Developmental reprogramming of splenic vasculature and homeostasis of B-1a cells in Nkx2.3 homeodomain transcription factor deficient mouse model

PhD thesis

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Introduction

The development, function and homeostasis of the immune system are strictly based on the firm interplay between its hematopoietic and mesenchymal components. Mobile cells of hematopoietic origin guide the development of distinct sessile cellular elements from their mesenchymal precursors in lymphoid organs. These organs then provide an essential environment for the sequential stages of the adaptive immune response, and provide homeostatic control for different hematopoietic cells of the immune system.

It has been shown that the spleen is involved in the maintenance and the function of a distinguished B-cell subset, the B-1a cells, although several aspects of this homeostatic relationship are still unknown. The pathogenic role of B-1a cells has been suggested in several mouse models of autoimmunity as well as their possible relationship with distinct haematological malignancies. Thus understanding the exact role of the spleen in homeostasis of B-1a cells under both steady-state and pathologic conditions seems reasonable.

The spleen is the largest, single secondary lymphoid tissue with the capacity to generate adaptive immune responses against blood-borne pathogens/antigens. Its histological structure is unique among secondary lymphoid tissues in many respects. The lymphoid compartment of the spleen, the white pulp, is arranged around central arterioles, the terminal branches of the splenic artery. The central white pulp consists of the periarteriolar lymphatic sheeth (PALS, T-cell zone) and the follicles (B-cell zone), while the marginal zone (MZ) forms the outermost edge of white pulp separating it from (or connecting it to) the red pulp. Between the MZ and the central white pulp the marginal sinus (MS) is situated, which is assumed to serve as the entry port of circulating lymphocytes from the blood stream to the spleen. The red pulp venous sinusoid network is composed of heterogeneous endothel subsets with characteristic spatial distribution. The microarchitectural frame of white pulp as well as the red pulp is built up of fibroblasts which, similarly to the endothelial cells, are heterogeneous, and the distribution of their subsets has been well characterized in the spleen.

The spleen develops from the mesenchymal cells of the dorsal mesogastrium. Its development requires many cell-intrinsic factors (mainly transcription factors) as well as receptor-ligand pairs. Interactions between receptors and ligands mediate the effect of cellular interactions between stromal precursors of the spleen and haematopoietic cells that enter the splenic anlage at distinct stages of the development. Several transcription factors act during the early phase of the spleen development, and their isolated absence leads to asplenia. However, the absence of later-arising transcriptional factors may differentially influence the development of distinct compartments of the spleen. It has been shown that the lack of the homeodomain transcription factor Nkx2.3 results in complex structural alterations of the spleen. These include the lack of MZ, MS and the proper PALS-follicule segregation. Furthermore, Nkx2.3 deficiency alters the development of the fibroreticular meshwork of both the white and the red pulp, and severely impairs the development of the red pulp endothelial sinus network. These alterations have been well characterized previously. Therefore Nkx2.3 KO mutant mice may serve as suitable model in understanding the tissue region-specific role of the spleen in a variety of immunological processes.

Based on developmental and functional features, the cells of the immune system can be classified into two distinct groups: (1) those first encountering with and reacting to invading pathogenic organisms make up for the innate immune system, whereas (2) those that react later in an antigen-specific manner, amplify the overall efficiency of the immune response and form immunological memory belong to the adaptive immune system. The distinction between innate and adaptive immune system have been blurred with identification of a group of cells, termed innate-like lymphocytes (ILL-s). These cells, similarly to the cells of the adaptive immune system, express clonally rearranged antigen receptors. However the

molecular features and the low diversity of their antigen receptors, as well as their pre-activated, “ready for responding” status renders them similar to the cells of the innate immune system. One of the ILL-s is the group of B-1a cells.

B-1a cells represent a unique lymphocyte population. In mice these cells mainly accumulate in serosal cavities and also dwell in the spleen, and express CD5 which is unique among B-lymphocyte subsets. A distinctive developmental feature of B-1a cells is that their major pool is generated during fetal life mainly from precursors of fetal liver origin. After their formation during embryonic life B-1a cells represent a self-maintaining population. Although adult bone marrow retains the potential of B-1a cell formation under steady-state conditions, B-1a cell population is overwhelmingly maintained by self-division, and B-1a influx from the bone marrow is negligible. Importantly, the self-renewal capacity of these cells largely depends on the spleen possibly mainly by their recirculation for the acquisition of survival / division signals. However, the nature of this putative signal is still unknown. Similarly to other B-cell subsets, B-1a cells undergo BCR gene rearrangement during maturation, and subsequently express BCR. Furthermore, they produce natural antibodies that are formed without any obvious previous antigen exposure. Natural antibodies have low affinity binding against a wide range of microbial antigens largely conserved among species, as well as cross-reactive features targeting them against self antigens. Similarly to the self-renewal capacity of B-1a cells, their antibody production also depends on the spleen.

