Article is available online at http://www.webio.hu/por/2007/13/3/0227

ARTICLE

Complex Organizational Defects of Fibroblast Architecture in the Mouse Spleen with Nkx2.3 Homeodomain Deficiency

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The capacity of secondary lymphoid organs to provide suitable tissue environment for mounting immune responses is dependent on their compartmentalized stromal constituents, including distinct fibroblasts. In addition to various members of the tumor necrosis factor/lymphotoxin β family as important morphogenic regulators of peripheral lymphoid tissue development, the formation of stromal elements of spleen is also influenced by the Nkx2.3 homeodomain transcription factor in a tissue-specific fashion. Here we extend our previous work on the role of Nkx2.3-mediated regulation in the development of spleen architecture by analyzing the structure of reticular fibroblastic meshwork of spleen in inbred Nkx2.3-deficient mice. Using immunohistochemistry and dual-label immunofluorescence we found both distributional abnormalities, manifested as poor reticular compartmentalization of T-zone and circumferential reticulum, and developmental blockade, resulting in the absence of a complementary fibroblast subpopulation of white pulp. The disregulated distribution of fibroblasts was accompanied with an increased binding of immunohistochemically detectable complement factor C4 by T-cell zone-associated reticular fibroblasts, distinct from follicular dendritic cells with inherently high-level expression of bound C4. These data indicate that the impact of Nkx2.3 gene deficiency on fibroblast ontogeny within the spleen extends beyond its distributional effects, and that the formation of various white pulp fibroblast subsets is differentially affected by the presence of Nkx2.3 activity, possibly also influencing their role in various immune functions linked with complement activation and deposition. (Pathology Oncology Research Vol 13, No 3, 227–235)

Key words: spleen, fibroblast heterogeneity, Nkx2.3

Introduction

The formation of peripheral lymphoid organs is a crucial event contributing to the establishment of adaptive immune responsiveness. In this process, several key regulators have been identified that promote diverse forms of cellular interactions needed for the development of lymph nodes, gut-associated lymphoid tissues and the spleen.[1] These include several members of tumor necrosis factor/lymphotoxin β (TNF/LT) family and their NF-κB-mediated signaling pathways, adhesion molecules, soluble cytokines, chemokines and their receptors.[2,3] The endogenous regulation of cellular maturation and expansion involves the activity of distinct transcription factors, promoting either the differentiation of lymphoid tissue inducer cells from hemopoietic precursors, or the inductive transformation of mesenchymal/stromal cells. Several components involved in the process of embryonic lymphoid organogenesis can also be observed in different pathological conditions, including the formation of tertiary lymphoid tissues in chronic inflammation, or the ectopic differentiation of stromal elements during the progression of lymphoid malignancies.[4]

Received: March 27, 2007; *accepted:* July 20, 2007

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JB and TC contributed equally to this publication. PB is a recipient of Öveges József Research Fund from the National Office for Research and Technology of Hungary.

List of abbreviations: BSA: bovine serum albumin; CFR: circumferential reticulum; FDC: follicular dendritic cell; mAb: monoclonal antibody; MAdCAM-1: mucosal addressin cell adhesion molecule-1; PALS: periarteriolar lymphoid sheath; PBS: phosphate-buffered saline; PE: phycoerythrin; TNF/LT: tumor necrosis factor/lymphotoxin

The spleen has a number of unique developmental, structural and functional features that distinguish it from other structured peripheral lymphoid organs. Its mesenchymal anlage develops from the dorsal mesogastrium, under the influence of several transcription factors including Pbx1, Hox11 and Nkx3.2, whose absence causes complete asplenia.[5] However, the null-mutation of either of these factors does not affect the formation of lymph nodes. In contrast, the absence of receptor for lymphotoxin β (LTβR) results in a near-complete lack of lymph nodes, without affecting the initial formation of spleen until the proper compartmentalization of its lymphoid and stromal constituents into PALS, follicles and marginal zone.[1,2] Furthermore, the development of red pulp appears intact in the absence of LTβ signaling.[6,7] Importantly, the developmental potential to generate mature lymphocytes and accessory cells with LTβR deficiency from hemopoietic precursors in bone marrow is preserved, therefore the compromised immune responsiveness is due to the defective formation of peripheral lymphoid tissues, including the spleen.[1,2]

In addition to the role of the LTβ/NF-κB axis in directing the segregation of lymphoid compartments of the spleen, the homeodomain (HD) transcription factor Nkx2.3 is also involved in the control of spleen formation.[8,9] Mice with ablated Nkx2.3 HD lack Peyer's patches and have a substantially reduced size spleen, with histological abnormalities closely resembling those found in various forms of LT deficiency, such as lack of polarized follicles and T/B segregation, absence of organized marginal zone, including the absence of MZ macrophages, B cells and MAdCAM-1 expression by sinus-lining cells. However, in contrast to deficiencies related to the LT pathway, the lymph nodes in Nkx2.3-deficient animals are indistinguishable from normal controls and in the absence of the Nkx2.3 HD, follicular dendritic cells (FDC) can still develop, even though their distribution relative to B cells in spleen is abnormal.[10] Furthermore, our recent observations indicate a range of severe vascular malformations affecting the red pulp sinus network in Nkx2.3-deficient mice, which is quite distinct from the intact red pulp architecture in LT-deficient animals.[11]

