Transcription Factor Nkx2-3 Controls the Vascular Identity and Lymphocyte Homing in the Spleen

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Transcription Factor Nkx2-3 Controls the Vascular Identity and Lymphocyte Homing in the Spleen

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The vasculature in the spleen and peripheral lymph nodes (pLNs) is considerably different, which affects both homing of lymphocytes and antigenic access to these peripheral lymphoid organs. In this paper, we demonstrate that in mice lacking the homeodomain transcription factor Nkx2-3, the spleen develops a pLN-like mRNA expression signature, coupled with the appearance of high endothelial venules (HEVs) that mediate L-selectin–dependent homing of lymphocytes into the mutant spleen. These ectopic HEV-like vessels undergo postnatal maturation and progressively replace MAdCAM-1 by pLN addressin together with the display of CCL21 arrest chemokine in a process that is reminiscent of HEV formation in pLNs. Similarly to pLNs, development of HEV-like vessels in the Nkx2-3–deficient spleen depends on lymphotixin-β receptor-mediated signaling. The replacement of splenic vessels with a pLN-patterned vasculature impairs the recirculation of adoptively transferred lymphocytes and reduces the uptake of blood-borne pathogens. The Nkx2-3 mutation in BALB/c background causes a particularly disturbed splenic architecture, characterized by the near complete lack of the red pulp, without affecting lymph nodes. Thus, our observations reveal that the organ-specific patterning of splenic vasculature is critically regulated by Nkx2-3, thereby profoundly affecting the lymphocyte homing mechanism and blood filtering capacity of the spleen in a tissue-specific manner. The Journal of Immunology, 2011, 186: 000–000.

The spleen in mammals is the largest single peripheral lymphoid organ, contributing to both innate and adaptive immune responses against invading pathogens. Its development and tissue architecture are strikingly different from other secondary lymphoid organs, including the peripheral lymph nodes (pLNs) and Peyer’s patches. Different lymphoid regions in the spleen are arranged around various vessels. The T cell zone is located around central arterioles, and the marginal zone is adjacent to terminal arterioles in human or marginal sinuses in rodents, whereas the red pulp is richly perfused by a sinus network lined by different endothelial subsets (1, 2). In contrast to the leukocyte homing in pLNs and Peyer’s patches, the spleen lacks high endothelial venules (HEVs) and requires no L-selectin binding for subsequent leukocyte extravasation (3, 4).

The development of the spleen is initiated by the formation of its mesenchymal anlage, controlled by the concerted action of multiple transcription factors, including Pbx1, Nkx3-2 (also known as Bapx1), TLX1 (formerly known as HOX11), and Nkx2-5 (5, 6). In the absence of any of these factors, development of the spleen is arrested early, resulting in asplenia. TLX1 may be regulated independently by the homeodomain transcription factor Barx1, which is also involved in stomach development (7). Importantly, loss of TLX1 does not prevent the formation of lymph nodes (8). Embryonic lethality of Pbx1- and Nkx2-5–deficient mice and neonatal death of Nkx3-2–null mutants precludes the analysis of their potential roles in lymph node development (9–11).

Following formation of the splenic primordium, spleen progenitor cells undergo expansion and local hematopoiesis starts, controlled by the transcription factor Tal-1 (also known as Scl) (12). Subsequently, CD4+/CD3−/CD45−/IL-7Rα+ lymphoid cells accumulate around the developing blood vessels in close proximity with stromal cells that express MAdCAM-1 and VCAM-1 (13). This close cellular association is thought to be necessary for efficient interaction between members of the TNF/lymphotoxin (LT) family displayed by lymphoid cells (termed lymphoid tissue inducer) and their cognate receptors on stromal cell precursors (14–16). In contrast to the spleen-autonomous defect in TLX1–deficient mutant mouse (8), loss of either LTα or the receptor for LTβ (LTβR) prevents the formation of lymph nodes and Peyer’s patches, whereas the spleen develops, although with severely disturbed architecture of white pulp and marginal zone (17). Distinct lymph node and spleen phenotypes elicited in mutants lacking LT ligands or corresponding receptors and in TLX1 mutants suggest that development of the various peripheral lymphoid organs follows distinct regulatory programs. In this process, the factors for cell lineage specification related to vascular patterning in distinct lymphoid organs are mostly unknown.

More recently, the homeobox transcription factor Nkx2-3 has been identified as an important regulator of spleen ontogeny (18–
20) without affecting pLN development. The 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, and gastrointestinal tract, in a highly complex expression pattern (21). Nkx2-3<sup>−/−</sup> mice lack the marginal sinus and have atrophic red pulp sinus network, two specific vascular structures in the spleen that are absent in other peripheral lymphoid tissues (22). In this paper, we report that Nkx2-3 deficiency in the mouse transforms the vasculature in a mutant spleen structurally and functionally toward the typical vascular pattern of pLNs. The disruption of the Nkx2-3 gene results in the formation of splenic pLN addressin (PNAd)-positive HEVs that display CCL21 arrest chemokine and mediate L-selectin–dependent homing. The development of ectopic HEV-like structures in a mutant spleen requires LTβR-mediated signaling, similar to the HEVs in pLNs. Moreover, the mutants show reduced marginal zone-associated capture of pathogens. Thus, our observations provide evidence that specification of the vascular pattern in the spleen and its consequences for lymphocyte homing are critically dependent on the transcription factor Nkx2-3.

