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Absence of Nkx2-3 Homeodomain Transcription Factor Induces the Formation of LYVE-1–Positive Endothelial Cysts without Lymphatic Commitment in the Spleen

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Summary

In contrast to peripheral lymph nodes possessing lymphatic and blood vasculature, the spleen in both humans and rodents is largely devoid of functioning lymphatic capillaries. Here it is reported that in mice lacking homeodomain transcription factor Nkx2-3, the spleen contains an extensive network of lymphocyte-filled sacs lined by cells expressing LYVE-1 antigen, a marker associated with lymphatic endothelium cells (LECs). Real-time quantitative PCR analyses of Nkx2-3 mutant spleen revealed a substantial increase of LYVE-1 and podoplanin mRNA levels, without the parallel increase of mRNA for VEGFR-3 (vascular endothelial growth factor receptor Type 3) and Prox1 (Prospero homeobobox protein 1), two markers specific for LECs. Although these structures express VEGFR-2/flk-1, they lack Prox1 protein, indicating their non-LEC endothelial origin. The LYVE-1$^+$ structures are bordered with ER-TR7$^+$ fibroblastic reticular cells with small clusters of macrophages expressing MARCO and sialoadhesin. Short-term cell-tracing studies using labeled lymphocytes indicate that these LYVE-1$^+$ cysts are largely excluded from the systemic circulation. Cells expressing LYVE-1 glycoprotein as putative precursors for such structures are detectable in the spleen of late-stage embryos, and the formation of LYVE-1$^+$ structures is independent from the activity of lymphotoxin β-receptor. Thus the splenic vascular defects in Nkx2-3 deficiency include the generation of LYVE-1$^+$ cysts, comprised of endothelial cells without being committed along the LEC lineage. (J Histochem Cytochem 59:690–700, 2011)

Keywords

Nkx2-3, spleen, LYVE-1, endothelium, development

The architecture of the various secondary lymphoid organs is closely coupled to the circulation of lymphocytes at these tissues. The main elements of blood vasculature in lymph nodes and Peyer's patches are the high endothelial venules (HEVs), which mediate leukocyte homing through the binding of L-selectin expressed on leukocytes. Besides their blood circulation, lymph nodes are also richly perfused by lymphatic vasculature, characterized by the expression of LYVE-1 hyaluronan receptor (Prevo et al. 2001) and Prox1 (Prospero homeobox protein 1) transcription factor (Wigle and Oliver 1999; Wigle et al. 2002), a fate-determining regulator for the embryonic differentiation of lymphatic endothelial cells (LECs).

The vascular pattern and thus the structure of the spleen considerably differ from those of peripheral lymph nodes or Peyer's patches. The splenic artery divides into smaller branches termed central arterioles, which are surrounded by T-cell area. Subsequent segments of these central arterioles

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continue through the B-cell follicles to the terminal arterioles (in human) or marginal sinuses (in rodents). These vessels end in the highly fenestrated network of sinuses in the red pulp, lined by different endothelial subsets (Balogh and Lábadi, 2011; Mebius and Kraal 2005). In the red pulp of human spleens, blood capillaries expressing CD34 are located in the central parts of fibroblastic cords, without making direct anastomoses with sinuses identifiable through their endothelial CD141 expression. In addition, these vessels display CD8 and mannose receptor (Giorno 1984; Martinez-Pomares et al. 2005; Steiniger et al. 2007). In mice, our own observations indicate a similar complexity by using endothelium subset-reactive monoclonal antibodies (Balázs et al. 1999; Balázs et al. 2001). Although capillaries with the characteristics of lymphatic vessels were described in mouse, it is uncertain whether real lymphatic vessels exist in the murine or human spleen and to what extent these contribute to lymphocyte recirculation or interstitial fluid flow (Pellas and Weiss 1990).

Several transcription factors, including homeobox transcription factor Nkx2-3, have been identified as important regulators of spleen ontogeny (Brendolan et al. 2007; Pabst el al. 1999; Pabst et al. 2000; Wang et al. 2000). Nkx2-3 transcription factor belongs to the structurally conserved Nk family of 18 members sharing a common DNA recognition sequence. This group of transcription factors is involved in the cell type specification of visceral mesoderm, including heart, lungs, pancreas, gastrointestinal tract, central nervous system, and skeleton, in a highly complex expression pattern (Stanfel et al. 2005). Furthermore, various members of the lymphotoxin/tumor necrosis factor (LT/TNF) family also crucially contribute to the formation of lymphoid compartments within the spleen, including the development of marginal sinus influenced by signaling through the receptor for lymphotoxin β (LTβR) (Fütterer et al. 1998). In contrast to the vascular defects restricted to the marginal sinus in mice deficient for LTβR, however, *nkx2-3*$^{-/-}$ mice display an extensive range of vascular disorganization, manifested in the absence of the marginal sinus and an atrophic red pulp sinus network (Balogh et al. 2007; Pabst et al. 2000; Wang et al. 2000). Other roles of Nkx2-3 related to altered lymphoid tissue induction outside the spleen have been suggested by the SNPs in patients with inflammatory bowel diseases, often associated with ectopic lymphoid tissue formation (Franke et al. 2008; Parkes et al. 2007).

Here, we report that the spleen in *nkx2-3*$^{-/-}$ mouse contains LYVE-1^{+} vascular-like structures, without expressing Prox1 transcription factor. We also determine their structural, phenotypic, and developmental characteristics. Cell tracing experiments demonstrate that they are excluded from the systemic circulation. The formation of these structures is independent from the activity of LTβR, and the absence of Prox1 transcription factor suggests that these vessels may represent embryonic endothelial remnants in the spleen of adult Nkx2-3 mutant mice.

Materials and Methods

Mice

Nkx2-3$^{-/-}$ mice from 129SvxB6 mixed background (Pabst et al. 1999) were backcrossed with BALB/c mice (obtained from Charles River, Gödöllő, Hungary) through 14 generations. C57Bl/6 and 129Sv Nkx2-3 mutants were maintained at the Technical University of Braunschweig. For homing studies, BALB/c mice from the faculty's SPF breeding unit were used as lymphocyte donor for adoptive transfer. LTβR-deficient mice (Fütterer et al. 1998) were kindly provided by Drs. Klaus Pfeffer and Falk Weih. All procedures involving live animals were conducted in accordance with the guidelines set out by the Ethics Committee on Animal Experimentation of the University of Pécs. The Nkx2-3/LTβR double knockout mice were identified in the F2 generation by simultaneous PCR amplification of the *nkx2-3, neomycin phosphotransferase*, and *ltbr* loci from genomic DNA using the following primer pairs: *nkx2-3:* forward GCGGGAGACTGTAAGACGAG, reverse TTATCCTGCCGCTGTCTCTT, amplicon size: 238 bp; *neomycin phosphotransferase*: forward GTCGATCAGGA TGATCTGGAC, reverse AAGGCGATAGAAGGCGATGC, amplicon size: 321 bp, *ltbr*: forward GCATGTAGCCAT GAAGACAGGAT, reverse CGCAAAGACAAACTCGC CTAT, amplicon size: 150 bp.