In this work we sought to determine the role of Nkx2.3 in the organization of reticular fibroblasts as basic scaffolding elements of the white pulp using immunohistochemical analysis. Our data indicate that distinct fibroblast subsets are variably affected by the absence of Nkx2.3 activity, resulting in both distributional and developmental defects. This irregular organization of reticular fibroblasts in spleen with Nkx2.3 deficiency is coupled with a moderately aberrant formation of FDCs, which may be related to a shift towards an increased deposition of complement components onto fibroblasts with preferential accumulation in the T-cell zone.

Materials and Methods

Mice. Wild-type BALB/c mice were obtained from Charles-River Hungary and housed under SPF conditions. Nkx2.3$^{-/-}$ mice from a mixed B6x129Sv mixed background (developed at the Technical University of Braunschweig, Department of Cell and Molecular Biology, Institute for Biochemistry and Biotechnology)[8] were backcrossed onto BALB/c background through 10 generations. The mice were genotyped by the PCR amplification of genomic DNA isolated from tail biopsy, using primers designed for the simultaneous detection of the Nkx2.3 mutant allele, and neomycin resistance cassette by using primers corresponding to the second exon of the Nkx2.3 gene (Nkx2.3-37, 5'-GCGGGAGACTGTAAGACGAG-3'; Nkx2.3-256, 5'-TTATCCTGCCGCTGTCTCTT-3; amplicon size: 238 bp) and to the neomycin resistance cassette (sense: 5'-GTCGATCAGGATGATCTGGAC-3'; antisense: 5'-AAGGCGATAGAAGGCGATGC-3'; amplicon size: 321 bp) with the following cycling profile: 95°C 3 min, 35 cycles of 94°C 45 s, 55°C 30 s, 72°C 1 min and a final extension at 72°C 10 min.[11] The mice were maintained under conventional conditions in the departmental animal breeding unit on pelleted rodent chow and water ad libitum, under the license issued by the Committee on Animal Experimentation of the University of Pécs. Mice between the age of 6-8 weeks of either sexes of Nkx2.3$^{-/-}$ or Nkx2.3$^{+/-}$ genotypes were sacrificed by cervical dislocation, their spleens were removed and placed in Killik cryoembedding medium, and kept frozen until processing.

Hybridoma cells and immune reagents. IBL-10 and IBL-11 hybridoma cells producing rat mAbs against mouse splenic fibroblasts were generated as described[12] and maintained in DMEM medium containing 5-10% FCS. PE-labeled goat anti-rat IgG was purchased from BD Pharmingen (San Jose, CA). FDC-M2 rat mAb against mouse C4[13] was kindly provided by Dr. Marie Kosco-Vilbois (NovImmune SA, Geneva, Switzerland) and Dr. Andras K Szakal (Virginia Commonwealth University, Richmond, VA). ER-TR7 anti-fibroblast mAb[14] was kindly supplied by Dr. Willem van Ewijk (Leiden University Medical Center, The Netherlands). For the flow cytometric enumeration of splenic B-cell subsets, PE-conjugated rat mAb against mouse CD21/35 (clone 7G6) and FITC-tagged anti-mouse CD23 (B3B4) were purchased from BD Pharmingen. The IgM profile of B-cell subpopulations was determined using biotinylated anti-IgM (clone B7.6) in conjunction with Streptavidin-CyChrome (BD Pharmingen). For immunohistochemistry the unlabeled rat mAbs against fibroblasts were detected by biotinylated mouse anti-rat kappa chain mAb (clone MRK-1) from BD Pharmingen, and were developed using ExtrAvidin-alkaline phosphatase conjugate (Sigma-Aldrich Kft, Budapest). For dual Immunofluorescence, IBL-10 and IBL-11 mAbs were