**Materials and Methods**

**Mice**

Nkx2-3<sup>−/−</sup> mice from a 129sv × B6 mixed background were backcrossed with BALB/c mice (obtained from Charles River Laboratories, Budapest, Hungary) through 14 generations and genotyped as described previously. C57BL/6 and 129sv Nkx2-3 mutants were maintained at the Technical University of Braunschweig (Braunschweig, Germany). For homing studies, BALB/c mice from the faculty’s specific pathogen-free breeding unit were used as a lymphocyte donor for adoptive transfer. LTβR-deficient mice (23) were provided by Drs. K. Pfeffer (Heinrich Heine University, Düsseldorf, Germany) and F. Weih (Leibniz Institute for Age Research, Fritz Lipmann Institute, Jena, Germany). 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 (Pécs, Germany). The double-knockout (KO) mice were identified in the F<sub>2</sub> generation by simultaneous PCR amplification of the Nkx2-3, neomycin resistance cassette, and LTβR loci from genomic DNA by using the following primer pairs: Nkx2-3, 5′-GCGGGAGACTGTAAGACGAG-3′ (forward) and 5′-TTATCTGCAGGCTGTCTCTT-3′ (reverse), amplicon size: 238 bp; neomycin resistance cassette, 5′-TGGCTAGAAGGTATGAC-3′ (forward) and 5′-AAGGGATAGAAGGGAGTGAC-3′ (reverse), amplicon size: 321 bp; and LTβR, 5′-GCATGTAGCCGTAAAGACAGGAT-3′ (forward) and 5′-CGCAAGAGCAACTGCGCTAT-3′ (reverse), amplicon size: 150 bp.

**Abs and reagents**

Rat mAbs against mouse fibroblastic reticular cell markers (I-BL-1) and Thy-1 (I-BL-2) were developed in our laboratory (24). For flow cytometry and dual immunohistochemistry, I-BL-1 mAb was conjugated with sulfo-N-hydroxysuccinimidyl biotin ester (Sigma-Aldrich, Hungary, Budapest). Rat hydridoma cell lines secreting anti-CD3 (KT-3), anti–MAdCAM-1 (MECA-367), L-selectin/CD62L (MEL-14), and B220 (RA3-6B2) were obtained from the bridoma cell lines secreting anti-CD3 (KT-3), anti–MAdCAM-1 (MECA-79), and FITC-labeled anti-mouse IgG were obtained from R&D Systems (Biomedica Hungary, Cambridge, MA) and was injected in newborn Nkx2-3 KO mice as described previously (26). Pooled human IgG was used as control.

**Flow cytometry**

Lymphocytes were isolated from the spleen and lymph nodes by teasing apart the organs between the frosted ends of two microscopic slides and filtered through a 70-μm pore-size cell strainer. The cells were incubated with a mixture of fluorescein-labeled anti-B220 and biotinylated anti-CD3 or anti-CD62L mAb in the presence of 2-4G anti-CD16/32 mAb. The biotinylated mAbs and the biotinylated cell in homing studies were detected with PE-streptavidin. Dead cells were excluded based on their light scattering properties. At least 20,000 live cells were collected by a BD Biosciences FACSCalibur cytometer and analyzed using the CellQuest software.

**Immunohistochemistry and immunofluorescence**

The single and dual-label immunofluorescence and immunohistochemical procedures are previously described (24, 26) for control staining normal rat IgG at 10 μg/ml concentration was used. After mounting, the sections were viewed under an Olympus BX61 fluorescent microscope. The acquisition of digital pictures with a charge-coupled device camera was performed using the analySIS software.

**Adaptive cell transfer and bacterial injection**

Lymphocytes from pLNs or mesenteric lymph nodes (mLNs) were isolated and labeled with CFSE (Invitrogen) or sulfo-N-hydroxysuccinimidyl biotin ester (Sigma-Aldrich) as described previously (27). For lymphocyte homing studies, 200 μl cell suspension at 5 × 10<sup>6</sup> CFSE-labeled cells was injected i.v. in the tail vein, followed by the removal of the spleen at various intervals. The distribution of labeled cells was tested cells by immunofluorescence using anti-PNAd or IBL-11 mAb against white pulp fibroblasts (24) in conjunction with PE-labeled anti-rat IgG. For competitive homing, two different cellular labeling procedures were used in which the CFSE-labeled cells were subsequently incubated with MEL-14 IgG (purified by Protein G FPLC) or IBL-10 control rat mAb. After washing, the cells were counted and mixed at 1:1 ratio with biotinylated cells as reference population, followed by the i.v. injection of mixed cells. The mice were sacrificed 30 min after the injection, and their distribution of CFSE-labeled and biotinylated lymphocytes in different lymphoid tissues was determined by flow cytometry or immunofluorescence. The CFSE:biotin ratio of the cells in lymphoid organs was calculated following the normalization of labeled cell frequencies with the preinjection CFSE:biotin ratio; thus, the CFSE:biotinylated cell ratio recovered from each organ was divided by the CFSE:biotinylated cell ratio in the preinjection cell mixture. For bacterial capture, mice were i.v. injected with 10<sup>6</sup> Escherichia coli previously conjugated with FITC in 250 μl PBS.