Antibodies and Reagents

Rat monoclonal antibody (mAb) against mouse fibroblastic reticular cell markers (clone ER-TR7) was kindly provided by Dr Willem van Ewijk. Anti-Thy-1/CD90 (clone IBL-1), IBL-11 mAb against reticular fibroblasts, anti-MARCO (clone IBL-12), and anti-sialoadhesin/CD169 (clone IBL-13) mAbs were developed in our laboratory (Balogh et al. 1992; Balogh et al. 2004; Kvell et al. 2006). For multicolor immunofluorescence, IBL-1 mAb was conjugated with FITC (Sigma-Aldrich Hungary, Budapest) according to standard procedures. Rat hybridoma cell line secreting anti-B220 (clone RA3-6B2) was obtained from ATCC and was conjugated with Alexa 647 (Invitrogen, Csertex Ltd, Budapest). Rat mAb against LYVE-1 (clone 223322) was obtained from R&D Systems (Biomedica Hungary Kft, Budapest) and was biotinylated with sulfo-*N*-hydroxysuccinimide biotin ester (Sigma-Aldrich Hungary, Budapest). Anti-VEGFR-2/flk1 (vascular endothelial growth factor receptor Type 2) mAb (clone Avas 12α1) was obtained from BD Biosciences (Soft Flow Ltd, Pécs, Hungary). Rabbit antibodies against Prox1 were purchased from AngioBio (Del Mar, CA) and were detected with FITC-conjugated goat anti-rabbit IgG (Sigma-Aldrich, Hungary). Streptavidin-Alexa Fluor 350 was purchased from Invitrogen. Unlabeled rat mAbs were detected with phycoerythrin-conjugated goat anti-rat IgG purchased from BD.

Table 1. Primers Used for Quantitative PCR Analyses

No.	Forward	Reverse
Prox1	TGTCCGACATCTCACCTTATTCA	GCGGGTGTAAAAGAACATGAGTT
Pdpn/Gp38 (Podoplanin)	GTGCTACTGGAGGGCTTAATGAA	GCTTCGTCCCACGCTCTCT
Flt4/Vegfr3	GGTTCCTGATGGGCAAAGG	TCAGTGGGCTCAGCCATAGG
Lyve1	GGAGCAGCATTCAAGAACGAA	GCGGCAGCACCAAAGAAG
Nkx2-3	TTATCCTGCCGCTGTCTCTT	GCGGGAGACTGTAAGACGAG

Immunohistochemistry and Immunofluorescence

Frozen and acetone-fixed sections were blocked with 5% BSA in PBS for 20 min. Single immunofluorescence was performed using anti-LYVE-1 mAb followed by phycoerythrine (PE)–conjugated goat anti rat IgG. For multiple immunofluorescence, unlabeled rat mAbs were detected using PE-conjugated anti-rat IgG, followed by saturation with 5% normal rat serum. Subsequently, FITC-conjugated and biotinylated antibodies were added in cocktail, followed by the visualization of latter reagents with Streptavidin-Alexa Fluor 350. Control staining included irrelevant rat IgG at 10 μg/ml purified from normal rat serum using protein G affinity chromatography. After mounting, the sections were viewed under an Olympus BX61 fluorescent microscope. The acquisition of digital pictures with a CCD camera was performed using the analySIS software. The pictures were edited with Adobe Photoshop, using adjustments for brightness contrast and black level for the entire images.

Electron Microscopy

After removal the spleens were cut in 2 to 3 pieces at a volume less than 1 mm^3. The tissue was placed in 2.5% buffered glutaraldehyde for 3 hr at room temperature under continuous agitation. Following fixation, the tissue was washed several times with phosphate buffer. The blocks were placed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH: 7.2) for 1 hr. After osmication and washing, the tissue blocks were dehydrated in an ascending ethanol series, with uranyl acetate (1%) included in the 70% ethanol stage. The tissues were placed into aluminum-foil boats containing Durcupan resin (Sigma-Aldrich, Budapest) and then embedded in gelatin capsules containing the same resin. Semithin and serial ultrathin sections were cut with a Leica ultramicrotome. Semithin sections (1 μm thick) were mounted on glass slides and stained with toluidine blue. Ultrathin sections (80 nm thick) were mounted either on mesh or on collodion-coated (Parlodion, Electron Microscopy Sciences, Fort Washington, PA) single-slot, copper grids. Additional contrast was provided to these sections with uranyl acetate and lead citrate, and they were examined in JEOL 1200EX-II electron microscope. The negative photographs were developed and scanned into computer, from which digitalized pictures were generated.

Adoptive Cell Transfer

Lymphocytes from lymph nodes were isolated and labeled with 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Invitrogen) as described (Nolte et al. 2002). For lymphocyte homing studies, 200 μl of cell suspension at 5×10^7 CFSE-labeled cells was injected intravenously, followed by the removal of spleen at various intervals. The distribution of cells was tested by immunofluorescence using anti-LYVE-1 in conjunction with PE-labeled anti-rat IgG.

Quantitative RT-PCR (qPCR)

Total RNA was isolated with RNeasy Plus Mini Kit (Qiagen, Budapest) and was treated with DNase I (Sigma-Aldrich, Budapest). cDNA was prepared with a High Capacity cDNA Archive Kit (Applied Biosystems, Budapest). PCR primers used for real-time quantitative amplification of Prox1, Pdpn, Flt4, Lyve1, 18S ribosomal RNA, and Hprt1 were designed by Primer Express Software (Applied Biosystems, Table 1). PCR reactions were run in triplicates using Power Sybr Green Master Mix (Applied Biosystems) on an ABI 7500 Real Time PCR System (Applied Biosystems). Standard curves were generated for each transcript and expression levels were normalized to β-actin.

Statistical Analysis

Normal distributions of means were tested with one-sample Kolmogorov-Smirnov test. Means were compared with one-way ANOVA followed by Bonferroni test. *P* values less than 0.05 were considered statistically significant. Statistical analyses were performed with SPSS 14.0 software.

Results

Presence of LYVE-1$^+$ Endothelial Sacs in the Spleen of Mice Lacking Nkx2-3

Our previous work established the substantial alteration of blood vasculature of the red pulp in mice with deleted Nkx2-3 (Balogh et al. 2007). Further histological and electron microscopic analyses of *nkx2-3*$^{-/-}$ spleen revealed dilated vessel-like structures tightly filled with lymphocytes, reminiscent of lymphatic vessels (Figure 1A and B).