The implication and aims of the study

Although it has been demonstrated that the spleen plays an indispensable role in the maintenance of B-1a cells, the exact cell type or tissue compartment responsible for B-1a homeostasis is not known. Therefore animal models with distinct alteration in splenic structure may prove useful in understanding the microenvironmental requirement of the self-maintenance of B-1a cells. Therefore I further examined in detail the splenic vascular alterations of the Nkx2.3 KO mouse, and used this model to examine the B-1a lymphocyte homeostasis.

(1) Earlier microarray analysis and the peripheral lymph node (pLN)-like lymphocyte composition of the mutant spleen as well as the lack of MS prompted us to identify vascular elements responsible for lymphocyte homing in Nkx2.3-deficient spleen.

- I examined the transcription pattern of maker genes associated with High Endothelial Venule (HEV) formation in the mutant spleen, and compared it to wild-type (wt) spleen and to wt and mutant LN-s.
- Morphological characterization of the HEV-like vessels in the mutant spleen.
- Examining the functional relevance of the ectopic HEV-like vessels in lymphocyte extravasation in the Nkx2.3 KO spleen.

(2) I aimed to evaluate the homeostatic features and preferential tissue redistribution of B-1a cells under steady-state conditions as well as after lipopolysaccharide (LPS) stimulation, necessitating the development of a novel approach with *in situ* carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling technique in wt mice.

(3) I wished to characterize the consequences of Nkx2.3 mutation on B-1a cell homeostasis and function over time.

- With a panel of cell surface marker and flow cytometry, I analysed the identical phenotypic features of CD5+ peritoneal B lymphocytes in Nkx2.3 KO mice to establish their *bona fide* B-1a cell derivation.
- I compared the peritoneal B-1a cell homeostasis of mutant and wt mice with flow cytometry, and examined the changes in serum natural antibody level with ELISA over time.
- To examine the contribution of the Nkx2.3 KO environment to the altered homeostasis of B-1a cells, adoptive cell transfer experiment of B-1a cells to Nkx2.3 KO recipient mice has been performed.

Materials and Methods

Mice

Nkx2.3$^{-/-}$ mice from a 129Sv x B6 mixed background were backcrossed with BALB/c mice (obtained from Charles River Laboratories, Budapest, Hungary) through 14 generations and genotyped using conventional duplex PCR method amplifying Nkx2.3 and neomycin resistance gene. For homing and peritoneal cell transfer studies, BALB/c mice from our faculty's specific pathogen-free breeding unit were used as a lymphocyte donor for adoptive transfer. 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.

In situ CFSE labeling

CFSE was dissolved sterile PBS without Ca^{2+} and Mg^{2+} ions, and was i.p. injected to BALB/c or Nkx2.3 KO mice. After various time points, the mice were sacrificed and the frequency and fluorescence intensity of CFSE-labeled cells in the peritoneum, pleural cavity, and in various peripheral lymphoid organs were investigated.

Intraperitoneal LPS administration

LPS was dissolved in sterile PBS without Ca^{2+} and Mg^{2+} ions at a concentration of 100 μg/ml. Two hundred and fifty microliter of this solution was injected i.p. to the mice 2 h after i.p. CFSE administration.

Single-cell suspension preparation

Lymphocytes were isolated from the spleen and lymph nodes by tearing apart the organs between the frosted ends of two microscopic slides and filtered through a 70-μm pore-size cell strainer. Peritoneal cells were isolated by flushing with incomplete DMEM from the peritoneal cavity of mice. Pleural cells were isolated through a right parasternal incision by flushing with 1 ml incomplete DMEM from the pleural cavity.

