ROLE OF NKX2-3 TRANSCRIPTION FACTOR IN THE VASCULAR DEVELOPMENT AND IDENTITY OF VISCERAL LYMPHOID TISSUES IN MICE

Doctoral (PhD) thesis

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I. Introduction

I.1. Secondary lymphoid organs

The function of the immune system is the recognition of and distinction between pathogenic and harmless structures, regardless of their origin (self or foreign), followed by either the elimination of damaged self-cells and harmful microbes, or tolerance of benign symbiotic antigens. Secondary lymphoid organs provide the appropriate microenvironment and meeting point for mature lymphocytes and antigens. A crucial step in this process is the homing of circulating lymphocytes and antigen-presenting cells as well as the transport of antigens to, and subsequently within, these tissues. Peripheral lymphoid organs include the spleen, the peripheral lymph nodes (pLN), mucosa-associated lymphoid tissue (MALT, consisting of mesenteric lymph nodes [mLN] and Peyer's patches [PP]), and skin-associated lymphoid tissue (SALT).

The development of various secondary lymphoid organs follows two main schemes. Lymph node development is based on interactions between hematopoietic lymphoid tissue inducer (LTi) cells displaying lymphotoxin alpha/beta heterotrimer ($LT\alpha\beta_2$) and stromal lymphoid tissue organizer (LTo) cells producing the receptor for this ligand ($LT\beta R^+$, [van de Pavert and Mebius 2010]). This interaction leads to recruitment and accumulation of of lymphoid cells, which in adult mice are indispensable for maintaining lymph node structure. In Peyer's patches an additional cell type termed lymphoid tissue initiator (LTin) cell is also required in PP formation [Hashi *et al* 2001]. Meanwhile, spleen formation is based on the sequential interplay of various transcription factors and although lymphotoxin ligands play a role in structural compartmentalization, they are not needed for the spleen, as a distinct organ, to form [Brendolan *et al* 2007].

In adult lymph nodes T cells reside in the interfollicular area while B cells are found in follicles in the outer cortex [Crivellato *et al* 2004]. This segregation is achieved by the differential expression of the appropriate homeostatic chemokines. Lymphocytes reach LNs via high endothelial venules (HEVs). PPs have a similarly compartmentalized structure with B cells in follicles and T cells in less pronounced interfollicular areas. In addition, the epithelial surface of PPs contains specialized M cells capable of mediating contact between pathogens in the intestinal lumen and various immune cells within PPs [Corr *et al* 2008]. In contrast, T cells in the spleen reside in the periarteriolar lymphoid sheath (PALS) surrounding central arterioles, with B cells located in follicles. Fibroblastic reticular cells (FRCs) present in the white pulp have an important role in establishing the conduit system [Nolte *et al* 2003]. The marginal zone (MZ) is a distinct ring surrounding the white pulp and separating it from the red pulp [Mebius *et al* 2004]. Here two main macrophage subsets and also the marginal zone B cells can be found. The spleen is void of HEVs and lymphocytes reach the white pulp through the marginal zone.

I.2. Lymphocyte homing to peripheral lymphoid organs

Tissue-specific homing of lymphocytes is determined by the expression profile of endothelial addressins on specialized vascular segments in various tissues, which are recognized by various homing receptors expressed on lymphocytes. In pLNs the main addressin is peripheral node addressin (PNAd), which is recognized by L-selectin on lymphocytes and can be identified by MECA-79 monoclonal antibody [Streeter *et al* 1988]. PNAd consists of various backbone proteins that undergo different

posttranslational modifications performed by various fucosyltransferases and sulfotransferases. Meanwhile, the main addressin expressed in mucosal tissues is mucosal addressin cell adhesion molecule (MAdCAM-1), recognized by α4β7 integrin expressed on lymphocytes and identified by the MECA-367 monoclonal antibody [Berlin *et al* 1993]. Importantly, MAdCAM-1 is also expressed on lamina propria vessels outside lymphoid areas. Interestingly, in neonatal mice HEVs in all tissues express MAdCAM-1, and in pLNs PNAd only appears during the first postnatal month as it gradually replaces MAdCAM-1 [Mebius *et al* 1996].