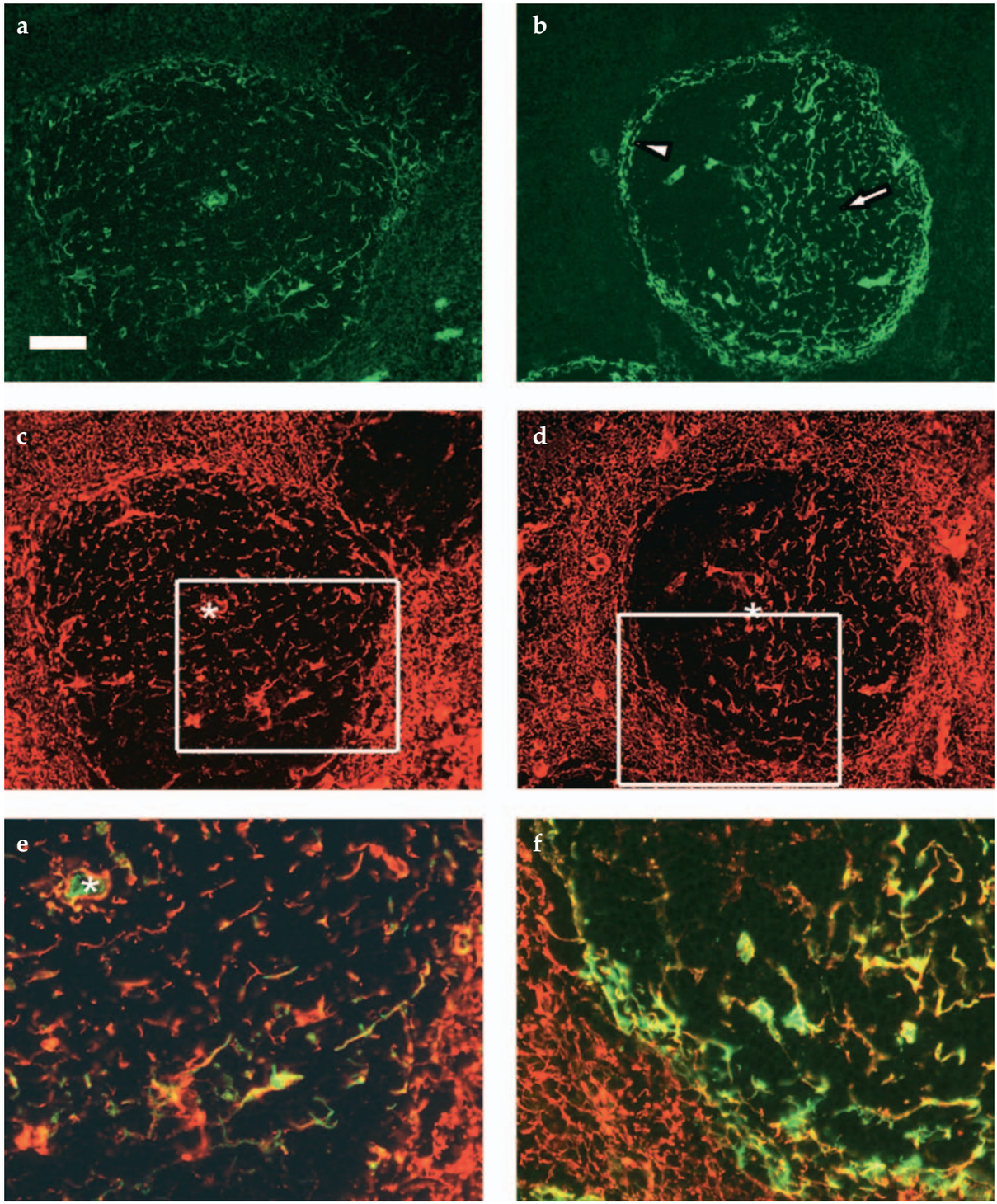


Figure 1. *IBL-10 and IBL-11 markers are expressed by two distinct fibroblast subsets in spleen. Dual stained spleen sections from normal BALB/c mice for immunofluorescence with IBL-10/green fluorescence* ***(a)*** *and ER-TR7/red fluorescence* ***(c)*** *or IBL-11/green fluorescence* ***(b)*** *and ER-TR7/red fluorescence* ***(c** and **d)*** *mAbs were observed through FITC or PE-filters, and digitally merged* ***(e, f),*** *corresponding to the smaller rectangular area in insets* ***c/d.*** *Dual stained cells appear yellow. In insets* ***c/d/e*** *the asterisks indicate the position of central arteriole, in inset* ***b*** *the arrowhead points to the circumferential reticulum, arrow identifies PALS. Bar size corresponds to 100 μm. Results are representative of experiments repeated three times on a cohort of at least 4 mice each occasion.*