Flow cytometry

Isolated cells from LN-s and spleen were incubated with mixtures of appropriate unlabeled, fluorescently labeled, or biotinylated primary antibodies. Unlabeled antibodies were detected with fluorescently labeled species-specific secondary antibodies, or with fluorochrom coupled 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 BD Cell-Quest software, or WinMDI 2.8 software.

Immunohistochemistry and immunofluorescence

Single and dual-label immunofluorescence and immunohistochemical procedures were performed in our experiments. For control staining normal rat IgG 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.

In vitro CFSE labeling of lymphocytes isolated from peripheral lymph nodes, and adoptive cell transfer of the labeled cells

Lymphocytes from peripheral lymph nodes (pLN-s) or mesenteric lymph nodes (mLN-s [mesenteric lymph nodes]) were isolated and labeled with CFSE (Invitrogen) or sulfo-N-hydroxysuccinimide biotin ester. For lymphocyte homing studies, 200 µl cell suspension at 5 x 10^7 CFSE-labeled cells was injected intravenously (i.v.) in the tail vein, followed by the removal of the spleen at various intervals. The distribution of labeled cells was tested by immunofluorescence using anti-PNAd (Peripheral node addressin) or IBL-11 monoclonal antibody (mAb) against white pulp fibroblasts 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 purified MEL-14 IgG or IBL-10 control rat mAb. After washing, cells were counted and mixed at 1:1 ratio with biotinylated cells as reference population, followed by the i.v. injection of mixed cells. 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. To be able to perform these measurements, biotinylated cells were labeled with streptavidin-phycoerythrin following the single cell suspension preparation from the isolated tissues. 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.

In vitro CFSE labeling of peritoneal cells, and peritoneal cell transfer

Peritoneal cells were isolated by lavage and the cells were incubated at a 10^6/ ml cell density in either 1 µg/ml, or 6 µg/ml CFSE dissolved in PBS/0.1% BSA for 10 min at 37°C on a rotator. The appropriate working concentration of CFSE during the labeling was chosen according to the respective experiments performed. Reaction was stopped by washing the cells with ice-cold complete DMEM medium. A total of 5 x 10^6 CFSE-labeled cells per recipient were i.p. injected to mice.

ELISA

ELISA plates were coated with 23 µg/ml Pneumovax-23, with 10 µg/ml phosphorylcholine or with 5 µg/ml monoclonal rat α-mouse IgM (clone IBL-16) to measure the relative serum level of pneumococcus polysaccharide (PPS)-, phosphorylcholine-specific IgM, IgG and that of total IgM antibodies, respectively. Diluted sera of mice were then added, followed by the addition of horseradish peroxidase conjugated rabbit α-mouse IgM or IgG. Ortho-phenylene diamine was added and the reaction was stopped with 4M H_2SO_4. Transmitted light was measured at 490 nm.

Quantitative RT-PCR

Total RNA was isolated with the RNeasy Plus Mini Kit and was treated with DNase I. cDNA was prepared with the High Capacity cDNA Archive Kit. PCRs were run in six parallel samples using the Power Sybr Green Master Mix on an ABI 7500 Real Time PCR System. 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. Independent sample t-test was used for the pairwise comparison of the data, where it was appropriate. For multiple datasets 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

Vascular reprogramming of splenic vasculature in Nkx2.3 KO mutant mice

Lymphocyte composition of the spleen of Nkx2.3 KO mutant mice is similar to that of lymph nodes.

In Nkx2.3 KO mutant spleen, in spite of the lack of vascular components that may have potential role in the splenic homing of B and T lymphocytes, a significant accumulation of lymphoid cells is still preserved, suggesting the presence of tissue structures that are able to mediate lymphoid entry. Therefore to define the lymphocyte subpopulations present in the spleen, I used flow cytometry. Interestingly, the distribution of T/B lymphocyte populations in the mutant spleen was more similar to that of lymph nodes than to the wild-type spleen, with a higher frequency of T lymphocytes. In addition, lymphocytes present in the mutant spleen had a somewhat higher L-selectin expression, also similar to lymph nodes in this respect.