**Quantitative RT-PCR**

Total RNA was isolated with the RNeasy Plus Mini Kit (Qiagen) and was treated with DNase I (Sigma-Aldrich). cDNA was prepared with the High Capacity cDNA Archive Kit (Applied Biosystems). PCR primers used for real-time quantitative amplification of Glycam1, Madcam1, B3gnt3, Gctn1, Chs2, Chs4, and Fat7 were described previously (27). PCR primers for Hprt1 were designed by Primer Express Software (Applied Biosystems). PCR primers for Cd34, endomucin, Cd300lg, podocalyxin-like protein, 185 rRNA, and Hprt1 were designed by Primer Express Software (Applied Biosystems). Reactions were run in triplicates using the 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; the relative expressions were calculated using β-actin normalized expression levels of wild-type spleens as reference samples.

**Statistical analysis**

Normal distributions of means were tested with a one-sample Kolmogorov-Smirnov test. Means were compared with one-way ANOVA, followed by a Bonferroni test. A p value < 0.05 was considered statistically significant. Statistical analyses were performed with SPSS 14.0 software.

**Results**

Altered expression of marker genes associated with HEV development in the spleen and lymph nodes

Previous studies of Nkx2-3<sup>−/−</sup> mice in our laboratory and elsewhere already revealed a broad range of structural alterations of the spleen (18, 22), affecting the marginal sinus and red pulp sinuses, two highly specific segments of the splenic vasculature.
involved in splenic recirculation in mice (reviewed in Ref. 2). Despite the lack of these vessels in mutants, the accumulation of lymphocytes in the mutant spleen indicates the existence of a selective homing process to that organ, prompting an analysis of those endothelium-associated genes that have been implicated in tissue-specific homing. Microarray analysis of the Nkx2-3–mutant spleen suggested that expression of several genes associated with endothelium-mediated tissue-specific homing (28–33) was altered (data not shown). In this study, we performed real-time PCR to quantify expression of some of these Nkx2-3–regulated candidate genes in wild-type and mutant spleens as well as in pLNs and mLNs. The analysis demonstrated profound reduction of MadCAM-1 expression in the spleen of the homozygous mutant mouse in agreement with previous reports (19, 20). Importantly, expression of GlyCAM-1 in the mutant spleen was dramatically upregulated (~10, 000-fold), whereas transcript levels for other marker genes, such as CD34, endomucin, CD300 Ag-like family member G (Cd300lg, also known as nepmucin), and podocalyxin-like protein, were also increased, although to a lesser extent (between 4- and 16-fold) in comparison with wild type (Fig. 1A). Particularly, the upregulation of the endothelial marker GlyCAM-1 and the reduction of MadCAM-1 expression in the mutant spleen appeared more similar to pLNs than in wild-type spleens. In comparison, the pLNs from Nkx2-3–deficient mice showed a slightly elevated expression of MadCAM-1 and an equal expression of GlyCAM-1 compared with wild-type mice, whereas mLNs had less MadCAM-1 and a higher level of GlyCAM-1 mRNA expression than wild-type controls. The differences between the expression level of other core proteins genes was less dramatic, although the general tendency was an increased mRNA production for all genes investigated. Pdxl, a lesser investigated PNAd core protein, showed an increased expression in all lymphoid tissues of Nkx2-3 mutants, whereas its expression in pLNs and mLNs of wild-type mice was reduced compared with wild-type spleens.