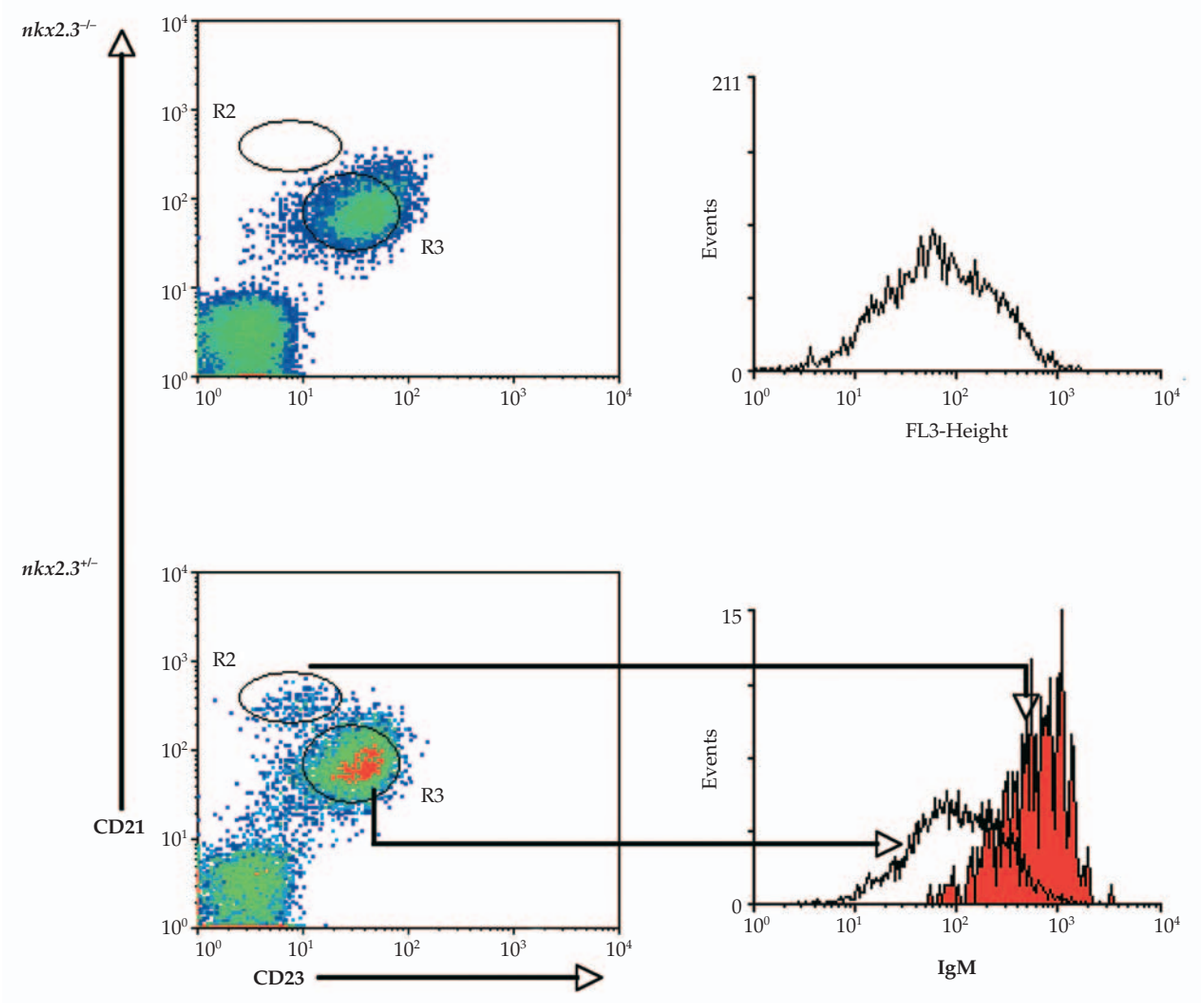
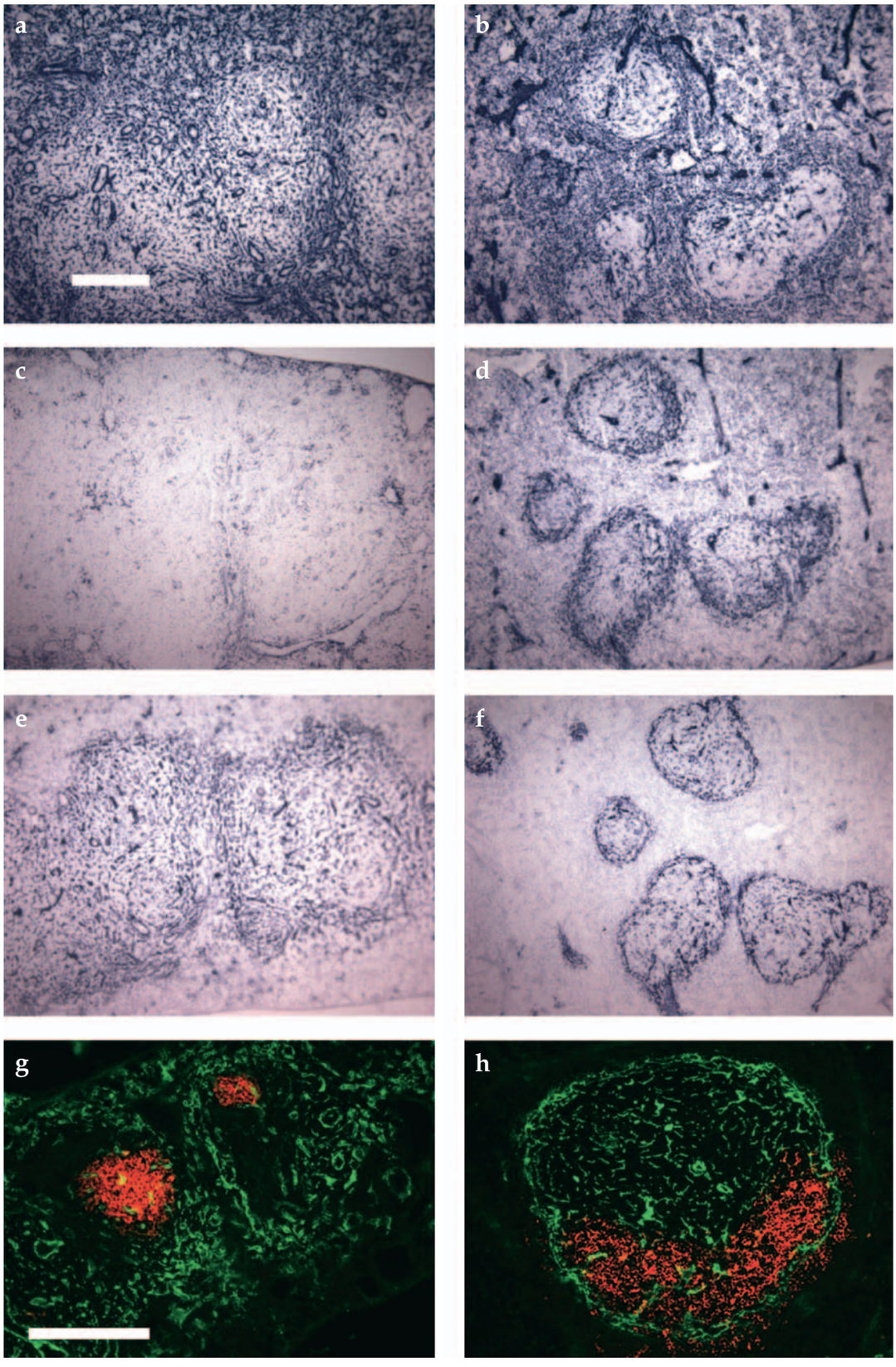