Altered expression of marker genes associated with high endothelial venule (HEV) development in the spleen and lymph nodes

The broad structural alterations of Nkx2.3 KO mutant spleen together with the lymph node-like accumulation of lymphocytes prompted us to examine which molecules / processes could be responsible for the selective accumulation of these cells. Earlier microarray analysis of the Nkx2.3 KO mutant spleen suggested that expression of several genes associated with endothelium-mediated tissue-specific homing was altered. In this study, we performed real-time PCR to quantify expression of particular genes involved in endothel specific homing in wt and Nkx2.3 KO spleen, pLN and mesenteric LN (mLN). These genes encode PNAd core proteins (MAdCAM-1, GlyCAM-1, CD34, endomucin, nepmucin, podocalyxin-like protein) as well as modifying enzymes that glycosylate, fucosylate or sulfatate the core proteins during the process of PNAd formation (glycosyltransferases: B3gnt3 and Gcnt1; fucosyltransferase: Fut7; sulfotransferases: Chst2 and Chst4). The most remarkable changes in the gene expression profile of the Nkx2.3 KO mutant spleen were the robust GlyCAM-1 upregulation and MAdCAM-1 downregulation compared to the wt spleen. The mutations induced expression changes of other core protein genes and of modifying enzymes showed a general tendency, indicating that the pattern of endothelial marker gene expression in mutant spleens was reminiscent of HEVs in pLN-s

Ectopic formation of HEV-like vessels in the Nkx2.3 KO mutant spleen

The pLN-like signature of transcripts for HEV-related genes and the described alterations of the marginal zone and white pulp in the Nkx2.3–deficient spleen prompted us to investigate the vasculature in more detail by using MECA-79 anti-PNAd and anti–MAdCAM-1 mAbs. In contrast to the wild-type spleen, which never contains PNAd-positive HEVs, in the spleens of young adult Nkx2.3 KO mice, PNAd-positive HEVs were prominently present. Similarly to the HEVs of pLNs, PNAd positive HEV in the mutant spleen co-expressed Chst4 sulfotransferase. Additionally, the PNAd epitope was expressed on the luminal (recirculating lymphocyte accessible aspect) of the HEVs as revealed by intravenous (i.v.) administration of MECA-79 antibody followed by immunofluorescent imaging.

Ectopic HEV-like vessels in Nkx2.3 KO mutant spleen mediate lymphocyte homing in an L-selectin-dependent manner, and express CCL21 arrest chemokine.

To test whether ectopic HEV-like vessels of Nkx2.3 KO mutant spleen serve as lymphocyte exit ports, CFSE-labeled lymphocytes were i.v transferred into mutant and wt mice followed by fluorescent examination of the animals spleen and pLN. After injection the accumulation of labeled lymphocytes in the mutant spleen showed similar kinetics as either in wt or mutant pLNs over time, with an initial association of lymphocytes with ectopic HEV-like vessels in mutant spleen, and with HEV in pLNs.

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, I performed short-term competitive homing experiments. Equal numbers of CFSE-labeled lymphocytes treated with MEL-14 (anti L-selectin) mAb and mock-treated biotinylated control cells were injected into Nkx2.3 KO mutant and wt recipients. MEL-14 mAb have been reported to prevent the homing of lymphocytes to pLNs *in vivo* but this mAb does not eliminate the cells. Thirty minutes after the injection, spleen and pLN cells were isolated and subjected to flow cytometry. I observed that homing of the CFSE-labeled cells to the mutant spleen was significantly blocked compared with wild-type recipients as indicated by the decrease in the CFSE:biotin ratio in the Nkx2.3-deficient spleen but not in the wild-type spleen. The CFSE:biotin ratio in the mutant spleen was very similar to that in pLN-s of wild-type or mutant mice, suggesting that the ectopic $PNAd^+$ HEV-like vessels in the mutant spleen use L-selectin for lymphocyte homing.

After the arrest of lymphocytes in HEV, mediated by PNAd and L-selectin interaction, lymphocyte transmigration is dependent on CCL21 arrest chemokine expressed by the HEV of LNs. To investigate, whether ectopic HEV of the spleen of Nkx2.3-deficient mice express CCL21, double fluorescent labeling was performed with MECA-79 and anti-CCL21 mAb-s While in wild-type spleen, CCL21 labeling was restricted to the fibroreticular network of PALS, in Nkx2.3-deficient spleen CCL21 co-localized with PNAd, similarly to LNs.