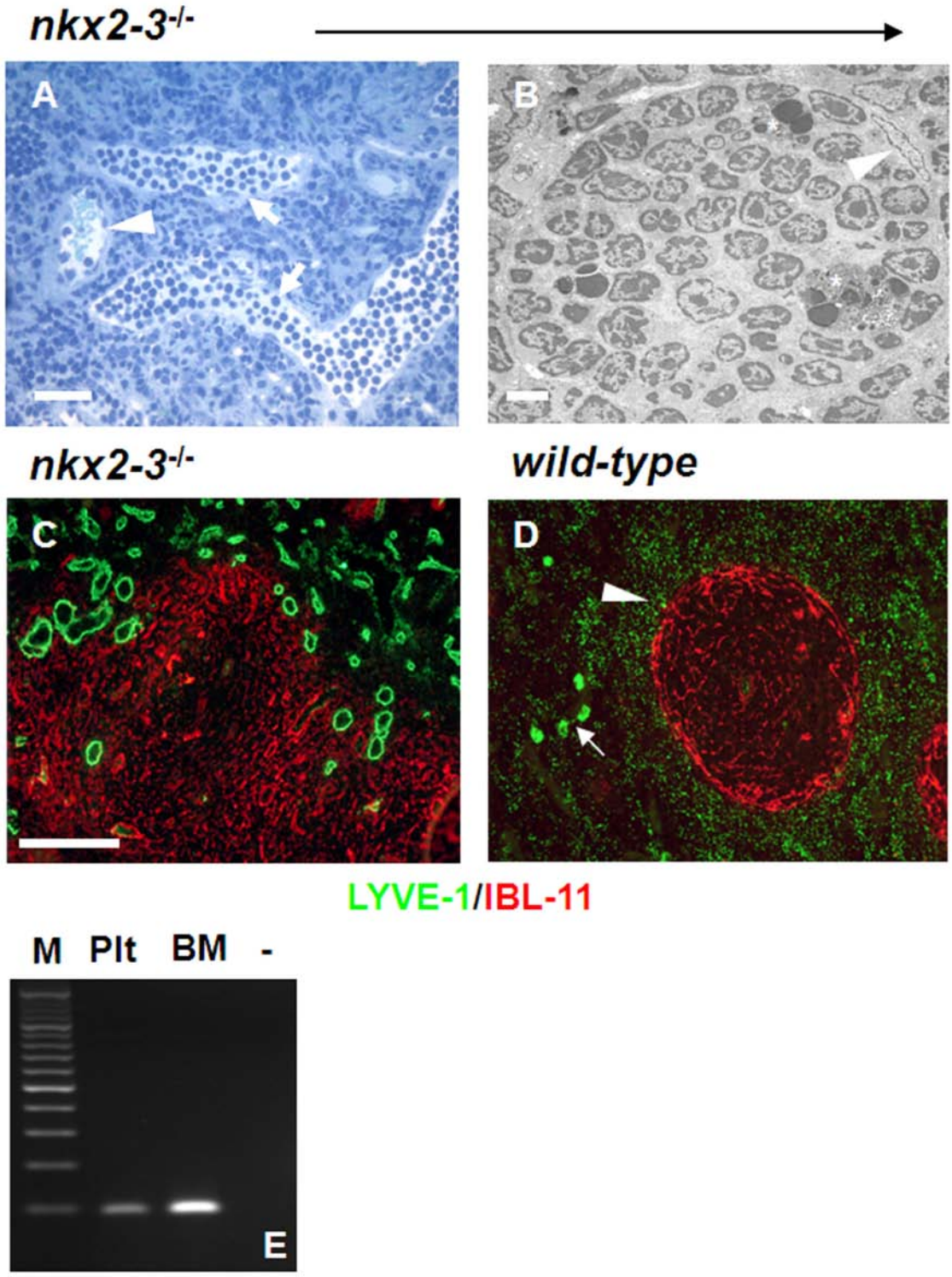

Figure 1A-E. Deficiency of Nkx2-3 induces the formation of LYVE-1$^+$ vessels. Semi-thin section stained with toluidine blue (A) demonstrates two lymphocyte-filled vascular segments (arrows) and a blood capillary (arrowhead; scale bar = 50 µm). Transmission electron microscopy (B) shows lymphoid cells filling a sac-like structure, lined by mesenchymal cells with elongated nucleus (arrowhead; scale bar = 7.5 µm). LYVE-1-positive structures in the spleen of *nkx2-3*$^{-/-}$ mice (C) accumulate mainly in the peripheral segment (upper part) of the spleen, without IBL-11-positive fibroblastic cuff. The scale bar = 200 µm. In wild-type spleen (D), LYVE-1 reactivity reveals platelets (arrowhead) in the marginal zone and megakaryocytes (arrow) in red pulp; the white pulp is outlined by IBL-11$^+$ fibroblasts. The scale bar = 200 µm. (E) LYVE-1 mRNA expression is detected by RT-PCR in platelet-enriched plasma (Plt) and bone marrow (BM) from wild-type mouse. Negative control (–) contained water (M: 100 bp ladder).

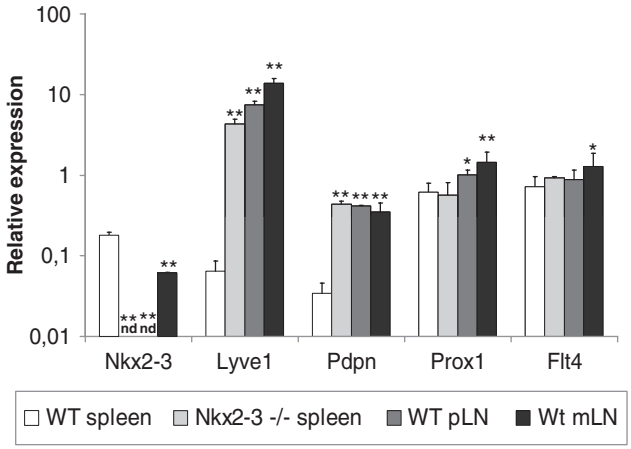

Figure 2. Comparison of the expression pattern of lymphatic endothelium associated genes and Nkx2-3 by quantitative PCR normalized to β-actin. Samples included spleens from wild-type (WT) and Nkx2-3–deficient mice and also peripheral lymph nodes (pLN) and mesenteric lymph nodes (mLN). The target genes were Nkx2-3, Lyve1, podoplanin/gp38 (Pdpn), Prox1, and Flt4/Vegfr3. Bar diagram shows the mean ± SD of six parallel samples of three mice. *$p < 0.05$; **$p < 0.001$; nd, not detectable.

Extended analysis using a battery of endothelium-subset specific antibodies included anti-LYVE-1 antibody, a marker frequently used to delineate lymphatic capillaries (Prevo et al. 2001), in conjunction with IBL-11 mAb against circumferential reticulum cells in marginal zone as topographic marker (Balogh et al. 2004). Staining spleen sections from Nkx2-3 mutant and wild-type samples with anti-LYVE-1 antibody resulted in intense labeling of mutants, completely delineating these structures. These vascular–cystic structures were mostly located at the periphery of the mutant organ (Figure 1C). In contrast, LYVE-1 immunofluorescence in wild-type spleen was limited to megakaryocytes in red pulp and platelets in the marginal zone outside the IBL-11-positive fibroblastic ring, without revealing any lymphatic capillaries (Figure 1D). The presence of LYVE-1 mRNA expression detectable by RT-PCR in platelet-enriched plasma and in bone marrow from wild-type mice is also consistent with its protein expression in these cell types (Figure 1E).