Besides the selective expression of addressins, the chemokine profile of HEVs also significantly contributes to lymphocyte subtype-specific extravasation into secondary lymphoid organs. CCL21 is expressed on pLN and PP HEV and directs T cells into lymphoid tissues by binding to CCR7 chemokine receptor found on T cells. The chemokine CXCL13 plays a critical role in B cell trafficking to follicular HEVs in PPs as it binds to CXCR5 expressed on B cells [Warnock *et al* 2000].

Although HEV development and maintenance is not completely characterized, the important role of lymphotoxins has been described. LTβR is expressed by HEVs and interfering with LTβR activation leads to dedifferentiation and loss of function of both $PNAd^+$ and $MAdCAM\text{-}1^+$ HEVs [Browning *et al* 2005]. Meanwhile, enhanced LTβR signaling leads to ectopic PNAd appearance [Drayton *et al* 2003].

I.3. Nkx2-3 transcription factor

Nkx2-3 (Nk2 transcription factor related, locus 3) homeodomain-containing transcription factor has been identified as an important regulator of spleen and PP ontogeny without affecting pLN development. Mice with targeted Nkx2-3 gene disruption have morphological alterations in their small intestine with delayed villus formation in fetuses [Pabst *et al* 1999]. $Nkx2\text{-}3^{-/-}$ mice also have significantly smaller spleens with a highly disorganized structure. The T and B cell segregation characteristic for normal spleens cannot be observed and the marginal zone is absent. Peyer's patches are smaller and less numerous. Also, endothelial MAdCAM-1 is absent and Nkx2-3 was shown to directly activate MAdCAM-1 transcription [Pabst *et al* 2000; Wang *et al* 2000]. B-cell development and T-dependent immune responses are also affected [Tarlinton *et al* 2003]. Previous work in our lab revealed alterations in the vasculature and stromal network of $Nkx2\text{-}3^{-/-}$ spleen [Balogh et al 2007; Bovári et al 2007].

Recently, genome-wide association studies have revealed single nucleotide polymorphisms in the coding region of human Nkx2-3 gene sequence in patient samples from both main types of inflammatory bowel disease (IBD), Crohn's disease and ulcerative colitis [Cho 2008; WTCCC 2007]. Interestingly, Nkx2-3 mRNA expression was significantly increased in both B cells and inflamed intestinal tissues from Crohn's disease patients, but not ulcerative colitis patients [Yu *et al* 2012].

II. Aims

We wished to examine the role Nkx2-3 homeodomain transcription factor plays in the development and maintenance of endothelial cells and vascular structures. We studied the two main sites where Nk2-3 has been reported to be expressed: the spleen and the intestine.

The first part of my PhD thesis focuses on the spleen. Previously, our lab investigated the blood vasculature of Nkx2-3$^{-/-}$ spleens. Results included alterations in the red pulp and the presence of ectopic, PNAd-expressing high endothelial venules in Nkx2-3$^{-/-}$ spleens. Now, we wished to answer the following questions:

- Is there other evidence for altered endothelial differentiation and stromal connections in the spleen of Nkx2-3-deficient mice?
- Does LTβR have a similar role in the development of the splenic PNAd^{+} venules as in PLNs?

In the second part of my work we focused on the intestine-associated lymphoid tissues of Nkx2-3 mutant animals. Although endothelial cells in Nkx2-3$^{-/-}$ mice lack MAdCAM-1, lymphocytes still reach mucosal lymphoid tissues. We wished to characterize and analyze the various compensatory mechanisms participating in this process.

- What addressin(s) do lymphocytes utilize to home to gut-associated lymphoid tissues?
- How does the altered addressin profile affect the lymphocyte composition?
- How do the presence of lymphocytes and the activity of LTβR affect the vascular patterning in the intestines of Nkx2-3-deficient mice?

III. Materials and methods

Mice

Nkx2-3$^{-/-}$ mice from 129SvxB6 mixed background were backcrossed with BALB/c mice (obtained from Charles River Hungary) through 14 generations and genotyped as described. For homing studies either BALB/c, GFP-BALB/c or FvBN mice from the Faculty's SPF breeding unit were used as lymphocyte donor for adoptive transfer. LTβR-deficient mice were kindly provided by Drs. Klaus Pfeffer and Falk Weih. RAG-1$^{-/-}$ mice were obtained from Jackson Laboratories. The Nkx2-3/LTβR and Nkx2-3/RAG-1 double KO mice were identified in the F2 generation by simultaneous PCR amplification of *nkx2-3, neomycin phosphotransferase* and *ltbr* loci or *nkx2-3, neomycin phosphotransferase* and *rag* loci, respectively. 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.