Figure 2. *Flow-cytometric analysis of the B-cell composition in BALB/c Nkx2.3$^{-/-}$ mice. Spleen cells from mice with different genotypes (indicated on the left) were labeled with direct conjugates against CD23/FITC, CD21/PE and IgM/biotinylated, revealed with avidin-CyChrome. The fluorescent density plots depict lymphocyte-gated distribution. The R2 oval area corresponds to the marginal zone (CD21^{hi}/CD23^{lo}) population, R3 to the follicular B cells with the opposite phenotype, also confirmed by the differential expression of IgM by these two B-cell subsets (histogram overlays linked to follicular/marginal zone B cells, respectively). Data are representative of experiments repeated three times on a cohort of at least 4 mice each occasion.*

purified from tissue culture supernatant by FPLC using Protein G affinity chromatography, and labeled by water-soluble sulfo-NHS biotin ester (Pierce Chemicals, Rockford, IL). The biotinylated reagents were detected using avidin-FITC conjugate (Dako, Glostrup, Denmark).

Flow cytometry. After removal of the spleen, lymphocytes were released by mincing the spleen between frosted ends of two slides. The tissue debris was removed by filtration with cell strainer, followed by pelleting the cells in chilled PBS containing 0.1% BSA and 0.05% Na-azide. 2x10^5 cells/sample were incubated in a cocktail of PE-conjugated anti-mouse CD21/35, FITC-labeled anti-mouse CD23 and biotinylated anti-IgM (at 1 μg/ml concentration

Figure 3. *Aberrant organization of reticular fibroblasts in BALB/c Nkx2.3$^{-/-}$ mice and spatial relationship between FDCs and IBL-11$^+$ fibroblasts. Spleen sections from Nkx2.3 KO mice* ***(a/c/e)*** *or heterozygous controls* ***(b/d/f)*** *were labeled with ER-TR7* ***(a/b),*** *IBL-10* ***(c/d)*** *or IBL-11* ***(e/f)*** *mAbs, detected with alkaline phosphatase immunostaining. The relationship between FDCs and IBL-11$^+$ fibroblasts in Nkx2.3 KO* ***(g)*** *and heterozygous controls (H) was determined by dual immunofluorescence with CD21/35-PE conjugate and biotinylated IBL-11 mAb revealed with FITC-avidin. Bar size corresponds to 200 μm. The red fluorescence is digitally reduced to exclude the B-cell associated expression of CD21/35, to accentuate FDCs. Data are representative of experiments repeated three times on a cohort of at least 4 mice each occasion.*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each). After 20-min incubation on ice, the cells were repeatedly washed and further incubated with streptavidine-CyChrome conjugate (100x diluted). After washing, cells were fixed with 1% paraformaldehyde in PBS. The samples were analyzed on a BD FACSCalibur flow cytometer using the CellQuest Pro software. Data acquisition was set according to the size/granularity of cells, and 50,000 electronic events were collected.

Immunohistochemistry and immunofluorescence. Frozen tissue sections at 6-8 μm thickness were cut using a Leica cryostat, and allowed to dry for several hours, followed by fixation in chilled acetone. After drying the sections were processed as described previously.[12] Briefly, after blocking with PBS-5% BSA the sections were incubated with primary mAbs against various fibroblast markers as neat tissue culture supernatants, followed by secondary reagents (either biotinylated MRK-1 mAb against rat kappa chain or PE-conjugated goat anti-rat IgG at 1:100 dilution in PBS). For immunohistochemistry the sections were developed by subsequent incubation with ExtrAvidin-AP conjugate (100x diluted) employing NBT-BCIP as chromogenic substrate in the presence of levamisole. For dual immunofluorescence the remaining free binding sites of anti-rat IgG were saturated using 20x diluted normal rat serum, followed by the addition of biotinylated reference mAbs IBL-10 or IBL-11 (at 1μg/ml concentration). After thorough washing the specimens were reacted with Avidin-FITC conjugate (at 1:50 dilution). The sections were mounted and viewed under an Olympus BX61 epifluorescent microscope, and the digital images were captured using the AnalySIS software and a CCD camera.