Biased Expression of mRNA of Lymphatic Endothelium-associated Marker Genes

Next we investigated whether the absence of Nkx2-3 affects the expression of mRNA for other lymphatic endothelium-associated genes using quantitative real-time PCR (qPCR) in adult mutant spleen and then compared it with wild-type controls. Our findings revealed a substantial upregulation of mRNA for LYVE-1 (66.8-fold) and podoplanin (12.1-fold, Pdpn also known as gp38; Breiteneder-Geleff et al. 1999) compared with Wild-type (Figure 2). Both genes in mutant spleen reached expression levels comparable with peripheral lymph nodes (pLN) and mesenteric lymph nodes (mLN). In contrast, Prox1 (Wigle 1999) and Flt4 (Kaipanen et al. 1995) (also known as VEGFR-3), two other specific marker genes for mouse lymphatic endothelium, did not differ between the wild-type and mutant spleen. We also investigated the mRNA expression of Nkx2-3. We found that in wild-type pLN Nkx2-3, mRNA was undetectable, while in mesenteric lymph nodes (mLN) it was expressed at a lower level compared with wild-type spleen.

Dual immunofluorescent labeling for Prox1 and LYVE-1 showed overlapping expression in normal pLNs but failed to reveal any Prox1 protein in Nkx2-3 mutant spleen (Figure 3A-D), in agreement with our results on the absence of upregulated Prox1 mRNA. However, the double staining of these structures with VEGFR-2/flk-1 and LYVE-1 indicated their endothelial, albeit non-LEC, origin (Figure 3E-G), although the LYVE-1^+ compartment of splenic vessels had a weaker VEGFR-2 staining compared with the LYVE-1^- segments (Figure 3F).

Internal Content and Stromal Tissue Microenvironment in LYVE-1+ Sacs

To investigate whether the LYVE-1^+ sacs have any ordered arrangement of enclosed lymphocyte subpopulations, we stained mutant spleens for T and B cells. In addition, we also analyzed their mesenchymal and stromal surroundings using mAbs against fibroblastic reticular cells (FRCs) and resident splenic macrophage subsets. We found that similar to the T-cell dominance in these mutant spleens (Czömpöly et al. in press), the LYVE-1^+ structures overwhelmingly contained T cells. The B lymphocytes were typically arranged in a scattered pattern (Figure 4A). Macrophage subsets expressing MARCO or sialoadhesin/CD169 that in normal spleen delineate the marginal zone (Mebius and Kraal 2005) formed small clusters adjacent to the LYVE-1^+ structures in mutant spleens (Figure 4B and C). In contrast, ER-$TR7^+$ FRCs closely surrounded the LYVE-1^+ endothelial cells. However, no ER-TR7 reactivity could be found within the sacs, indicating the absence of FRCs, in agreement with the cyst-like architecture of these structures (Figure 4D).

Limited Entry of Lymphocytes into LYVE-1^+ Cysts

To test whether circulating lymphoid cells can enter the LYVE-1^+ cysts, adoptive cell transfer experiments were performed. Tracing of CFSE-labeled lymphocytes after intravenous injection revealed only occasional transplanted cells within the LYVE-1^- positive compartment after 20 min and only a few scattered cells after 8 hr (Figure 5). Cell counting revealed that the ratio between the cells located

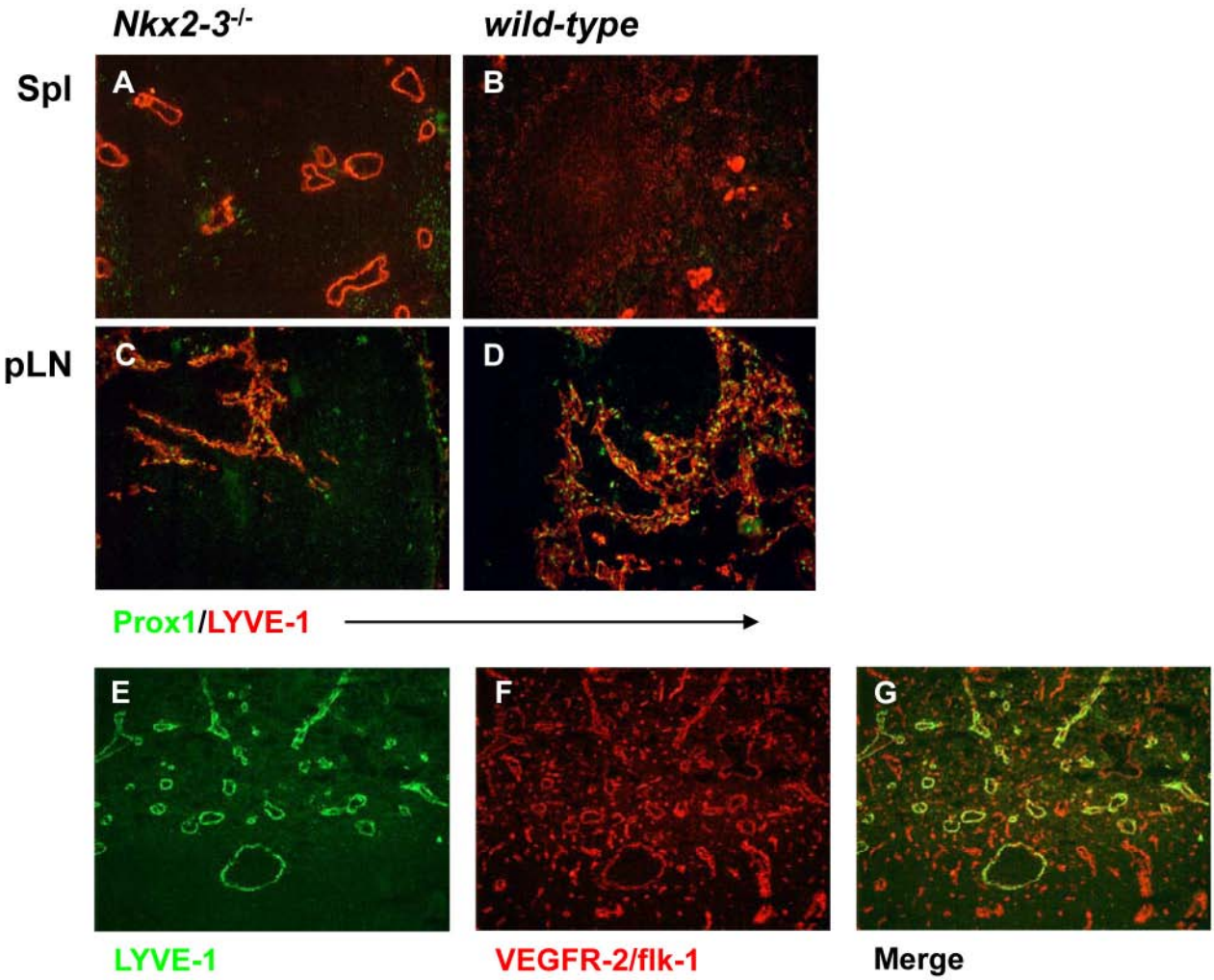

Figure 3. Nonlymphatic endothelial characteristics of LYVE-1$^+$ structures. LYVE-1$^+$ structures (red) in mutant spleen (A) do not express Prox1 transcription factor (green); in wild-type spleen (B) only platelets and megakaryocytes display LYVE-1, whereas lymph node lymphatic endothelium cells in both Nkx2-3 mutants and wild-type samples (C/D) coexpress both markers (merged as yellow). Cells lining LYVE-1$^+$ sacs coexpress VEGFR-2/flk-1 endothelial marker. (E, LYVE-1; F, VEGFR-2/flk-1; G, Merge.) Scale bar, 200 μm.

within and outside the sacs was initially 1.4:58.6 cells (approximately 2.3%), whereas after 8 hr it rose to 55.3:455.8 (12.1%) in a 1.4-mm^2 area of spleen section. Together with the absence of erythroid cells, this finding indicates that these LYVE-1$^+$ structures are not directly linked to the systemic blood circulation; however, some lymphocyte exchange occurs.