Immunohistochemistry and immunofluorescence

Lymphoid organs were removed and snap-frozen. Cryostat sections were fixed with acetone. Single, dual, and multiple immunofluorescence was performed with various antibodies and unlabeled antibodies were detected using labeled secondary antibodies. Normal rat IgG was used as control. For chemokine stainings fixation was performed with 1% paraformaldehyde. After mounting, sections were viewed under an Olympus BX61 fluorescent microscope. Acquisition of digital pictures was performed with AnalySis® software. For the analysis of luminal MAdCAM-1 and PNAd expression a cocktail containing both anti-PNAd and anti-MAdCAM-1 at 0.5 mg/ml was injected intravenously into mice, which were sacrificed 30 mins later.

Confocal microscopy

Confocal fluorescence images were taken using an Olympus Fluoview FV-1000 laser scanning confocal imaging system. For quantification the samples were visualized using a 20x dry objective at 80 μm wide confocal aperture in photon count mode, excited with multi line argon and helium-neon lasers for 10 μs/pixel in sequential mode creating 1024x1024 pixel single layer confocal images. Total pixel intensity of the images was determined by Fv10-ASW 01.07.03.00 software.

Electron microscopy

After removal spleens were cut in pieces at a volume less than 1 mm^3, then placed in 2.5% buffered glutaraldehyde for 3 hours at room temperature under continuous agitation. Subsequently semithin and ultrathin sections were prepared according to standard protocols in the Central Electron Microscope Laboratory (University of Pécs). Sections were examined in a JEOL 1200EX-II electron microscope. Negative photographs were developed and scanned into computer, from which digitalized pictures were generated.

Flow cytometry

Single-cell suspensions from lymphoid tissues were incubated with various labeled or unlabeled antibodies for 20 minutes on ice. Unlabeled antibodies were

detected using conjugated secondary antibodies. Flow cytometric measurements were performed by a BD Biosciences FACS-Calibur cytometer and analyzed using the CellQuest Pro software or WinMDI 2.8 software. Dead cells were excluded based on size and granularity. At least 20,000 live lymphocytes were analyzed per sample.

***In vitro* labeling and adoptive cell transfer**

Lymphocytes were isolated from GFP-transgenic mice or BALB/c mice and then labeled with 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE). Recipient mice received $5x10^7$ cells intravenously in tail veins and were sacrificed 30, 60, or 120 minutes later. With flow cytometry we determined the rate of homed $CFSE^+$ cells while the distribution and localization of the cells was analyzed by immunofluorescence.

***In vivo* blocking of lymphocyte homing**

To block lymphocyte homing, recipient mice were injected with anti-endothelial monoclonal antibodies, followed 30 minutes later by injection of $5x10^7$ lymphocytes from FvBN mice. Recipient mice were sacrificed 1 hour after lymphocyte injection and cells from spleen, pLN, mLN, and PP were isolated for flow cytometry. Donor lymphocytes were identified using mouse mAb DaB1-FITC conjugate against $H\text{-}2K^b$ cross-reacting with $H\text{-}2K^q$ haplotype expressed by FvBN mice.

Quantitative RT-PCR (qPCR)

Total RNA was isolated with RNeasy Mini Kit and was treated with DNase I. cDNA was prepared with High Capacity cDNA Archive Kit. PCR reactions were run in triplicates using Power Sybr Green Master Mix on an ABI 7500 Real Time PCR System. Expression levels were normalized to beta-actin.

Statistical analysis

Data analysis was performed using SigmaPlot software and graphs were created with the help of GraphPad Prism. Shapiro-Wilk test was performed to assess normality of data distribution. Between-group analysis for normally distributed variables was tested using parametric one-way ANOVA and *post-hoc* Tukey test. In the case of variables that violated assumption of normality, differences were tested with non-parametric Kruskal–Wallis test followed by pairwise multiple comparison procedures (Dunn's and Student-Newman-Keuls method). T-test or Mann-Whitney U test were employed to compare two groups with normally and non-normally distributed data, respectively. Data are presented as mean and standard error of the mean (mean±SEM). Quantitative rtPCR data were analysed with the help of REST 2009 software. Accepted statistical significance was for $p<0.05$.