Both GlyCAM-1 and MadCAM-1 may serve as backbone proteins for PNAd in a process depending on glycosylation and sulfation, mediated by the glycosyltransferases B3gnt3 and Gcnt1, the fucosyltransferase Fut7, and the sulfotransferases Chst2 and Chst4, the latter also known as HEC-GlcNAc6ST, respectively (34). Quantitative RT-PCR (qPCR) analysis for these genes in wild-type and mutant spleens revealed that the majority of these transcripts were upregulated in the Nkx2-3–/– mutant, although to different degrees (Fig. 1B). B3gnt3 glycosyltransferase in Nkx2-3–/– mutant spleen was expressed at a lower amount compared with wild-type spleen; however, this reduced mRNA transcription was also observed at various degrees in lymph nodes from both mutant and wild-type samples. Gcnt1 glycosyltransferase enzyme mRNA was the only transcript showing differences in expression in lymph nodes between Nkx2-3 mutants and wild-type samples in which, in both pLNs and mLNs of Nkx2-3–mutant mice, mRNA for Gcnt1 glycosyltransferase was expressed at a robustly increased amount, similarly to the mutant spleen. Chst4 sulfotransferase mRNA showed a similar degree of increased expression in the mutant spleen as well as in the pLNs and mLNs from both mutant and wild-type mice. Taken together, these observations suggest that, in the absence of the transcription factor Nkx2-3, endothelial MadCAM-1 expression in the spleen is essentially replaced by GlyCAM-1, and the modifying enzymes required to generate functional PNAd are also upregulated. Thus, this pattern of endothelial marker gene expression in mutant spleens is reminiscent of HEVs in pLNs.

**Ectopic formation of HEV-like vessels in the Nkx2-3–/– mutant spleen**

The pLN-like signature of transcripts for HEV-related genes and the described alterations of the marginal zone and white pulp (19–22, 26) in the Nkx2-3–deficient spleen prompted us to investigate the vasculature in more detail by using MECA-79 anti-PNAd (35) and anti–MadCAM-1 mAbs. In contrast to the wild-type spleen, which never contains HEVs, in the spleens of young adult Nkx2-3–/– mice, PNAd-positive HEVs in close proximity of B cell-rich foci were prominently present. Dual immunofluorescence for HEC-GlcNAc6ST sulfotransferase (25) and the PNAd epitope showed coexpression on HEV-like vessels in the mutant spleen, similarly to pLNs from either wild-type or mutant mice (Fig. 2A). To test whether ectopic HEVs develop only in BALB/c background, we also tested Nkx2-3 mutants from both 129Sv and C57BL/6 strains. We found that spleen sections from these haplotypes also contained PNAd-positive HEVs (data not shown). These vessels were lined with plump endothelial cells that express CD31 endothelial Ag in which transmigrating lymphocytes could also readily be observed (Fig. 2A). It is interesting to note that elevated expression of PNAd on ectopic HEV-like vessels in the Nkx2-3–deficient mouse mutant appeared to be limited to the spleen, because HEVs in Peyer’s patches or in mLNs did not show any increased reactivity for this marker (data not shown).

To test whether the PNAd epitope is accessible for recirculating lymphocytes, MECA-79 mAb against PNAd was administered to Nkx2-3–/– mutants and wild-type mice i.v. We found a strong luminal binding in mutant spleens to a degree similar to pLNs from either mutant or wild-type mice, demonstrating that this L-selectin ligand is accessible for recirculating lymphocytes in the mutant spleen, similarly to pLNs, whereas wild-type spleens had no discernible reactivity (Fig. 2B). We also found that, unlike Nkx2-3 mouse mutants on other genetic backgrounds (129Sv × B6 [F1] or C57BL/6), ~75% of

**FIGURE 1.** qPCR analysis of mRNAs for PNAd core proteins (A) and modifying enzymes (B) in the Nkx2-3–/– and wild-type (WT) spleens, pLNs, and mLNs. Values in different organs are normalized to the ratio of target mRNA to β-actin in the WT spleen, represented by the line drawn at y = 1, and are expressed on a log scale. Bar diagram shows the mean ± SD of six parallel samples from three mice per organ; *p < 0.05, **p < 0.001.
FIGURE 2. Absence of the Nkx2-3 gene in the BALB/c mouse leads to HEV formation and pLN-like lymphocyte composition in the spleen. A, Frozen sections of the spleen and pLNs from mutant and wild-type mice were stained with fluorescence-labeled Abs as indicated. The purple color represents the merging of complement receptor (CR) expression by FDCs and IgM reactivity of adjacent B cells. Scale bar, 100 μm. H&E staining (upper right) shows plump endothelial cells (asterisks) and lymphocytes either closely associated with (arrowhead) or transmigrating between (arrow) these cells (scale bar, 25 μm). Endothelial cells in the mutant spleen coexpress CD31 and PNAd (lower right). Scale bar, 100 μm. B, The PNAd epitope is detected at the luminal surface of the endothelium in HEVs in the mutant spleen (upper left) and pLNs from mutant (upper right) or wild-type (lower right) mice following i.v. administration of MECA-79 anti-PNAd mAb without any detectable reactivity in the wild-type spleen (lower left). Dashed lines. Edges of tissue; inset, higher magnification of the marked rectangular area in left panel (60× objective). Ellipses in the pLN sections demarcate follicles. Scale bar, 100 μm. Lower panels, PNAd-positive HEVs that strongly coexpress HEC-GlcNAc6ST in the Nkx2-3−/− spleen (lower panel). Scale bar, 50 μm. The figures are representative from a cohort of three to five mice repeated three times.