Results

IBL-10 and IBL-11 mAbs identify distinct fibroblast subsets within the splenic white pulp

Our previous reporting on the tissue reactivity of IBL-10 and IBL-11 mAbs revealed cells with branching fibroblastic morphology positioned at different locations within the white pulp relative to both each other and the lymphoid cells forming distinct lymphoid compartments (PALS, follicles and marginal zone, respectively).[12] In this work, we found a substantial co-localization with both IBL-10 and IBL-11 mAbs in conjunction with ER-TR7 mAb against a fibroblast-derived intracellular and/or secreted antigen as reference,[14,15] corresponding to the respective tissue distribution of these two subsets within the white pulp. Thus a strong dual staining could be observed for IBL-10 and ER-TR7 labeling within the follicles. Due to the paucity of IBL-10 reactivity, the overlap was weaker within the PALS, and just a negligible co-staining was seen at the periphery of follicles. In contrast, the bulk of ER-TR7-positive fibroblasts co-expressing the IBL-11 marker was found primarily at the PALS, and at a narrow rim corresponding to the circumferential reticulum (*Fig. 1*). Taken together, these data confirm our previous interpretation of IBL-10 and IBL-11 being two topically restricted markers for white pulp fibroblast subsets with distinct topographic pattern.

Aberrant reticular organization of spleen in Nkx2.3 deficiency

NKX2.3 as a vertebrate homologue of *tinman*, a homeobox gene belonging to a family of homeodomain-containing transcription factors, has been demonstrated to influence the formation of several tissue components in the developing spleen.[8,9,11]

Our preliminary immunohistochemical analyses revealing the absence of marginal zone macrophages (marginal zone macrophages, and metallophilic macrophages, respectively, not shown) and flow cytometric data showing the lack of marginal zone B cells in spleen of BALB/c Nkx2.3$^{-/-}$ mice (*Fig. 2*) indicated no noticeable difference from the published phenotype of original mutants with mixed B6x129Sv background. To further analyze the extent of impaired reticular organization caused by Nkx2.3 deficiency, we compared the labeling pattern of pan-fibroblast mAb ER-TR7 and subset-reactive IBL-10 and IBL-11 mAbs between BALB/c-backcrossed Nkx2.3$^{-/-}$ and heterozygous controls. Parallel labeling of cryosections from BALB/c Nkx2.3 KO and heterozygous littermates with ER-TR7 mAb revealed a severely disturbed reticular meshwork in homozygous deficient samples as evidenced by the diffuse distribution of stained cells (*Fig. 3a,b*). In addition, the fibroblast subset reactive with IBL-10 mAb was barely detectable, with no indication of fibroblastic "hedge" formation within the white pulp between the T- and B-cell zones (*Fig. 3c,d*). Furthermore, the typical compartmentalized pattern of IBL-11$^+$ cells was no longer discernible, as the central compartment of spleen contained diffusely scattered IBL-11$^+$ cells. As a result, the circumferential reticulum compartment of IBL-11$^+$ elements was also absent (*Fig. 3e,f*).

In normal mice the IBL-11$^+$ reticular meshwork in the PALS and the FDC network identifiable with CD21/35 immunolabeling in the follicles are complementary stromal territories of the white pulp. Next we investigated the relationship between these tissue constituents between BALB/c KO and heterozygous mutants. We found that in Nkx2.3$^{+/-}$ mice the regions delineated by FDC staining and IBL-11$^+$ fibroblasts complement each other in a tightly fitting pattern, whereas in Nkx2.3$^{-/-}$ KO mice areas with paucity of both labelings can be observed between the compacted FDC reticula and the disorganized IBL-11$^+$ meshwork. These findings indicate a previously undetected complex pattern of abnormalities that affects the lymphoid stromal organization of spleen in Nkx2.3 deficiency.