In situ CFSE labeling reveals mutual kinetic exchange of peritoneal B-1 cells with B-1 cells of other niches

After the detailed characterization of the vascular changes in Nkx2.3 KO mutant spleen, my aim was to examine the B-1a lymphocyte homeostasis in these mice. However, even in wt mice, many features of the homeostasis of B-1a cells remain unknown under steady-state condition. The major reason is that the studies addressing these questions have been relied on the adaptive cell transfer of B-1a cells, thus altering the B-1a pool. Therefore, my aim was to develop an *in situ* fluorescent labeling technique of the peritoneal B-1a lymphocyte. Furthermore, by using this approach, I examined the peritoneal B-1a cell homeostasis under steady-state conditions, and after LPS stimulation of these cells.

Efficient and selective labeling of peritoneal leukocytes in vivo

CFSE is a versatile fluorescent cell-tracing compound; after its diffusion into the cells, it is covalently entrapped in the intracellular compartment without interfering with cellular physiology. Its ease of use makes it suitable for stable *in vitro* and *in vivo* fluorescent labeling of cells. Consistently, we found that a single intraperitoneal injection of CFSE at an optimized dose and volume was sufficient to label the overwhelming majority of lymphoid and myeloid cells residing there. Furthermore, the tracer did not reach leukocytes in other lymphoid organs, including blood, spleen, peripheral and mesenteric lymph nodes and pleural cavity, but the labeling remained restricted to the cells of the peritoneal cavity.

Comparing this *in situ* labeling technique, with the adoptive intraperitoneal transfer of *ex vivo* labeled cells we found that *in situ* intraperitoneal CFSE injection confers a considerably more representative labeling of peritoneal lymphocyte. Furthermore the result was consistently reproducible.

Intraperitoneal administration of CFSE reveals different exchange kinetics of peritoneal leukocyte sub-populations under steady-state conditions

Next, I investigated whether various peritoneal leukocyte subsets show different tissue replacement kinetics by determining the proportion of *in situ* labeled $CFSE^+$ cells over time. Using flow cytometry, peritoneal lymphocyte sub-populations (B-1, B-2 and T) were identified and the percentage of $CFSE^+$ cells was examined within these populations from 6 h to 4 weeks after *in situ* CFSE labeling. Among peritoneal lymphocytes, T cells showed the fastest exchange kinetics with extraperitoneal sites. Six hours after *in situ* CFSE labeling, 50% of the peritoneal T cells were $CFSE^+$ on average, reflecting an exchange of 50% of peritoneal T lymphocytes to extraperitoneal T cells during this period. After 1 week, the frequency of $CFSE^+$ T cells remained constant at 2 to 5% of the total peritoneal T-cell pool. For B cells, this event was substantially slower and showed a strong correlation with the B-1/B-2 phenotype of B cells. Post-labeling follow-up revealed that the 50% exchange ratio was achieved over 2 days for B-2 and 2 weeks for B-1 cells after CFSE administration, respectively. Within the B-1 population, no significant differences were observed between the exchange kinetics of B-1a and B-1b cells during this period. The ratio of $CFSE^+$ B-1 cells is stabilized after 2 weeks, at 45 to 50% of total B-1 cells with detectable CFSE label, and it remained stable for at least two more weeks. In contrast, the decrease of $CFSE^+$ ratios of B-2 cells continued during the 4-week interval. These results indicate that under steady-state conditions, T cells have the fastest exchange rate among peritoneal lymphocytes. B-2 cells are replaced at a slower pace. B-1 cells reside for the longest period in the peritoneal cavity and are exchanged for extraperitoneal B-1 (CFSE-negative) cells at the slowest kinetics but remain stable after reaching equilibrium.

LPS induced egress and division of peritoneal B cells is associated with enhanced preferential homing of B-1 cells in the pleural cavity

In vitro and *in vivo* data indicate that stimulation of B cells through toll-like receptor 4 (TLR-4, LPS receptor) has a complex effect on B cells including enhanced proliferation and rapid egress of B-1 cells from the peritoneal cavity. Therefore, I examined the effect of a single intraperitoneal LPS injection on peritoneal B-cell homeostasis delivered 2 h after *in situ* CFSE labeling. LPS induced a dramatic egress of both peritoneal B-2 and B-1 cells 24 h after stimulation, reflected by an 50 and 40% decrease in the absolute number of both subsets, respectively At 1 week, the absolute number of peritoneal B-1 and B-2 cells was restored. Restoration of the B-cell compartment by this time is attributed both to LPS-induced proliferation and enhanced homeostatic immigration of B cells from extraperitoneal niches following LPS-induced egress. The extent of LPS-induced proliferation and the immigration of both B-1 and B-2 cells could be determined by quantifying the changes in $CFSE^{dim}$ and $CFSE^-$ cells respectively, within these populations.
In addition to peritoneal accumulation, B-1 cells are also present in the pleural cavity, although the relationship to their peritoneal counterparts has remained elusive. Therefore, I tested whether following their *in situ* labeling, peritoneal B-1 cells would relocate in the pleural cavity and how their inter-cavity distribution can be modulated by exposure to LPS. I found that the frequency of both $CFSE^+$ B-1 and $CFSE^+$ B-2 cells within the pleural cavity