Homing of lymphocytes in the Nkx2-3−/− spleen involves the ectopic HEV-like vessels producing CCL21 and depends on L-selectin/CD62L.

Next, we investigated whether the ectopic PNAd+ vessels formed in the Nkx2-3−/− mutant spleen function as lymphocyte exit ports. CFSE-labeled lymphocytes isolated from wild-type pLNs or mLNs were injected i.v., and their localization was determined at various times after injection using double immunofluorescence microscopy. Shortly after, injection-labeled lymphocytes were found to be closely associated with PNAd+ HEVs in the spleen of mutant animals, whereas in wild-type spleens, they were located at the marginal zone (Fig. 4A). The mutant spleens appeared to receive significantly fewer lymphocytes than the wild-type organ; however, their accumulation within the HEVs in the mutant spleen was almost indistinguishable from pLNs, and by 12 h, the lymphocytes showed the same degree of tissue dispersion as in peripheral lymph nodes (data not shown).

The initial binding of lymphocytes to marginal sinus-lining cells in the spleen occurs independently of L-selectin (2, 3), but in pLNs, binding to HEVs critically requires L-selectin. To determine whether the presence of PNAd+ HEVs in the spleen is coupled with an altered dependence on L-selectin during homing, we performed short-term competitive homing experiments in which wild-type lymphocytes were treated with the specific Ab MEL-14 to block L-selectin (36). This mAb prevents the homing of lymphocytes to pLNs in vivo but does not eliminate the cells (37). Equal numbers of CFSE-labeled lymphocytes treated with MEL-14 mAb and mock-treated biotinylated control cells (27) were then injected into Nkx2-3−/− mutant and wild-type recipients. Thirty minutes after the injection, spleen and pLN cells were isolated and subjected to flow cytometry. We observed that the

homologous BALB/c mice carrying the Nkx2-3 mutation exhibited spleens with nearly no red pulp or had spleens with macroscopically detectable red pulp only in a segment of the organ (Fig. 3A). The lack of MAdCAM-1+ marginal sinus-lining cells or aberrant distribution of topographic markers, including marginal zone-associated sessile macrophages or white pulp-associated fibroblastic cells (20, 22), excluded the precise determination of the distribution of various lymphocyte subpopulations. These “redless” spleens or the spleen segments devoid of red pulp are entirely filled with T and B cells without forming discrete territories (Fig. 3B). Thus, the formation of red pulp sinuses seems to be particularly sensitive to the Nkx2-3 mutation in BALB/c mice; however, HEV-like vessels develop in homologous Nkx2-3 mutants irrespective of the genetic background.

The lymphocyte composition of mutant spleens was tested by flow cytometry. Our analyses revealed a slightly increased proportion of lymphoid cells expressing L-selectin/CD62L. Homing receptors together with a relative increase of T cells in a mutant spleen in BALB/c background, thus approaching a T/B cell ratio that is more characteristic for pLNs than for the spleen (Fig. 3D). Taken together, these observations suggest that both the vasculature and lymphocyte composition in the Nkx2-3-deficient spleen resemble that of peripheral lymph nodes without, however, the typical tissue compartmentalization of pLNs. To test whether the absence of organized follicles is caused by the lack of CXCL13 homeostatic chemokine production by follicular dendritic cells (FDCs), we stained sections with anti-CXCL13 and anti-CD21/35 Abs. We found that, although the FDC network in mutant spleens was considerably more compact compared with wild-type controls (Fig. 2A), FDCs showed detectable CXCL13 production, similarly to the wild-type samples (Fig. 3C).
homing of mock-treated biotinylated lymphocytes in the spleen of Nkx2-3−/− mutants was reduced compared with that in wild-type recipients, but more importantly, the homing of MEL-14 mAb-treated CFSE-labeled cells to the mutant spleen was significantly blocked compared with wild-type recipients (Fig. 4B).

The impaired homing activity of lymphocytes with blocked L-selectin function was indicated by a 3-fold decrease in the CFSE:biotin ratio in the Nkx2.3-deficient spleen but not in the wild-type spleen. The ratio in the mutant spleen was very similar to that in pLNs of wild-type or mutant mice, suggesting that the ectopic PNAd+ HEV-like vessels in the mutant spleen use L-selectin for lymphocyte homing. As expected, blocking L-selectin significantly reduces the ratio of CFSE:biotin-labeled cells in both wild-type and mutant pLNs, confirming that lymph node use L-selectin-dependent homing mechanism, regardless of the Nkx2-3 genotype. Interestingly, the biodistribution of variously labeled lymphocytes without MEL-14 blockade also revealed a striking parallel between the mutant spleen and pLNs, because the biotinylation of lymphocytes favored their splenic homing in wild-type mice, whereas in pLNs, the frequency of CFSE cells was higher, resulting in a CFSE:biotin ratio < 1 in the wild-type spleen, and CFSE:biotin ratio > 1 in pLNs as well as in the mutant spleens, respectively.