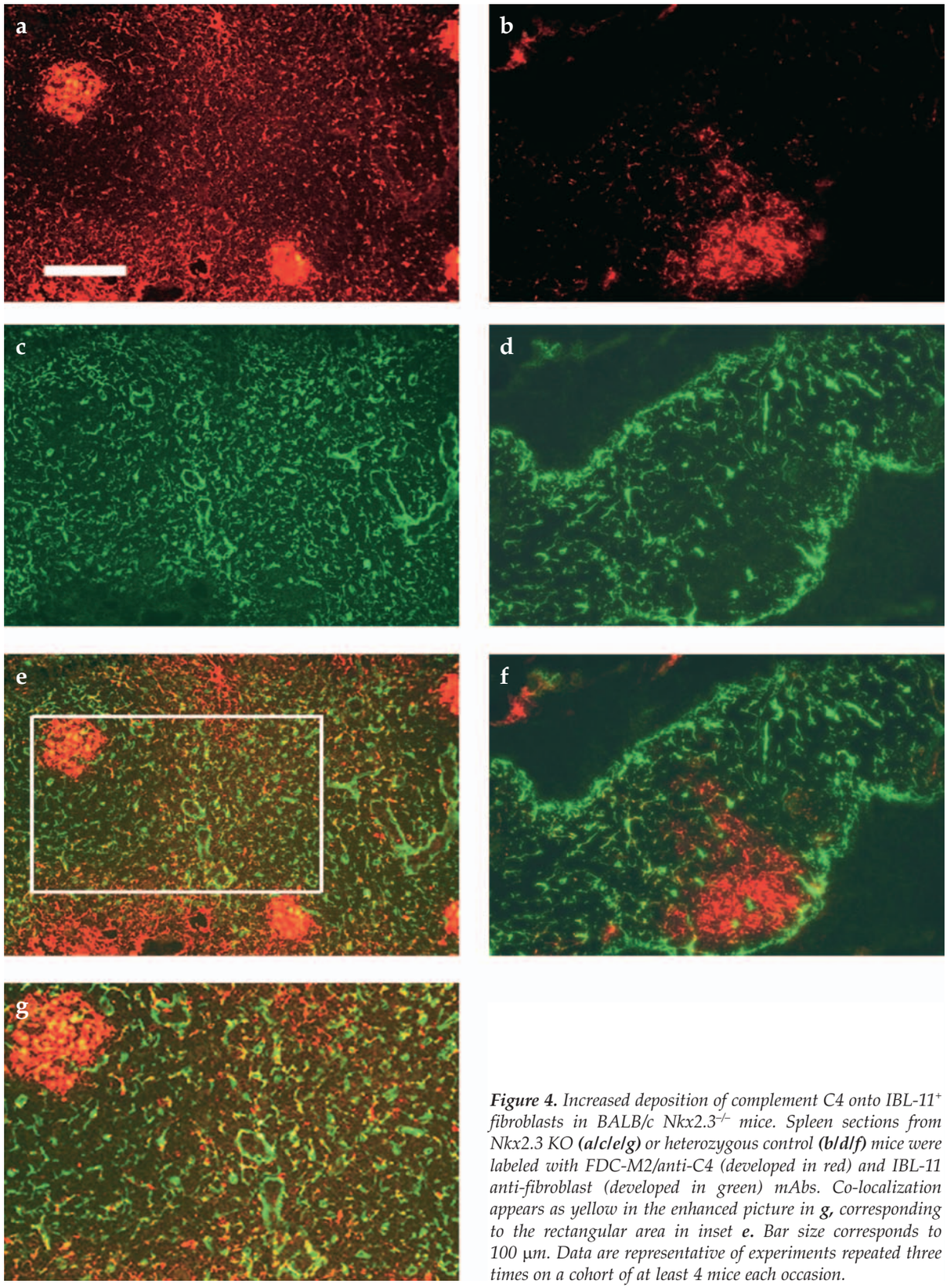

Figure 4. *Increased deposition of complement C4 onto IBL-11^{+} fibroblasts in BALB/c Nkx2.3$^{-/-}$ mice. Spleen sections from Nkx2.3 KO* ***(a/c/e/g)*** *or heterozygous control* ***(b/d/f)*** *mice were labeled with FDC-M2/anti-C4 (developed in red) and IBL-11 anti-fibroblast (developed in green) mAbs. Co-localization appears as yellow in the enhanced picture in* ***g,*** *corresponding to the rectangular area in inset* ***e.*** *Bar size corresponds to 100 μm. Data are representative of experiments repeated three times on a cohort of at least 4 mice each occasion.*

Table 1. **Characteristics of abnormal stromal organization in SCID and Nkx2.3$^{-/-}$ mutants**

	Central white pulp reticular architecture and tissue composition				*Marginal zone (MZ) reticular architecture and tissue composition*			
Mutation	*IBL-10 subset/distribution*	*IBL-11 Subset/distribution*	*Follicles*	*FDC formation*	*IBL-11 CFR*	*MAdCAM-1 expression*	*MZ macrophages*	*MZ B cells*
Nkx2.3$^{-/-}$	↓/–	+/–	–	+	–	–	–	–
SCID	↓/–	+/–	–	–	+	+	+	–
WT	+/+	+/+	+	+	+	+	+	+

Features present/absent are indicated as (+) or (–), respectively; diminished expression is indicated as ↓. FDCs were tested by anti-CD21/35 mAb, MZ macrophages were tested using anti-MARCO and anti-MOMA-1 mAbs, respectively

Increased deposition of complement C4 onto T-cell zone-associated fibroblasts in Nkx2.3 deficiency