rose gradually after labeling, with a faster kinetics of pleural accumulation of B-1 cell. This was a 3-fold increase accelerated by LPS stimulation while the accumulation of $CFSE^+$ B-2 cells did not change significantly. These data indicate that although both peritoneal B-1 and B-2 cells have the preference for pleural migration, LPS promotes an increased activity only for the B-1 cells to target pleural cavity.

Homeostasis of B-1a cells in Nkx2.3 KO mutant mice

In the third part of my work I addressed the homeostasis of peritoneal B-1a cells in Nk2.3 KO mutant mice. Studies examining the role of the spleen in B-1a cell homeostasis are either based on asplenic mice, or investigate this question after splenectomy. No detailed examination of B-1a cell homeostasis in these mice with well-defined splenic alteration has been performed so far.

Peritoneal B-cell subsets of identical phenotype are formed in Nkx2.3 KO mutant and BALB/c mice

It is well established that the spleen plays an important role in the terminal differentiation stage of B lymphocytes during which several phenotypic changes occur. Furthermore, under certain conditions, CD5 expression can be achieved on B-2 lymphocytes as well. Therefore it is imperative to ensure that in Nkx2.3 KO mutants peritoneal $CD5^+$ B lymphocytes are indeed B-1a cells, rather then B-2 cells with altered phenotype caused by a yet unidentified effect of the splenic structural alterations in these mice. Therefore a panel of a cell surface marker specific mAbs (anti-IgM, anti-IgD, anti-CD43, anti-CD23 and anti-CD21, anti-MAC-1) was used and flow cytometry was performed to ensure the cellular identity of B-1a cells in Nkx2.3 KO mutant mice. My results showed that distinct peritoneal B-lymphocyte subsets (B-1a, B-1b, B-2) in Nkx2.3 KO mutant mice corresponded the respective subsets in wt mice according to their cell surface marker expression pattern.

The ratio of peritoneal $CD5^+$ B cells of Nkx2.3 KO mutant mice shows an age-dependent progressive decrease

To examine the effect of the absence of Nkx2.3 transcription factor on the peritoneal $CD5^+$ B-cell homeostasis, peritoneal cell composition of the mutants of various ages were examined with flow cytometry, and compared to age-matched wt mice. As a result of the examination, an age dependent decrease in the ratio of $CD5^+$ peritoneal B cells could be seen in mutants. Initially (until 2 months) decrease in the B-1c population was more prominent. This was followed by an accelerated decrease in the proportion of B-1a cells (between 2 and 4 months). My findings are consistent with studies demonstrating the dependence of B-1a cell self-maintenance on the spleen, probably due to the preserved red pulp. Furthermore, similar changes were observed in the frequency of pleural $CD5^+$ (B-1a and B-1c) lymphocyte subsets over time.

Progressive decrease in the serum level of natural antibodies of distinct specificities in Nkx2.3 KO mutant mice

B-1a lymphocytes have been held responsible for the production of natural antibodies of IgM isotype, which provide an early, innate-like protection against invading bacteria and viruses. B-1a cells do not form antibodies in the peritoneal cavity, but their antibody production is dependent on the splenic environment.

I next examined whether changes in B-1a lymphocyte homeostasis of Nkx2.3 KO mutant mice affect natural autoantibody production. For this total serum IgM, total serum IgG, pneumococcus polysaccharide (PPS) -specific IgM and phosphorylcholine (Pc) - specific IgM levels were measured at 1, 2 and 4 month of age in Nkx2.3 KO mutant and wt mice with ELISA technique.
I observed that the total serum IgM level was significantly lower in 1 month old mutant than in wt mice. In contrast to the total serum IgM level, the PPS- and Pc-specific IgM levels decreased only later, mostly between 1 and 2 month. Total serum IgG level did not differ considerably between the mutant and the wild-type mice at any time point.
In conclusion concerning PPS and Pc specificity, the natural antibody production of B-1a cells did not seem to completely depend on the intact splenic structure, but rather showed a correlation with the decrease in the ratio peritoneal $CD5^+$ cells over time, with an enhanced decrease in young mice between the age of 1 and 2 month.