Formation of LYVE-1$^+$ Structures in Nkx2-3 Mutant Spleen Is Initiated in the Embryonic Period and Does Not Require LTβR Signaling

In addition to adult LECs, murine embryonic vessels have recently been demonstrated to display LYVE-1 glycoprotein (Gordon et al. 2008). To investigate whether the embryonic spleen contains LYVE-1$^+$ vessels, we stained E18.5 spleens from both wild-type and Nkx2-3–deficient mice for LYVE-1 expression. We found that in Nkx2-3–deficient embryos, LYVE-1–positive structures were present although did not yet form discernible sacs. In contrast, in wild-type mice no such structures could be identified (Figure 6A and B). Shortly after birth in mutant mice, similar structures could be found typically within the central region of the spleen; however, by the second week the LYVE-1$^+$ structures achieved adult-type appearance, including their repositioning to the peripheral segment of the organ (Figure 6C-F).

In addition to promoting the maturation of blood vasculature in embryonic peripheral lymphoid organs into adult-type HEV, the sprouting of lymphatic capillaries may be influenced

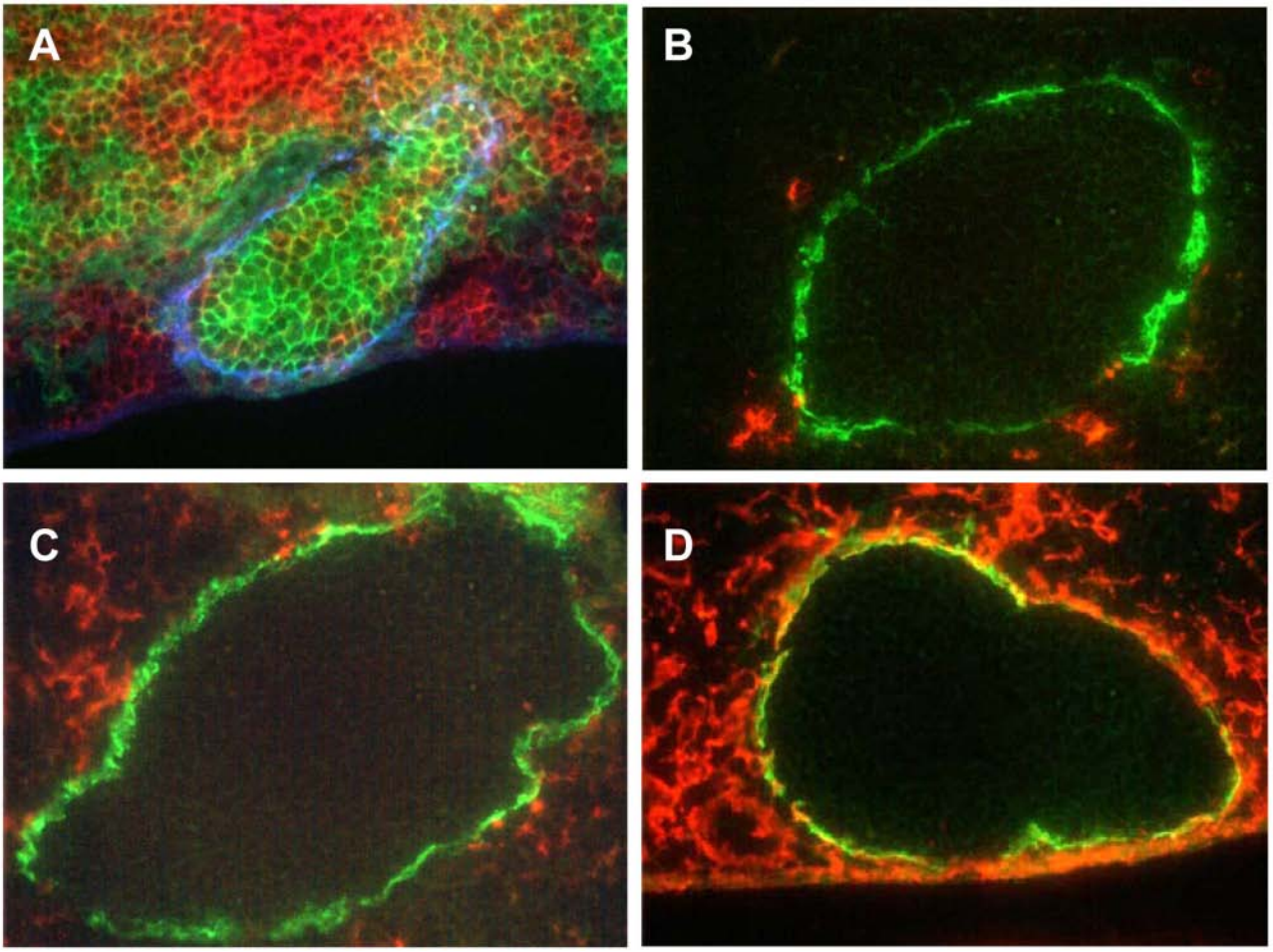

Figure 4. Internal and external composition of LYVE-1$^+$ structures. (A) The LYVE-1$^+$ sacs (blue) are mostly filled with T lymphocytes (green), with occasional B cells or their smaller groups (red). Macrophages (red) expressing MARCO (B) or sialoadhesin (C) form only occasional associations with LYVE-1$^+$ structures (green), which are tightly encircled with ER-TR7$^+$ fibroblastic reticular cells antigen (D, red). Scale bar, 100 μm.

by LTβR by inducing the production of vascular endothelial growth factor (VEGF)-C, a lymphangiogenic ligand for VEGFR-3 (Vondenhoff et al. 2009). To test whether the formation of splenic LYVE-1$^+$ structures in Nkx2-3 mutants requires LTβR, we established double mutants deficient for both Nkx2-3 and LTβR. We found that the LYVE-1-positive vessels were present in the double mutant spleens; thus, their formation is independent of LTβR signaling (Figure 6G and H). In addition, in double mutants the LYVE-1$^+$ structures were more numerous in the central region than in Nkx2-3 single mutant samples, and their appearance was more similar to vascular than cyst-like structures.

Discussion

The present report describes the appearance of aberrant endothelial structures formed by cells resembling lymphatic endothelium in the spleen of mice deficient for homeodomain transcription factor Nkx2-3. To our knowledge, no such aberrant structures have been reported in other mutations affecting the splenic vasculature; thus, their study offers a unique opportunity to facilitate the understanding of splenic endothelial maturation and vascular patterning.