Subsequent to the binding of L-selectin by PNAd, extravasation of lymphocytes in pLNs involves the arrest chemokine CCL21 that is produced by HEV endothelium (38). To test whether the HEV-like vessels in the mutant spleen also produce CCL21 chemokine, spleen sections were subjected to double immunofluorescence analysis by using anti-CCL21 and MECA-79 Abs. Similarly to pLNs, the majority of PNAd hi vessels in the mutant spleen coexpressed CCL21, which was additionally present on fibroblastic reticular cells. In a normal spleen with no PNAd reactivity, the CCL21 expression was restricted to the central arteriole and fibroblastic reticular cells in the T cell zone (Fig. 4C). Thus, the CCL21 chemokine is readily available in ectopic PNAd+ vessels in mutant spleens, and it is likely to play a similar role in homing as for the HEVs in pLNs.

**Impaired capture of blood-borne pathogens in the spleens of Nkx2-3−/− mice**

In addition to providing a microenvironment for the initial lodging of mobile lymphocytes during their splenic recirculation, the marginal zone also plays an important role in capturing blood-borne corpuscular Ags, including both encapsulated Gram-positive bacteria and Gram-negative *E. coli* (1). The abnormal vascular arrangement and the reported loss of marginal zone macrophages and a subset of B cells in Nkx2-3−/− mutant spleens (18–22, 26) prompted us to examine the clearance of bacteria by i.v. injection of fluoresceinated *E. coli* (39). Because of the lack of marginal zone macrophages and MApCAM-1+ positive sinu-lining cells as reference markers in the spleens of mice with...
disrupted Nkx2-3, IBL-11 mAb against white pulp fibroblastic reticular cells was used, and IBL-11 mAb detects circumferential reticulum in wild-type mice and perivascular stromal cells in mutants (22, 26). Although efficient retention of bacteria in the marginal zone of wild-type animals was observed, capture of bacteria in the mutant spleens was markedly reduced (Fig. 5). These data indicate that the replacement of splenic marginal sinus and other splenic vessels with ectopic HEVs and the attenuated recirculation of lymphocytes in the Nkx2-3–deficient spleen are accompanied by a considerably reduced capacity to collect blood-borne pathogens, presumably because of the severe reduction of marginal zone macrophages in this mutation (20), which cells are closely associated with the marginal sinus in wild-type mice (1).

Ectopic HEVs in the spleen of the Nkx2-3<sup>−/−</sup> mouse develop postnatally and require LTβR activity

It has been previously reported that in the secondary lymphoid organs of adult Nkx2-3<sup>−/−</sup> mouse MAdCAM-1 is absent, whereas it is still detectable in mLNs of newborn mutants (19, 20). Under physiological conditions, MAdCAM-1 is similarly downregulated during postnatal maturation of HEVs in pLNs (40). In contrast, PNA<sup>D</sup> HEVs can also be induced to occur at an ectopic location upon chronic infection coupled with accumulation of lymphoid cells (41). To investigate whether the appearance of ectopic HEVs in a mutant spleen follows the same postnatal developmental kinetics characterized by upregulated PNA<sup>D</sup> expression in place of MAdCAM-1 as in pLNs, we compared MAdCAM-1 and PNA<sup>D</sup> expression in mutant mice after birth using immunofluorescence analysis. At P0.5 (<1-d-old neonates), spleens of both Nkx2-3–deficient and wild-type mice contained cell clusters expressing MAdCAM-1 but no PNA<sup>D</sup> (Fig. 6A). Significantly, the spleen of the Nkx2-3<sup>−/−</sup> mutant at postnatal day 10.5 (P10.5) exhibited less MAdCAM-1, which was limited to a few dispersed vessels, simultaneously expressing PNA<sup>D</sup> (Fig. 6B). This was in marked contrast to the wild-type spleen at P10.5 in which a considerable
MAdCAM-1 reactivity was observed in the developing marginal sinus, but absolutely no PNAd-positive cells could be detected at any period. After the second postnatal week, no MAdCAM-1 expression was observed in the mutant spleen, similarly to young adults (19, 20) (data not shown). Comparison of MAdCAM-1 and PNAd expression in the mutant spleen and in normal age-matched pLNs revealed that the kinetics of early postnatal shift of addressin expression were similar in both lymphoid organs (Fig. 6B).

The appearance of pLN-like HEVs in Nkx2-3–mutant spleens in a fashion similar to the development of HEVs in pLNs prompted us to investigate whether this shift of programming is restricted to the vasculature, or other elements in the mesenchymal anlage in mutant spleens also switch to pLN-like features, characterized by their dependence on signaling mediated by LTβR (41, 42). To test whether LTβR signaling influences the development of a spleen in Nkx2-3–mutant mice, we generated double-mutant mice lacking both LTβR and Nkx2-3. These double-KO mice at 4 wk of age entirely failed to display PNAd-positive HEVs in their spleens in addition to lacking IBL-9/2 red pulp vessels (a trait shared with Nkx2-3–deficient mice) and MAdCAM-1 marginal sinus (a feature observed in both parent strains) (Fig. 7A). This result suggests that the formation of ectopic HEVs in a mutant spleen is dependent on LTβR function similarly to pLNs; however, as the spleen as a separate organ in these double mutants was present, we conclude that the nonvascular mesenchymal elements of splenic anlage can still develop, in contrast to the pLN formation blocked in the absence of LTβR.