IV. Results

IV.1. Effects of Nkx2-3 absence in the spleen

IV.1.1. Presence of LYVE-1$^+$ sacs in the spleen of Nkx2-3$^{-/-}$ mice

Upon detailed histological and electron microscopic examination of Nkx2-3$^{-/-}$ spleens we noticed sac-like structures located usually at the peripheral regions of the organ. These structures were filled with lymphoid cells, resembled lymphatic vessels and were LYVE-1$^+$ (indicating lymphatic endothelial origin), VEGF-R2$^+$ (indicating their endothelial origin); but Prox1$^-$ (a marker specific for lymphatic endothelium).

IV.1.2. Biased expression of mRNA of lymphatic endothelium-associated marker genes

We compared the levels of mRNA of various lymphatic endothelium-associated proteins in Nkx2-3$^{-/-}$ and wild type mice. In mutant spleen mRNA levels of LYVE-1 and podoplanin (Pdpn) were significantly increased compared to wild type and were similar to those seen in wild type pLN and mLN. Prox1 mRNA did not show any difference between wild type and mutant spleens. Levels of Flt4, another lymphatic endothelium marker, were similar to wild type spleen and also to wild type pLN.

IV.1.3. Internal content and stromal tissue microenvironment of LYVE-1$^+$ sacs

Immunofluorescence revealed small clusters of MARCO$^+$ macrophages and sialoadhesin/CD169$^+$ macrophages in the vicinity of the LYVE-1-positive sacs. The lymphatic sacs contained mostly T lymphocytes with only some scattered B cells and were tightly surrounded by fibroblastic reticular cells.

IV.1.4. Recirculating lymphocytes are excluded from LYVE-1$^+$ cysts

Intravenous injection of CFSE$^+$ lymphocytes revealed that after 20 minutes only ~2.3% of CFSE$^+$ cells were within the LYVE-1$^+$ structures in a 1.4mm^2 area of the spleen section. This ratio rose to 12.1% at 8 hours. This indicates that the sacs are not directly connected with the blood circulation and suggests other entry routes to these structures.

IV.1.5. Formation of LYVE-1$^+$ structures and ectopic high endothelial venules in Nkx2-3 mutant spleen is initiated in the embryonic period

Staining of E18.5 wild type and mutant spleens revealed that Nkx2-3$^{-/-}$ spleens showed only scattered LYVE-1 expression that did not form discernible sacs. At P0.5 LYVE-1$^+$ sacs were noticeable in the central regions of the spleen with the phenotype characteristic for adult Nkx2-3$^{-/-}$ spleens achieved by P10. These structures were completely absent from wild type embryos.

Lack of Nkx2-3 also leads to the appearance of ectopic PNAd$^+$ HEVs in spleens. We analyzed the development of PNAd$^+$ HEVs by comparing MAdCAM-1 and PNAd expression in mutant mice after birth. At P0.5 spleens of both Nkx2-3$^{-/-}$ and wild type mice contained cell clusters expressing MAdCAM-1. In Nkx2-3$^{-/-}$ spleens, similarly to wild type pLNs, endothelial MAdCAM-1 gradually diminished and was replaced by PNAd by the second postnatal week. In wild type spleen MAdCAM-1 reactivity was observed in the developing marginal zone sinus, with absolutely no PNAd$^+$ cells.

IV.1.6. LTβR activity is dispensable for formation of lymphatic sacs but is required for development of PNAd$^+$ high endothelial venules in Nkx2-3$^{-/-}$ spleens

We created Nkx2-3$^{-/-}$ x LTβR$^{-/-}$ mice to examine whether the LYVE-1$^+$ sacs or PNAd$^+$ HEVs require LTβR signaling for development. In adult double KO spleens LYVE-1$^+$ structures were present while PNAd$^+$ HEVs were absent suggesting that, similarly to wild type pLNs, the formation of HEVs in mutant spleens is dependent on LTβR function.

Administration of the LTβR-Ig fusion protein as decoy receptor to neonates effectively blocks postnatal vascular development in the murine spleen. LTβR-Ig fusion protein applied to Nkx2-3$^{-/-}$ mice on P1 and P4 resulted in a significant reduction of PNAd$^+$ HEVs in the spleen. Thus, the transformation of splenic vasculature to HEV-like vessels in Nkx2-3$^{-/-}$ mice seems to follow a similar program that underlies vessel formation in pLNs.