Beyond their importance as structural elements in peripheral lymphoid organs, the specification of vasculature is also essential for maintaining the immunological surveillance by simultaneously providing access during leukocyte homing, and entry routes for pathogens, via either lymphatic vessels in lymph nodes or bloodstream in spleen, respectively. In this process, several transcription factors and other morphogenic regulators have been identified that promote the embryonic specification toward lymphatic endothelium and the expansion of these LEC-committed cells. These factors include Prox1 and VEGFR-3, and their

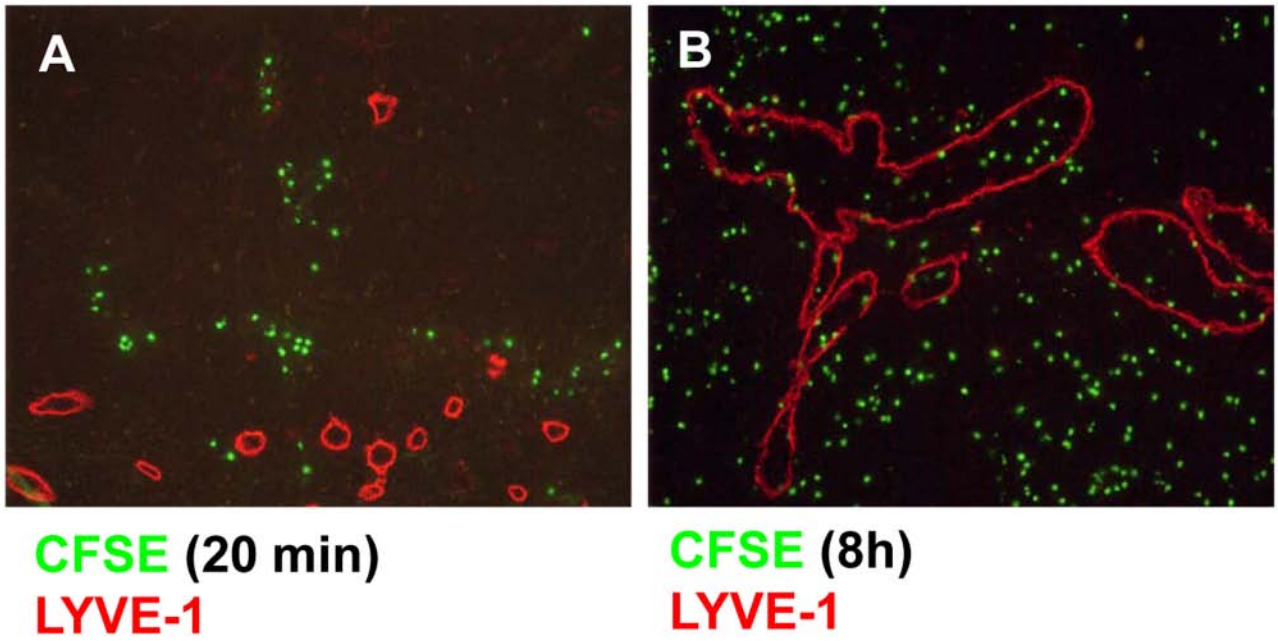

Figure 5. LYVE-1$^+$ vessels do not contribute to the early splenic recirculation of lymphocytes. The ratio of carboxyfluorescein diacetate, succinimidyl ester (CFSE)–labeled cells within/outside the sacs was 0.023 at 20 minutes (A), whereas at 8 hr (B) the ratio was 0.12 (1.4-mm^2 area). The picture is representative of three independent experiments performed on five mice; the scale bar = 200 µm.

effects are marked by the continued expression of LYVE-1 and the induction of podoplanin as LEC-associated markers (Oliver and Srinivasan, 2010). Furthermore, the blood vasculature in developing lymph nodes also undergoes substantial transformation into HEVs upon the engagement of LTβR (Browning et al. 2005), which also promotes the local expansion of LECs (Vondenhoff et al. 2009).

In contrast to the lymph nodes with detailed knowledge on the vascular specification, substantially less is known about the establishment of the splenic vasculature, mostly because of its complexity in both humans and mice (Balogh and Lábadi, 2011). In mice the process of red pulp vascular maturation is nearly completed by the time of birth, apparently independently from the activity of LTβR during the embryonic period (Balázs et al. 2001; Vondenhoff et al. 2008). In contrast, Nkx2-3 in a unique fashion blocks the development of the red pulp vasculature, and the absence of either Nkx2-3 or LTβR impairs the subsequent formation of marginal sinus in mice (Balogh et al. 2007). However, the expression of Nkx2-3 in spleen declines well before birth (Wang et al. 2000), indicating that its absence is also likely to manifest during the embryonic period, supported by our finding of the late embryonic appearance of LYVE-1$^+$ cell clusters in the spleen of Nkx2-3 mutants. In age-matched wild-type controls, LYVE-1$^+$ vascular segments could be observed only in the stomach but not in the spleen (not shown). It is not yet known how the absence of Nkx2-3 results in the upregulation of *Lyve1* mRNA, although in silico analyses for possible binding sites within the promoter region of *Lyve1* gene indicate a number of putative recognition sequences, thus raising a possible repressor effect of Nkx2-3 for *Lyve1* expression under normal conditions. Although the promoter region of *Prox1* also contains putative Nkx2-3 recognition sequences, its unaltered expression in mutant spleens indicates that its expression is either unrelated to the activity of Nkx2-3 or that other members of the Nkx family (e.g., Nkx2-5) may compensate its absence.

The formation of lymphatic vessels is coupled with phenotypic maturation of LEC-committed endothelial cells, which is critically influenced by Prox1 fate-determining transcription factor. This process is characterized by the appearance of several LEC-associated cell surface molecules, including LYVE-1 and podoplanin. Although the expression of podoplanin/gp38 mRNA in mutants is also elevated, it can be attributed to the expanded fibroblastic reticular meshwork associated with the enlarged T-cell pool in these mutants (Bovári et al. 2007). Importantly, LYVE-1 molecule can also be displayed by inflammatory macrophages (Maruyama et al. 2005; Schledzewski et al. 2006) similarly to podoplanin/gp38, another marker for LECs, which can be expressed by splenic macrophages (Hou et al. 2010). Together with our observation on the expression of LYVE-1 by megakaryocytes and platelets, this suggests that the usual phenotypic markers used for identifying LECs in lymph nodes and skin have other cellular reactivities in