During our studies on the topography of stromal compartments in Nkx2.3-deficient mice we used several markers to identify FDCs, including anti-CD21/35 as genuine FDC-derived antigen and FDC-M2 mAb directed against complement C4 as an acquired ligand deposited onto FDCs and, to a lesser extent, onto reticular cells.[13,16] The use of FDC-M2 revealed a striking difference in staining pattern between BALB/c Nkx2.3 homozygous and heterozygous mutants (*Fig. 4*). In Nkx2.3$^{-/-}$ mice a substantial increase of non-FDC-associated reactivity was observed, whereas in Nkx2.3$^{+/-}$ mice the deposition of complement C4 remained overwhelmingly confined to the FDC reticula, similar to wild-type controls. Although FDCs from Nkx2.3$^{-/-}$ mice maintained their ability to retain complement C4, the size of tissue attributable to FDC labeling also showed at least a 50% reduction compared to heterozygote control, confirming their shrunken appearance observed after CD21/35 immunolabeling. Furthermore, dual staining with FDC-M2 and IBL-11 mAbs revealed an extensive overlap between the fluorescent signals, indicating increased deposition of complement C4 onto fibroblasts that are normally excluded from the follicles.

Discussion

The reticular scaffolding composed of fibroblasts and extracellular matrix components constitutes an essential element for establishing a highly ordered compartmentalized structure by stromal and hemopoietic cells in peripheral lymphoid tissues. Despite their ubiquitous presence, these mesenchymal cells have proved quite heterogeneous in both phenotypic and functional characteristics.[17] To investigate the developmental regulation of these splenic fibroblasts, we analyzed the topography of fibroblast meshwork using subset-specific mAbs[12] in mice with the absence of Nkx2.3 transcription factor. As a result of this mutation, marginal sinus-lining cells of these mice fail to express MAdCAM-1 glycoprotein as the product of the only identified target gene of Nkx2.3 activity in peripheral lymphoid tissues.[8,9]

Previous studies in mice with Nkx2.3 null-mutation have hitherto indicated the lack of marginal zone macrophages and B cells in addition to the MAdCAM-1-expressing sinus-lining cells, without addressing the cellular composition of reticular meshwork. The formation of marginal sinus boundary between the marginal zone and central white pulp is related to the production of L1 adhesion glycoprotein by sinus-lining cells, although the exact mechanism remains to be clarified.[21] Nevertheless, the absence of L1 (despite the perturbed sinus architecture) from sinus-lining cells co-expressing MAdCAM-1 does not induce any noticeable alteration of marginal zone composition other than irregular distribution of laminin. Moreover, the intravenous administration of a function-blocking anti-MAdCAM-1 mAb has not been reported to result in any detectable cellular alteration, or influencing the splenic homing of recirculating lymphocytes.[22] Taken together, mechanisms other than the absence or inhibition of MAdCAM-1 glycoprotein produced by endothelial or other sinus-lining cells may also impair the topography of marginal sinus, without affecting the neighboring cells. Our data clearly indicate that Nkx2.3 has also an important role in guiding the proper fibroblast segregation both in this region to form the circumferential reticulum, and also towards other stromal elements, including follicular dendritic cells. Moreover, these structural aberrations without any apparent relationship to the tissue occurrence of MAdCAM-1 imply the involvement of other genes affected by the absence of Nkx2.3. Our previous studies on the ontogeny of splenic fibroblasts in immunodeficient SCID mice established that the differentiation of the same fibroblast subpopulation also requires the presence of T and B cells, whereas the complementary IBL-11^{+} subset and marginal zone macrophages develop independently of these cells.[12] The main structural differences between the two models are summarized in *Table 1*.[11,12]

In addition to the lack of a circumferential reticulum fibroblast element in the marginal zone of Nkx2.3 mice, our data indicate a considerable re-distribution of central white pulp reticular meshwork as well as a defect in forming the IBL-10-reactive subset. The near-complete absence of this subpopulation is associated with a diffuse arrangement of IBL-11$^+$ fibroblasts, which also showed an increased binding of complement C4, thus raising the possibility of an influence of fibroblast architecture on immune reactivities. These functions include the regulation of lymphocyte positioning and movement within the tissues, as well as their capacity to influence ongoing immune responses.[18,19] These reticular cells have also been implicated in the localization and retention of prion agents.[20] In normal mice IBL-10$^+$ fibroblasts were evenly distributed between the follicles and PALS, with an accentuated appearance at their border region resembling a hedge, whereas IBL-11$^+$ cells seemed mostly excluded from the follicles, but were abundant within the PALS. Their B-cell zone reactivity was restricted to the peripheral rim adjacent to the marginal zone. The lack of co-localization observed during subsequent dual staining for surface markers associated either with follicular dendritic cells (such as CD21/35) or marginal zone sinus-lining cells (such as MAdCAM-1) indicated that these tissue constituents represent distinct cellular components to the reticular cells labeled either with IBL-10 or IBL-11 mAbs.[12] On the other hand, the FDC network in Nkx2.3$^{-/-}$ mice appeared considerably shrunken, therefore it is tempting to speculate whether the increased C4-deposition onto reticular fibroblasts typically associated with T-cell zone may serve as a compensatory mechanism for antigen/immune complex retention, or reflects an impaired clearance. It remains to be seen how the above structural alterations influence these mutants' ability to mount immune responses requiring interactions between lymphoid cells and their stromal microenvironment.

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