B-1a cells are efficiently produced in Nkx2.3 KO mutant mice

Previous works addressing B-1a homeostasis has revealed that beside postnatal maintenance, fetal B-1a cell formation is also dependent on the spleen. I found however that a significant fraction of peritoneal B-1a cells exists in 1 month old Nkx2.3 KO mutant mice. This prompted me to examine the efficiency of fetal B-1a cell formation in Nkx2.3 KO mutants. Therefore peritoneal B-cell composition was examined with flow cytometry in 15 day old mutant and wt mice. I observed that within the peritoneal lymphocyte population, the majority of cells belonged to the $CD5^{dim}$ $B220^{dim}$ population (corresponding to $CD5^+$ B cells) in mutant and in wt mice. Furthermore the frequency was similar irrespectively of the genetic background. This reflects an efficient pre/perinatal $CD5^+$ B lymphocyte production in mutant mice, which is indistinguishable from that in wt mice.

Diminished cell proliferation of $CD5^+$ B cells may be responsible for altered homeostasis, and is the consequence of stromal alteration in Nkx2.3 KO mutant mice

Based on my observations after efficient fetal formation, a progressive net loss of $CD5^+$ B lymphocytes occurs postnatally in Nkx2.3 KO mutant mice. However, it is still unknown whether this process results from an intrinsic defect of this B-lymphocyte subset, or is caused by the splenic stromal changes in mutants. Furthermore, it is unknown whether reduced cell division or reduced cell survival is responsible for the net loss of this population over time**.**
To investigate this question, adoptive cell transfer experiment was performed. Total peritoneal washout cells from wt BALB/c mice were labeled *in vitro* with CFSE, and intraperitoneally injected either to Nkx2.3 KO mutant or wt recipients. Peritoneal cells were isolated from the recipients two weeks after cell transfer. The ratio of total $CFSE^+$ donor B-1a cells, and within this population, the CFSE decay have been determined.
I observed that in mutant mice the ratio of CFSE-labeled donor $CD5^+$ B lymphocytes was significantly lower than in wt recipients. Furthermore, based on the assessment of CFSE decay in labeled donor cells, B-1a cells divided at a lower frequency in Nkx2.3 KO mutant recipients.
These results suggest that the progressive postnatal loss of $CD5^+$ B lymphocytes in Nkx2.3 KO mutant mice is - at least partially - associated with diminished self-maintaining division of $CD5^+$ lymphocytes, and may be the consequence of the environmental alteration caused by the lack of Nkx2.3.

Discussion

In the development of lymphoid organs many transcription factors participate at distinct stages in a hierarchic order and in tissue-specific manner, including several members of the Nkx homeodomain-containing family. To address the function of, the Nkx2.3 HD transcription factor in the development of the vascular identity of the spleen, I examined the splenic vascular changes of the Nkx2.3 KO mutant mice backcrossed to BALB/c background. Earlier studies reported severe red pulp vascular and stromal changes, the lack of MS and MZ, and the lack of T/B zone segregation of the white pulp in the spleen of Nkx2.3 KO mutant mice. Importantly the red pulp was the most affected splenic tissue compartments. The molecular mechanism that causes such a widespread alteration in the mutant mice remains elusive. In the spleen the Nkx2.3 transcription factor is expressed during the fetal life when the red pulp develops. This may account for the prominent effect of the mutation on the red pulp, which on BALB/c genetic background may even result in the complete loss of this compartment ("redless spleen"). This observation may lead to the assumption that changes in the white pulp are indirect consequence of the loss of the red pulp in mutants. However on B6 genetic background the red pulp persists yet the white pulp is affected. Therefore changes in the white pulp may not be the exclusive consequence of the red pulp alterations.
In my work I have shown that in Nkx2.3 KO mutant mice ectopic HEV-like structures appear in the spleen. These ectopic vascular structures are structurally and functionally equal to HEVs in pLNs. The only known target gene of the Nkx2.3 HD transcription factor is *madcam-1*. However it is unlikely that the loss of MAdCAM-1 (together with the MS) by itself results in the ectopic development of an alternative exit port for lymphocytes to the mutant spleen. Indeed based on my gene expression profiling (microarray, qPCR) results it seems that the Nkx2.3 HD transcription factor act as a suppressor of the pLN-like vascular commitment in the splenic anlage. This assumption however requires further examinations.