IV.1.7. Defective formation of follicular conduit retaining MARCO in Nkx2-3$^{-/-}$ spleens

Previous observations indicated the disrupted architecture of marginal zone (MZ) macrophage layers in Nkx2-3$^{-/-}$ mice. Besides being present on a subset of MZ macrophages, we have found that in wild type spleens the scavenger receptor MARCO (macrophage receptor with collagenous structure) also displays a granular expression pattern arranged into fibrils within follicles. MARCO possesses a broad ligand binding capacity, and is also involved in the retention of MZ B cells in mouse spleen. Multiple immunofluorescence revealed that follicular MARCO expression in normal spleen is associated with follicular dendritic cells (FDCs), is separate from the T-cell zone, and is also distinct from the ER-TR7$^+$ conduit system established by T-zone specific FRCs.

We further examined the cellular requirements for the follicular MARCO expression by performing dual immunofluorescence with anti-MARCO and anti-CR1.2 antibodies in various genotypes with defects in either the white pulp or the MZ. In Nkx2-3$^{-/-}$ spleens FDCs are present; however, the MAdCAM-1$^+$ marginal sinus and the MARCO$^+$ MZ macrophages are absent. Immunofluorescence in these mice revealed FDCs that lacked follicular MARCO staining. We found only scattered MARCO$^+$ cells surrounding FDCs; this staining pattern did not resemble the MARCO$^+$ MZ macrophages seen in wild type spleens. Furthermore, we found that MARCO is deposited within the follicles without the involvement of MZ B cell movement. On the other hand, the CXCR5/CXCL13-driven B-cell migration into developing white pulp territory is necessary for the follicular deposition of MARCO through the induction of FDC reticula. Together, these findings indicate that follicular MARCO deposition requires the presence of both MZ macrophages and mature FDCs.

IV.2. Effects of Nkx2-3 absence in gut-associated lymphoid tissues

IV.2.1. Altered mRNA expression of high endothelial venule signature genes in GALT of adult Nkx2-3$^{-/-}$ mice

Leukocyte homing to Peyer's patches requires recognition of endothelial MAdCAM-1 by α4β7 integrin expressed on leukocytes. Nkx2-3$^{-/-}$ mice lack endothelial MAdCAM-1 expression and have fewer and smaller Peyer's patches. The presence of

structured lymphoid tissues indicates that lymphocytes reach these organs in a MAdCAM-1-independent fashion. We hypothesized that, similarly to Nkx2-3$^{-/-}$ spleens, PNAd^{+} HEVs might be present in the intestines of these mice.

We first analyzed the mRNA expression pattern of PNAd core proteins and the modifying enzymes that create the PNAd epitope in mutant GALT. Interestingly, MAdCAM-1 mRNA was significantly increased in mutant PPs, despite the absence of MAdCAM-1 reported earlier. The majority of backbone protein mRNAs were variably increased in Nkx2-3$^{-/-}$ PPs compared to normal. The levels of modifying enzymes showed variable alterations in mRNA expression, with some enzymes showing significant increases and others presenting with non-significant changes. Overall, while significant changes of mRNA for proteins involved in PNAd generation were detected, their extent was less robust compared to those observed in mutant spleen.

IV.2.2. PNAd is upregulated in the gut-associated lymphoid tissues of Nkx2-3$^{-/-}$ mice

Next we stained PP sections from both mutant and wild type young adult mice with various anti-endothelial antibodies. Staining with the pan-endothelial marker VEGF-R2 revealed a normal degree of vascularization in mutant PPs. As expected, MAdCAM-1 showed a near total absence of endothelial expression while it was present on stromal cells in lymphoid follicles, serving as a probable explanation for the increased levels of MAdCAM-1 mRNA in mutant tissues. PNAd had an intense presence in mutant PPs while it was rarely expressed in wild type samples. Interestingly, appearance of PNAd in mutant mice was restricted to lymphoid tissues.

Staining with anti-B220 (to identify B cells) and anti-Thy-1 (for identification of T cells) revealed that in Nkx2-3 deficient mice mutant HEVs have a similar distribution within PPs compared to MAdCAM-1^{+} HEVs in wild type samples.