We and others have shown that administration of the LTβR–Ig fusion protein to neonates effectively blocks postnatal vascular development in the murine spleen (26, 43). To test whether the pLN-like programming of splenic vasculature in Nkx2-3 mutants can be modulated postnatally, we applied this decoy receptor to

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** Impaired capture of blood-borne pathogens in the Nkx2-3–mutant spleen. A small number of i.v. injected fluoresceinated E. coli (green) are retained in the vessels (arrows) in the mutant spleen (left), whereas in control mice, a substantial accumulation can be observed in the marginal zone (arrowhead) outlined by IBL-11 mAb (red; scale bar, 500 μm; the results are representative for three independent experiments with at least three mice each).

![FIGURE 6](http://www.jimmunol.org/)

**FIGURE 6.** pLN-like postnatal switch of addressin expression in splenic HEVs in Nkx2-3–deficient mice. A, Frozen newborn (P0.5) mutant and wild-type spleen sections were immunostained as indicated. Nuclei were stained with DAPI (blue). B, By postnatal day 10.5 (P10.5), the expression of MAdCAM-1 in the mutant spleen is limited to a few vessels that coexpress PNAd (top row, the quadrangle indicates the same area). In the wild-type spleen (middle row), MAdCAM-1 outlines the boundary between white and red pulp; PNAd is not expressed. In wild-type pLNs (bottom row), most of the HEVs coexpress MAdCAM-1 and PNAd (left and middle panels). The pictures are representative of three independent experiments performed on three to five mice. Scale bars, 50 μm.

![FIGURE 7](http://www.jimmunol.org/)

**FIGURE 7.** The formation of ectopic HEVs requires the activity of LTβR. A, Frozen spleen sections from various genotypes from 4-wk-old mice were labeled with the Abs indicated at the top (representative result from staining performed on three separate occasions on three to five mice). Scale bar, 250 μm; n = 5. B, Postnatal treatment of Nkx2-3–deficient mice with the LTβR-Ig decoy receptor disrupts ectopic HEV formation and abolishes MAdCAM-1 expression (top row). HEVs in mice treated with control human IgG (bottom row) express both MAdCAM-1 and PNAd (arrowheads) in addition to stroma-associated MAdCAM-1 (asterisk; scale bars, 200 μm; n = 4; nuclei counterstained with DAPI).
Nkx2-3–deficient mice on P0.5 and P3.5 and harvested spleens on P10.5. We found that postnatal interference with LTβR activity also resulted in a significant reduction of PNAd expression indicative for less ectopic HEVs in the spleen (Fig. 7B). Thus, the transformation of splenic vasculature to HEV-like vessels in an Nkx2.3-deficient mouse seems to follow a similar program that underlies vessel formation in pLNs.

Discussion

A common theme in the postnatal organization of structured secondary lymphoid organs is their compartmentalization into discrete regions of T or B cell dominance as a consequence of lymphocyte extravasation from specialized vascular segments. In this study, we provide structural, molecular, and functional evidence that the transcription factor Nkx2-3 plays a key role in determining the vascular identity of the spleen. The spleen in Nkx2-3–deficient mouse acquires pLN-like vessels, which structures mediate L-selectin–dependent homing. These observations suggest an unexpected plasticity among the developmental programs that control the vasculature of peripheral lymphoid organs. The importance of the homeodomain transcription factor Nkx2-3 in the correct splenic vessel development is most strikingly illustrated by the redless spleen lacking nearly all of the red pulp.

In contrast to vessel formation in pLNs, little is known about vascular patterning in the spleen (1, 2, 4). A characteristic vascular segment formed during the early postnatal period in the rodent spleen is the marginal sinus composed of MadCAM-1–positive cells (2, 44, 45). Allocation of MadCAM-1–1 cells to the marginal sinus in the mouse spleen (43) is controlled by several signals and regulators, including Nkx2-3, which can probably activate the transcription of MadCAM-1 directly (19). Inactivation of Nkx2-3 function affects the expression of MadCAM-1 and also the formation of the spleen and Peyer’s patches. In contrast, loss of LTα or LTβR blocks embryonic development of virtually all peripheral lymphoid organs, including the white pulp stroma of the spleen; however, segregation of vasculature into red pulp and white pulp territories takes place independently from LTβR ligands (14, 15, 17). The Nkx2.3-null mutation causes a particularly severe defect in splenic vasculature affecting all vascular segments in the red pulp, marginal zone, and white pulp as well (26). It results in nearly complete loss of red pulp vascular beds and disturbed white pulp architecture, thus offering the opportunity to analyze the putative role of red pulp on morphogenesis of the white pulp.