To examine the relationship of the peritoneal B-1a lymphocytes with B-1a lymphocytes of other niches and to describe their exchange kinetics I developed a fluorescent intraperitoneal *in situ* labeling technique. This method is flexible, easy to perform and requires fewer animals than previous methods applied for the same purpose. Additionally it allows examining the peritoneal B-1a cells on a so far unexamined time scale (from 6 hours to at least 4 weeks post labeling) and under steady-state conditions.
Given the versatility of this technique, it may be useful in future studies addressing the serosal accumulation of B-1a cells. The exact reason of their unique distribution is still largely unknown. According to some theories their serosal "sequestration" is essential in controlling their autoreactive natural antibody formation, thus preventing their transformation into pathologic autoantibody forming cells. Other theories consider that their continuous serosal recirculation provides a natural patrol system with a specified set of B-cell receptor (BCR) repertoire to these sites. Both assumptions require further experimental evidence.

Major part of the B-1a cell pool is produced during embryonic life and – according to previous studies - its formation is dependent on the spleen. To my knowledge, my report on the efficient foetal formation of B-1a cells in a mouse model with gross splenic alterations is the first of its kind. I also found that a progressive postnatal loss of peritoneal B-1a cells follows the efficient fetal formation in Nkx2.3 KO mutant mice.
It has been shown recently that the adult bone marrow is also capable of producing B-1a cells, although the molecular structure of the BCR significantly differs from that of the fetal derived counterparts. Under steady-state conditions the B-1a efflux from the bone marrow is minimal, and becomes prominent after B-1a depletion. However the progressive net loss of B-1a cells

in Nkx2.3 KO mutant mice indicates that in these mice the adult B-1a formation can not compensate for the loss of fetal B-1a cells over time. These results suggest that fetal and adult B-1a cell formation have differential dependence on the spleen.

Short summary of the results

Endothelial reprogramming of the spleen in Nkx2.3 KO mutant mice results in the appearance of ectopic HEV-like vascular elements. These structures are lined by typical HEV endothelial cells, co-express PNAd with Chst4, a modification enzyme required for functional PNAd formation, and CCL21, an arrest chemokine indispensible for lymphocyte extravasation through HEV-s of the lymph nodes, respectively. Furthermore, these HEV-like vascular elements mediate lymphocyte extravasation in an L-selectin dependent manner. In summary, ectopic HEV-like structures that appear in the mutant spleen are structurally and functionally equal to HEVs in pLNs.

To investigate the peritoneal B-1 lymphocyte distribution in normal an Nkx2.3 deficient mouse, a novel fluorescence-based intraperitoneal *in situ* labeling/tracing method was developed. Using this technique I established the exchange kinetics of distinct peritoneal lymphocytes subsets (B-1, B-2 and T lymphocytes) on a so far unexamined time scale (from 6 hours to 4 weeks post-labeling), and demonstrated that there is a mutual exchange between the peritoneal and pleural B-1 cell pool which is enhanced by LPS stimulation.

Using this assay I found that the fetal B-1a cell production is not affected by the absence of Nkx2.3 HD transcription factor. However a progressive postnatal decrease in the ratio of peritoneal B-1a cell occurs in mutant mice. This decrease is at least partially due to the diminished self-maintaining division of B-1a cells caused by the stromal changes in Nkx2.3 KO mutants, and is accompanied by the decrease in serum level of natural antibodies of distinct specificities.

Publications the thesis is based on

First authorship

Lábadi A, Balogh P. Differential preferences in serosal homing and distribution of peritoneal B-cell subsets revealed by in situ CFSE labeling. *Int. Immunol.* 2009; 21:1047–56.DOI: 10.1093/intimm/dxp071.

IF: 3,403

Second authorship

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IF: 5,788 (from this 2,894 is used for this dissertation)

Publications not directly related to this essay

Shared first authorship

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Book chapter:

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