Luminal expression of PNAd was detected by intravenous injection of anti-PNAd and anti-MAdCAM-1 mAbs. Quantifying the PNAd:MAdCAM-1 ratio we found that this was significantly higher than in normal PPs and close to both wild type and mutant pLNs. These data indicate that the occasional MAdCAM-1 expression noticed in mutant Peyer's patches is most likely restricted to the abluminal aspect of endothelial cells and does not participate in lymphocyte recirculation.

IV.2.3. Normal chemokine profile in PPs of Nkx2-3$^{-/-}$ mice

Various homeostatic chemokines are also needed for lymphocytes to recirculate to the different lymphoid compartments within PPs. CCL21, responsible for recruiting T cells, showed a similar pattern in Nkx2-3$^{-/-}$ mice compared to wild-type mice while CXCL13, expressed mainly in follicles was slightly reduced in Nkx2-3$^{-/-}$ Peyer's patches.

IV.2.4. Cell composition of mutant PPs resembles peripheral lymph node-like composition

Using flow cytometry we analyzed the cellular content of mutant PPs. We found a significantly lower rate of B cells and somewhat higher number of T cells in Nkx2-3$^{-/-}$ PPs, numbers resembling those seen in pLNs. No differences in memory and naïve T cell numbers or follicular helper T cells were seen. Interestingly, in mutant PPs we observed a significant increase of regulatory T cells. Expression of L-selectin on mutant PP lymphocytes was increased, in line with proposed PNAd-mediated homing.

IV.2.5. Appearance of PNAd in mutant Peyer's patches follows the kinetics observed in wild type pLNs

Wild type neonatal mice first display MAdCAM on pLN HEVs, and this expression is gradually replaced by PNAd during the first postnatal month. Also, endothelial MAdCAM-1 expression in mLNs of newborn Nkx2-3$^{-/-}$ mice has been reported. We examined whether the disappearance of MAdCAM-1 and the appearance of PNAd follows a similar timeline in PPs of mutant mice. PP sections of Nkx2-3$^{-/-}$ and wild type newborn (P0.5), 1 week old, and 2 week old mice were analyzed with immunofluorescence. At P0.5 endothelial MAdCAM-1 was observed in both wild type and mutant mice in lymphoid tissues and lamina propria (LP) vessels, while PNAd was absent. In 1 week old mutant mice PNAd appears and is largely co-expressed with MAdCAM-1 in PPs, but not the non-lymphoid vessels of the LP. By the second week MAdCAM-1 expression in mutant mice was diminished and largely replaced by PNAd in PPs and mLNs. PNAd did not appear in the non-lymphoid vessels of the mucosa. In wild type mice MAdCAM-1 remained the dominant addressin.

IV.2.6. Appearance of PNAd is dependent on LTβR signaling but does not require mature B or T cells

Appearance and maintenance of PNAd^{+} HEVs in wild type pLNs is dependent on signaling through the LTβR pathway. In mutant mice, PNAd only replaces MAdCAM-1 in the lymphoid areas of the intestine. We examined the requirements of LTβR signaling and lymphocyte dependence of PNAd^{+} HEVs in mutant PPs. Nkx2-3$^{-/-}$ mice were crossed with RAG-1$^{-/-}$ mice to create double KO mice that lack Nkx2-3 and also have no mature B- and T-cells. In the intestines of Rag1$^{-/-}$ x Nkx2-3$^{-/-}$ double KO mice we found small primordial PPs consisting of clustered CD45^{+} cells and possessed minimal endothelial MAdCAM-1. Interestingly, in these mice PNAd was present on HEVs, although to a lesser extent compared to Nkx2-3$^{-/-}$ HEVs. Next, Nkx2-3$^{-/-}$ mice were treated with LTβR-Ig fusion protein, a decoy receptor that interferes with the activation of LTβR, at P1, P3, and P5. Mice were sacrificed at P14. As control, littermates were injected with normal human IgG. In mice treated with LTβR-Ig fusion protein both PNAd and MAdCAM-1 were absent from the small identifiable CD45^{+} clusters, indicating that LTβR signaling is needed for PNAd to appear in Nkx2-3$^{-/-}$ PP. This signal can be delivered by non-B non-T cells, however, mature B and T cells contribute to PNAd expression as their absence leads to weakened PNAd presence.