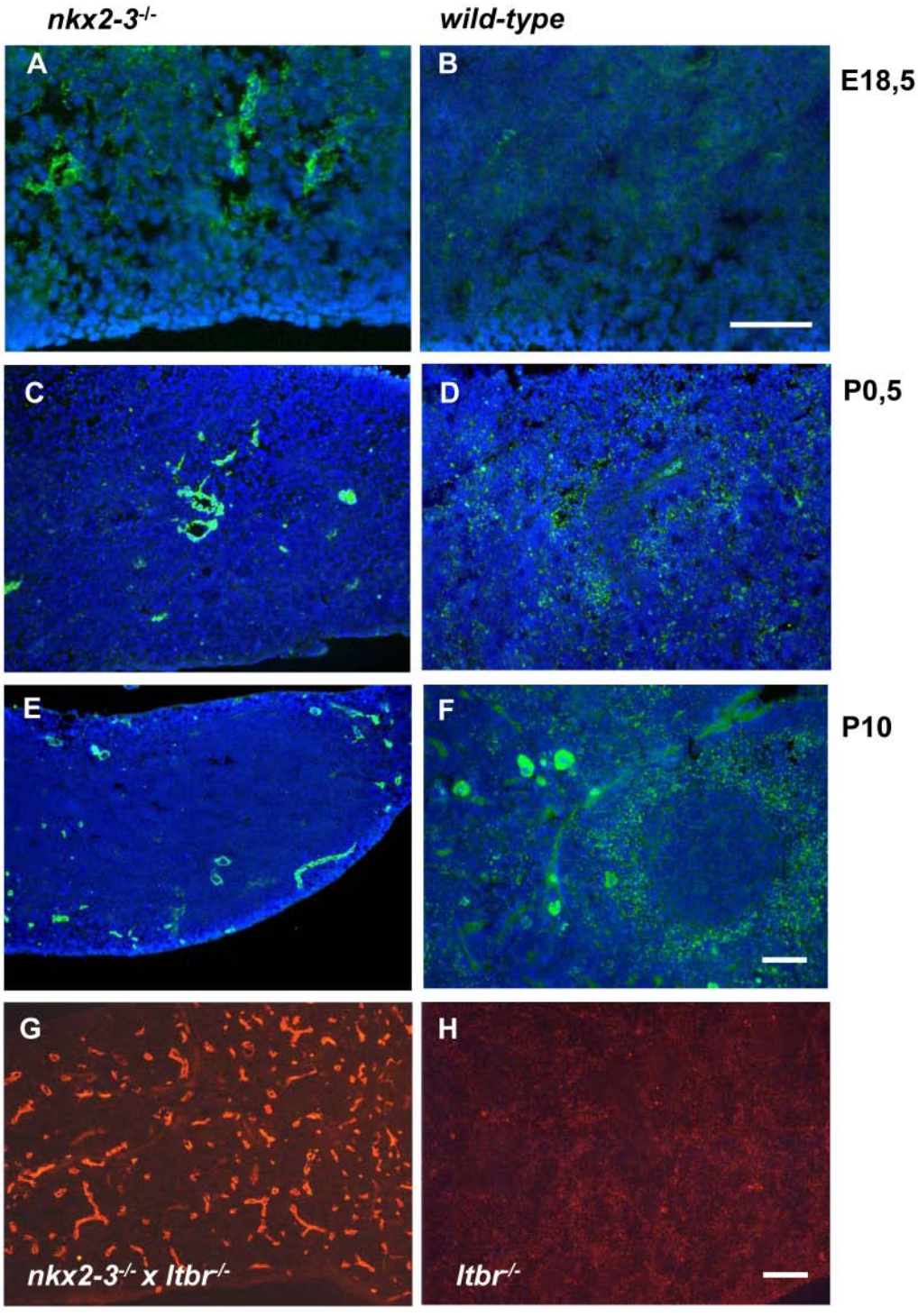

Figure 6. Formation of LYVE-1$^+$ structures and its independence from LTβR signaling. Late embryonic and early postnatal spleens (indicated on the left) from Nkx2-3–deficient mice (A, C, and E) contain LYVE-1$^+$ cells, compared with age-matched wild-type controls (B, D, and F, with DAPI counterstain). Spleens with combined mutation of Nkx2-3 and LTβR contain an increased number of LYVE-1 capillaries (G) compared with *ltbr*$^{-/-}$ mutants (H). The picture is representative of five mice per sample; the scale bar = 100 μm.

spleen. Thus we consider Prox1 as a cardinal LEC marker, and its absence together with that of VEGFR-3 suggests therefore that the postnatal persistence of LYVE-1$^+$ vessels is independent from the LEC commitment, so it probably represents the persistence of embryonic endothelium (Gordon et al. 2008), rather than the differentiation into

LEC-committed endothelia, in adult Nkx2-3 mutant spleens. However, the expression of VEGFR-2/flk1 confirms that these structures are composed of non-LEC endothelial cells. In contrast, cell tracing studies also suggest that these LYVE-1$^+$ cysts have only minimal exchange with either the systemic or lymphatic circulation. Although their formation is independent from the activity of LTβR, their increased number and capillary-like appearance in double mutants suggest some role for LTβR in their subsequent maturation.

The vascular malformations in human spleen are very rare and include various tumors and cysts (Kutok and Fletcher, 2003). The few reported cases of lymphatic endothelial cysts in human spleens do not correspond to the LYVE-1$^+$ structures in the Nkx2-3 mutants (Narita and Hizawa, 1995). In contrast, Nkx2-3 is also expressed in gut, and Nkx2-3 in humans has recently been associated with risk factors for inflammatory bowel diseases, although the underlying pathomechanism is still unknown (Franke et al. 2008; Parkes et al. 2007). It remains to be determined whether the formation of enteric lymphatic capillaries is also affected by the absence of Nkx2-3, similar to the reduction of Peyer's patches (Pabst et al. 2000; Wang et al. 2000). Nevertheless, our data raise the possibility that endothelial differentiation and maturation, including the early commitment from embryonic vessels, may be affected by perturbed expression of Nkx2-3, which may contribute to aberrant lymphoid neogenesis and immunological functions in these diseases (Drayton et al. 2006).

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References

Balázs M, Grama L, Balogh P. 1999. Detection of phenotypic heterogeneity within the murine splenic vasculature using rat monoclonal antibodies IBL-7/1 and IBL-7/22. Hybridoma. 18:177–182.

Balázs M, Horváth G, Grama L, Balogh P. 2001. Phenotypic identification and development of distinct microvascular compartments in the postnatal mouse spleen. Cell Immunol. 212:126–137.

Balogh P, Bebök Z, Németh P. 1992. Cellular enzyme-linked immunocircle assay: a rapid assay of hybridomas produced against cell surface antigens. J Immunol Methods. 153:141–149.

Balogh P, Horváth G, Szakal AK. 2004. Immunoarchitecture of distinct reticular fibroblastic domains in the white pulp of mouse spleen. J Histochem Cytochem. 52:1287–1298.

Balogh P, Lábadi Á. 2011. Structural evolution of the spleen in man and mouse. In: Developmental Biology of Peripheral Lymphoid Organs. P Balogh, editor, Berlin Heidelberg: Springer-Verlag; 121–141.

Balogh P, Balázs M, Czömpöly T, Weih DS, Arnold HH, Weih F. 2007. Distinct roles of lymphotoxin-beta signaling and the homeodomain transcription factor Nkx2.3 in the ontogeny of endothelial compartments in spleen. Cell Tissue Res. 328:473–486.

Bovári J, Czömpöly T, Olasz K, Arnold HH, Balogh P. 2007. Complex organizational defects of fibroblast architecture in the mouse spleen with Nkx2-3 homeodomain deficiency. Pathol Oncol Res. 13:227–235.

