center.

Single cell suspension

For CFU-F assays, isolated peripheral lymph nodes (which includes inguinal, brachial and axillary) and mesenteric lymph nodes, were cut into small pieces followed by enzymatic digestion in DMEM containing Blendzyme 2 (150µg/ml), DNAse I (200ug/ml, both Roche Applied Sciences, The Netherlands), 2% FCS, 2% antibiotics (digestion medium) for 15 minutes, 37°C while continuously stirring. Remaining fragments were further digested for 15 another 15 minutes, 37°C. Single cells were collected in Mesencult + stimulatory supplements (StemCell technology, Lyon, France) and 2% antibiotics, centrifuged (10min, 300g, 4°C) and resuspended in 1ml Mesencult + stimulatory supplements and counted.

For FACS-analysis, lymph node single cell suspensions were made according to the protocol described by Fletcher\(^3\). In short, isolated peripheral lymph nodes (which include axillary, brachial, inguinal and popliteal lymph nodes) and mesenteric lymph nodes were pierced with a 25g needle and placed in RPMI-1640 on ice. Upon start of digestion, lymph nodes were transferred to a tube containing 2 ml freshly prepared digestion medium (RPMI-1640) containing 0.8 mg/ml Dispase II, 0.2 mg/ml collagenase P and 0.1 mg/ml DNA-se I (all from Roche). Tubes were incubated at 37°C for 20 minutes and gently vortexed every 5 minutes. After 20 minutes, suspensions were gently pipetted to break the lymph node capsule. Upon settlement of large fragments, supernatant was transferred to a collection tube containing 10 ml icecold FACS-buffer (2% FCS, 5mM EDTA in PBS) and centrifuged (5 min, 300g, 4°C). 2 ml digestion medium was added to the digestion tube and incubated at 37°C while regularly mixing. After 10 minutes, digestion medium was robustly mixed with 1 ml pipette. Upon settlement of large fragments, medium with cells was transferred to previous collection tube with new FACS-buffer and centrifuged (5 min, 300g, 4°C). Again, 2 ml digestion medium was added to the digestion tube and mixture was robustly mixed every 5 min until all remaining fragments were digested. Medium with cells was transferred to previous collection tube with new FACS-buffer and centrifuged (5 min, 300g, 4°C). After centrifugation, all cells were resuspended in FACS-buffer without EDTA.

Flow Cytometry

Data were acquired on a Cyan ADP High Performance Research Flow Cytometer (Beckman Coulter) and were analyzed with Summit Software v4.3. Single stained cells were used to compensate for spectral overlap. Fluorescence Minus One (FMO) stained cells were used to set boundaries between positive and negative cells. 7-Amino-Actinomycin D (7AAD) or
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Sytox Blue (Molecular Probes) staining was used to exclude dead cells. Cell sorting was performed on a MoFlo High Speed Cell Sorter (DAKO Cytomation, Glostrup, Denmark) equipped with Summit Software. Sorted cells were collected in appropriate culture medium and further analyzed using in vitro cultures.

Colony-forming units-fibroblast assay
For colony-forming units-fibroblast (CFU-F) assay, cells were seeded in at least two different concentrations in 2 ml Mesencult + stimulatory supplements and 2% antibiotics/well in a 6-wells plate. CFU-F assays were always performed in duplo. After 2 weeks of culture, cells were washed twice with PBS, fixed in methanol and stained with Giemsa. CFU-F were counted using a stereo microscope. A group of ≥10 cells was contemplated as a colony.

Immunofluorescence Microscopy
Nestin-GFP<sup>pos</sup> embryos of 14.5 days after conception were fixed for 30 min in paraformaldehyde in PBS, cryoprotected overnight in sucrose (30% wt/vol in PBS) and subsequently embedded in OCT compound (Sakura Finetek, Europe) and stored at -80°C until sectioning. Lymph nodes and spleens from nestin-GFP and nestin-cre/Rosa-26 GFP mice were fixed in paraformaldehyde in PBS for 10 min, cryoprotected and subsequently embedded in OCT compound. For identification of primordial lymph nodes, embryos were serially sectioned (8 µm) on gelatin coated slides and every tenth slide was stained with anti-CD4 and anti-MAdCAM-1. Immunofluorescence staining was performed as described before. In short, Immunofluorescence staining was performed in PBS, supplemented with 0.1% (wt/vol) Bovine Serum Albumin (BSA) or Earle's Balanced Salt Solution (EBSS) in case of TRANCE staining. Biotinylated anti-TRANCE (IK22/5; eBioscience) was visualized with a Tyramide Signal Amplification kit plus horseradish peroxidase–streptavidin and Alexa Fluor 546–tyramide (Invitrogen). Sections were enclosed in Vinol (Air Products, Allentown, USA) supplemented with DAPI (Invitrogen) and analyzed on a Leica DM6000 fluorescence microscope or a Leica TCS-SP2-AOBS Confocal Laser Scanning Microscope (both Leica Microsystems) and images were obtained with Leica software.

 Antibodies
The following antibodies were used in variable combinations for flow cytometry or immunofluorescence: unconjugated anti-CD31 (ERMP12, kindly provided by P. Leenen, Erasmus University Rotterdam, The Netherlands), anti-GP38 (anti-podoplanin, clone 8.1.1 obtained from the Developmental Studies Hybridoma Bank (DSHB) at University of Iowa, Iowa City, IA), anti-VEGFR2 and anti-Lyve-1 (Millipore), TUJ-1 (antibody to neuron-specific βIII tubulin; ab18207, Abcam), anti-neurofilament (Sigma), anti-GFP (Invitrogen), anti-desmin (BD Biosciences, Breda, The Netherlands), anti-α-smooth muscle actin (α-SMA, clone 1A4, Sigma, Zwijndrecht, The Netherlands), MECA79 (anti-PNAd); biotinylated anti-CD35 (8C12), fluorescein isothiocyanate (FITC) labeled anti-ICAM-1 (CD54) and anti-VCAM-1 (CD106); Phycoerythrin (PE) labeled anti-ICAM-1; PE-Cy7 labeled anti-CD45 (clone 30F11); Alexa-Fluor 555 labeled anti-CD4 (clone GK1.5); Allophycocyanin (APC)
anti-VCAM-1; Alexa-Fluor 647 labeled anti-CD45 (clone MP33) and anti-MAdCAM-1 (MECA-367). Clones GK1.5, MP33 and MECA-367 were affinity purified from hybridoma cell culture supernatants with protein G-sepharose (Pharmacia, Uppsala, Sweden) and subsequently labeled with Alexa-Fluor 555 (GK1.5) or Alexa-Fluor 647 (MP33 and MECA-367) (Invitrogen). Unconjugated antibodies were detected with species specific secondary reagents, namely: goat anti-rat Alexa-Fluor 488 or 647, goat anti-rabbit Alexa-Fluor 488 or 647 and goat anti-hamster Alexa-Fluor 488 or 647 (all Invitrogen). Unless indicated otherwise, all antibodies were derived from eBioscience (San Diego, USA).
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