IV.2.7. Lymphocytes bind to PNAd^{+} high endothelial venules in Nkx2-3$^{-/-}$ Peyer's patches

To investigate the functional consequences of modified HEVs we performed cell transfer experiments. Donor lymphocytes were injected intravenously into wild type and mutant mice. Tissues were analyzed with immunofluorescence and flow cytometry at 30, 60, and 120 minutes following cell transfer. 1 hour after injection in BALB/c mice donor lymphocytes adhered to the MAdCAM-1^{+} HEVs, while in Nkx2-3$^{-/-}$ PPs transferred cells were observed bound to PNAd^{+} vessels. Cell count analysis revealed that in mutant mice a significantly higher number of donor lymphocytes were still associated to HEVs compared to wild type, indicative of a slower extravasation rate in mutant PPs.

Flow cytometry showed a significant dominance of T cells among transferred cells in both WT and mutant PPs. In mutant mice we found a significantly lower number of B cells. Interestingly, in both BALB/c and Nkx2-3$^{-/-}$ mice the ratio of transferred B cells was lower than the number of recipient B cells. Helper T cells gave the majority of homed cells in both genotypes while the number of cytotoxic T cells was significantly higher in Nkx2-3$^{-/-}$ PPs.

IV.2.8. Lymphocytes utilize PNAd to home to mutant Peyer's patches

Next, we investigated how interfering with PNAd and/or MAdCAM-1 addressins influences lymphocyte recirculation in Nkx2-3$^{-/-}$ mice. We injected anti-addressin antibodies either alone or in combination, followed 30 minutes later by the injection of allogenic lymphocytes. Normal rat IgG was used as control and relative inhibition was calculated by comparing the rate of homing to the various lymphoid organs in the two strains to homed cells in control-treated mice 1 hour after injection.

Lymphocyte homing to wild type spleen was not affected by either of the applied antibodies. Administration of MECA-79 against PNAd significantly blocked cell entry to mutant spleens, confirming our previous finding that cells home to Nkx2-3$^{-/-}$ spleens via ectopic PNAd^{+} HEVs. Blocking PNAd inhibited cell homing to both wild type and mutant pLNs, although the level of inhibition was smaller in mutant tissues. Anti-MAdCAM-1 treatment had no effect in pLNs. In BALB/c mice, lymphocyte recirculation to mLNs was significantly reduced by both antibodies while in mutant mLNs only anti-PNAd inhibited homing, consistent with the absence of endothelial MAdCAM-1 in Nkx2-3$^{-/-}$ mLNs.

Anti-MAdCAM-1 treatment significantly reduced homing of cells to wild type PPs, whereas blocking PNAd had no effect. In contrast, in Nkx2-3$^{-/-}$ PPs administration of anti-MAdCAM-1 mAb had no effect on lymphocyte homing while application of anti-PNAd mAb resulted in a large decrease in the number of donor cells. However, the rate of inhibition by anti-PNAd antibodies reached neither the effect of anti-MAdCAM-1 in wild type PPs nor of anti-PNAd treatment seen in wild type pLNs. Combining anti-PNAd treatment with anti-MAdCAM-1 did not further increase inhibition.

V. Discussion

In the absence of the Nkx2-3 homeodomain transcription factor both the blood and lymphatic vasculature of various lympohoid organs was affected, leading to different morphological and functional consequences. In the spleen, besides the formation of ectopic PNAd^{+} HEVs, we observed the presence of LYVE-1^{+} endothelial sacs localized to the peripheral regions of mutant spleens and containing mainly T cells. Direct connection to the systemic circulation was absent as transferred cells entered the sacs at a low rate.

Compared to LNs, significantly less is known about the vascular development of the spleen. In Nkx2-3$^{-/-}$ mice the development of red pulp vasculature is blocked, together with the subsequent formation of the marginal sinus [Balogh *et al* 2007]. Interestingly, Nkx2-3 is active only in the embryonic period and its expression in the spleen declines before birth [Wang *et al* 2000], indicating that its absence most probably manifests in the embryonic period. *In silico* analyses have revealed possible binding sites for Nkx2-3 within the promoter region of LYVE-1 gene, leading to the idea of Nkx2-3 being its negative regulator. The phenotypic maturation of LEC-committed endothelial cells is influenced by Prox1 fate-determining transcription factor and is characterized by the appearance of LYVE-1 and podoplanin. Real-time PCR results showed that in contrast to LYVE-1 expression, Prox1 was lower in Nkx2-3$^{-/-}$ spleens. We hypothesize that the postnatal persistence of LYVE-1^{+} vessels is not due to differentiation and commitment of LECs, but rather they are remnants of embryonic endothelial cells [Kaipainen *et al* 1995]. This issue can conclusively be defined by using Cre-Lox based fate-mapping approach with Cre recombinase driven by Prox1 promoter [Srinivasan *et al* 2007].