The Nkx2.3–/– mutant spleen contains vessels that fulfill the morphological and functional criteria of HEVs that are normally present only in pLNs. According to our qPCR data, these unusual splenic HEVs use predominantly GlyCAM-1 besides some other PNAd core proteins but no or very little MadCAM-1 as homing receptor (28–33). Replacement of MadCAM-1 by PNAd in the spleen of the Nkx2.3–/– mouse occurs postnatally, suggesting that early MadCAM-1 expression may either not be regulated by Nkx2-3 or, alternatively, may be regulated by coexpressed proteins such as Nkx2-5, providing redundant functions. Besides Nkx2-3, expression of MadCAM-1 is influenced by other regulators, such as Relβ/NF-κB (46), because NF-κB loss of function mutations in mice also lead to loss of marginal sinus-associated MadCAM-1 expression (46, 47). In this context, NF-κB appears to act downstream of LTβR-mediated signals (41, 48). Preliminary gene expression analysis in Nkx2.3–/– mice failed to provide evidence for loss of LT-related ligands or receptors in agreement with the presence of splenic FDCs (20, 49), which depend on LTβR activity (23). Similarly to previous findings, we also confirm that although in Nkx2-3–mutant mice FDCs are present and produce CXCL13 chemokine (20), yet no follicles are formed, indicating that CXCL13 production needs to be complemented with Nkx2-3–dependent processes to promote follicle development in the spleen.

According to this observation, Nkx2-3 does not seem to act upstream of lymphotoxin signaling, and it may indeed function in a parallel regulatory pathway. However, both regulators are dispensable for embryonic development of the mesenchymal spleen anlage, because in a double-mutant mouse the spleen is present.

It has been suggested that MadCAM-1 glycoprotein in wild-type mice may be instrumental for proper clustering of endothelial cells (43). Because HEV-associated MadCAM-1 is still present in the Nkx2.3–/– mutant spleen during the first two postnatal weeks when the white pulp and marginal zone architecture is established (25, 44, 45, 50), it is reasonable to assume that loss of additional, yet unidentified, proteins contributes to the adult spleen phenotype in this mutation. This view is in line with the observation that MadCAM-1–deficient mice exhibit apparently no major alterations of the splenic architecture (51). Importantly, the regulation of MadCAM-1 expression by Nkx2-3 appears to be different for endothelial cells and other cells, such as FDCs, because FDC-associated MadCAM-1 expression is preserved in mutant Peyer’s patches (20), whereas the HEV-associated MadCAM-1 production is abolished, similarly to HEVs in mLNs. Thus, the differential effects of the absence of Nkx2-3 on the expression of MadCAM-1 mRNA in pLNs of wild-type and Nkx2-3–mutant mice (where mutants had a higher level) compared with mLNs (where wild-type mice showed a higher level of expression) is probably caused by the enlarged germinal centers containing MadCAM-1–positive FDCs in pLNs of Nkx2-3 mutants and the presence of highly MadCAM-1–positive HEVs in the wild-type mLNs, respectively.

The transformation of splenic vasculature into pLN-like vessels as a result of the Nkx2-3 gene disruption emphasizes the importance of correct vessel formation for spleen ontogeny in mammals. The red pulp is formed during embryonic development when Nkx2-3 is expressed (15, 20), whereas expansion and compartmentalization of white pulp occur postnatally when Nkx2-3 expression is already downregulated. This scenario suggests that in the embryonic spleen Nkx2-3 may be directly responsible for the development of the red pulp sinus network that ascpreads blood supply essential for tissue growth. Nkx2-3 also seems to be required to set up early progenitor cells for the correct vasculature in white pulp. Whether or not the function of Nkx2-3 in this process is to actively promote the appropriate cell fate for white pulp endothelium or to suppress HEV-fated endothelial progenitors is currently unclear. Alternatively, Nkx2-3 may play a role that affects postnatal development of the splenic vasculature in a non–cell-autonomous fashion, for instance, by changing stromal signals. However, the transformation of splenic vasculature to contain HEV-like vessels in the mutant spleen is not simply the consequence of defective red pulp development, as it is observed in BALB/c mutants that retain some red pulp and in Nkx2-3 mutants on other genetic backgrounds where red pulp is also present. In addition to the altered vascular organization, other cellular compartments including marginal zone macrophage subsets are also affected, leading to major functional deficits in lymphocyte recirculation and pathogenic uptake, and may explain defective immune responses in Nkx2-3–mutant mice (49). Taken together, our observations clearly demonstrate that Nkx2-3 is an important factor for the distinct vasculature in spleens well beyond the regulation of MadCAM-1 by possibly determining the embryonic vascular patterning and recirculation activity in this secondary lymphoid organ.

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Disclosures

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