Breiteneder-Geleff S, Soleiman A, Kowalski H, Horvat R, Amann G, Kriehuber E, Diem K, Weninger W, Tschachler E, Alitalo K, et al. 1999. Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. Am J Pathol. 154:385–394.

Brendolan A, Rosado MM, Carsetti R, Selleri L, Dear TN. 2007. Development and function of the mammalian spleen. Bioessays. 29:166–177.

Browning JL, Allaire N, Ngam-Ek A, Notidis E, Hunt J, Perrin S, Fava RA. 2005. Lymphotoxin-beta receptor signaling is required for the homeostatic control of HEV differentiation and function. Immunity. 23:539–550.

Czömpöly T, Lábadi Á, Kellermayer Z, Olasz K,Arnold HH,Balogh P. 2011. Transcription factor Nkx2-3 controls the vascular identity and lymphocyte homing in the spleen. J Immunol, in press.

Drayton DL, Liao S, Mounzer RH, Ruddle NH. 2006. Lymphoid organ development: from ontogeny to neogenesis. Nat Immunol. 7:344–353.

Franke A, Balschun T, Karlsen TH, Hedderich J, May S, Lu T, Schuldt D, Nikolaus S, Rosenstiel P, Krawczak M, et al. 2008. Replication of signals from recent studies of Crohn's disease identifies previously unknown disease loci for ulcerative colitis. Nat Genet. 40:713–715.

Fütterer A, Mink K, Luz A, Kosco-Vilbois MH, Pfeffer K. 1998. The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. Immunity. 9:59–70.

Giorno R. 1984. Unusual structure of human splenic sinusoids revealed by monoclonal antibodies. Histochemistry. 81:505–507.

Gordon EJ, Gale NW, Harvey NL. 2008. Expression of the hyaluronan receptor LYVE-1 is not restricted to the lymphatic vasculature; LYVE/1 is also expressed on embryonic blood vessels. Dev Dyn. 237:1901–1909.

Hou TZ, Bystrom J, Sherlock JP, Qureshi O, Parnell SM, Anderson G, Gilroy DW, Buckley CD. 2010. A distinct subset of podoplanin (gp38) expressing F4/80+ macrophages mediate phagocytosis and are induced following zymosan peritonitis. FEBS Lett. 584:3955–3961.

Kaipanen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D, Breitman M, Alitalo K. 1995. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci U S A. 92:3566–3570.

Kutok JL, Fletcher CD. 2003. Splenic vascular tumors. Semin Diagn Pathol. 20:128–139.

Kvell K, Czömpöly T, Pikkarainen T, Balogh P. 2006. Species-specific restriction of cell surface expression of mouse MARCO glycoprotein in murine cell lines. Biochem Biophys Res Commun. 341:1193–1202.

Martinez-Pomares L, Hanitsch LG, Stillion R, Keshav S, Gordon S. 2005. Expression of mannose receptor and ligands for its cysteine-rich domain in venous sinuses of human spleen. Lab Invest. 85:1238–1249.

Maruyama K, Ii M, Cursiefen C, Jackson DG, Keino H, Tomita M, Van Rooijen N, Takenaka H, D'Amore PA, Stein-Streilein J, et al. 2005. Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. J Clin Invest. 115:2363–2372.

Mebius RE, Kraal G. 2005. Structure and function of the spleen. Nat Rev Immunol. 5:606–616.

Narita T, Hizawa Y. 1995. Lymphangiomatous hamartoma of the spleen. Pathol Res Pract. 191:1165–1167.

Nolte MA, Hamann A, Kraal G, Mebius RE. 2002. The strict regulation of lymphocyte migration to splenic white pulp does not involve common homing receptors. Immunology. 106:299–307.

Oliver G, Srinivasan RS. 2010. Endothelial cell plasticity: how to become and remain a lymphatic endothelial cell. Development. 137:363–372.

Pabst O, Zweigerdt R, Arnold HH. 1999. Targeted disruption of the homeobox transcription factor Nkx2-3 in mice results in postnatal lethality and abnormal development of small intestine and spleen. Development. 126:2215–2225.

Pabst O, Förster R, Lipp M, Engel H, Arnold HH. 2000. NKX2-3 is required for MAdCAM-1 expression and homing of lymphocytes in spleen and mucosa-associated lymphoid tissue. EMBO J. 19:2015–2023.

Parkes M, Barrett JC, Prescott NJ, Tremelling M, Anderson CA, Fisher SA, Roberts RG, Nimmo ER, Cummings FR, Soars D, et al. 2007. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. Nat Genet. 39:830–832.

Pellas TC, Weiss L. 1990. Deep splenic lymphatic vessels in the mouse: a route of splenic exit for recirculating lymphocytes. Am J Anat. 187:347–354.

Prevo R, Banerji S, Ferguson DJ, Clasper S, Jackson DG. 2001. Mouse LYVE-1 is an endocytic receptor for hyaluronan in lymphatic endothelium. J Biol Chem. 276:19420–19430.

Schledzewski K, Falkowski M, Moldenhauer G, Metharom P, Kzhyshkowska J, Ganss R, Demory A, Falkowska-Hansen B, Kurzen H, Ugurel S, et al. 2006. Lymphatic endothelium-specific hyaluronan receptor LYVE-1 is expressed by stabilin-1+, F4/80+, CD11b+ macrophages in malignant tumours and wound healing tissue in vivo and in bone marrow cultures in vitro: implications for the assessment of lymphangiogenesis. J Pathol. 209:67–77.

Stanfel MN, Moses KA, Schwartz RJ, Zimmer WE. 2005. Regulation of organ development by the NKX-homeodomain factors: an NKX code. Cell Mol Biol (Noisy-le-grand). 51:OL785–799.

Steiniger B, Stachniss V, Schwarzbach H, Barth PJ. 2007. Phenotypic differences between red pulp capillary and sinusoidal endothelia help localizing the open splenic circulation in humans. Histochem Cell Biol. 128:391–398.

Vondenhoff MF, Desanti GE, Cupedo T, Bertrand JY, Cumano A, Kraal G, Mebius RE, Golub R. 2008. Separation of splenic red and white pulp occurs before birth in a LTalphabeta-independent manner. J Leukoc Biol. 84:152–161.

Vondenhoff MF, Greuter M, Goverse G, Elewaut D, Dewint P, Ware CF, Hoorweg K, Kraal G, Mebius RE. 2009. LTbetaR signaling induces cytokine expression and up-regulates lymphangiogenic factors in lymph node anlagen. J Immunol. 182:5439–5445.

Wang CC, Biben C, Robb L, Nassir F, Barnett L, Davidson NO, Koentgen F, Tarlinton D, Harvey RP. 2000. Homeodomain factor Nkx2-3 controls regional expression of leukocyte homing coreceptor MAdCAM-1 in specialized endothelial cells of the viscera. Dev Biol. 224:152–167.

Wigle JT, Oliver G. 1999. Prox1 function is required for the development of the murine lymphatic system. Cell. 98:769–778.

Wigle JT, Harvey N, Detmar M, Lagutina I, Grosveld G, Gunn MD, Jackson DG, Oliver G. 2002. An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. EMBO J. 21:1505–1513.