We also found that in addition to these vascular alterations, the follicular accumulation of the macrophage scavenger receptor MARCO along a follicular conduit system we observed in wild type spleens was absent from these mice. FDC-associated granular MARCO was distinct from the ER-TR7^{+} conduit system created by FRCs of the T cell-zone and was only present in the follicles. We presume that this might be due to the MZ macrophage deficiency noticed in Nkx2-3$^{-/-}$ mice. Follicular MARCO was also absent in RAG1$^{-/-}$ mice which lack mature FDCs and in LTβR$^{-/-}$ mice with a complex marginal zone and follicular abnormality. Together these results indicate that the follicular deposition of MARCO requires the presence of mature FDCs in the follicles and also MARCO^{+} marginal zone macrophages. Based on these observations we propose that FDCs passively acquire MARCO from the MARCO-expressing marginal zone macrophages. Once expressed on FDCs, MARCO may support B cell responses via several molecular interactions [El Shikh *et al* 2007]. Thus, MARCO most likely plays an important role in the connection of the follicles with the MZ and enhances the transport of various antigens. In Nkx2-3$^{-/-}$ mice the absence of this transport system can be at least in part responsible for the diminished immune response [Tarlinton *et al* 2003].

In the intestine of Nkx2-3$^{-/-}$ mice endothelial MAdCAM-1 expression was absent, as shown previously. In lymphoid tissues, but not in the lamia propria vessels, this led to an increase in the mRNA of backbone proteins and modifying enzymes involved in creating the PNAd epitope. This, together with the HEV phenotype observed in Nkx2-3$^{-/-}$ x Rag1$^{-/-}$ double KO mice and in Nkx2-3$^{-/-}$ intestines following intraperitoneal injection of LTβR-Ig fusion protein, indicates the importance of LTβR in HEV development and

maintenance. In Nkx2-3$^{-/-}$ x Rag1$^{-/-}$ double KO mice, which lack mature B and T cells, possible sources for LT signals are CD11c^{+} dendritic cells that have been recently shown to influence HEV development and maintenance [Moussion and Girard 2011] and also RORγt^{+} innate lymphoid cells, which have been reported to express LT [Tumanov *et al* 2011].

The neonatal presence of endothelial MAdCAM-1 and then the switch of MAdCAM-1 to PNAd in HEVs of Nkx2-3$^{-/-}$ PPs resemble the physiological switch observed in pLNs of wild type mice during the first postnatal month [Mebius *et al* 1996]. Intriguingly, at this age Nkx2-3 is not expressed in these tissues. Thus it is probable that Nkx2-3 acts as a repressor of the MAdCAM-1 to PNAd switch. In line with this hypothesis, Nkx2-3 mRNA is highly present in PP and mLN endothelial cells, but only minimally expressed in wild type adult pLN HEVs. It has been recently shown that in PPs of wild type mice both HEV and non-HEV capillary cells express Nkx2-3 mRNA, while MAdCAM-1 is only expressed on HEVs [Lee *et al* 2014]. This, together with the presence of FDC-associated MAdCAM-1 in follicles, also emphasizes the differential effect of Nkx2-3 in different cell lineages. In the light of these recent findings and the presence of MAdCAM-1^{+}/PNAd^{+} vessels in early postnatal Nkx2-3$^{-/-}$ PPs, it is more probable that the PNAd^{+} HEVs in adult PPs develop from MAdCAM-1^{+} segments, rather than MAdCAM-1^{-} capillary cells.

Treatment of Nkx2-3$^{-/-}$ mice with anti-PNAd mAbs did not inhibit homing of lymphocytes to PPs to the same degree as either in wild type or mutant pLNs, and also did not reach the efficiency of anti-MAdCAM-1 antibodies in wild type PPs. This suggests that the loss of endothelial MAdCAM-1 in the absence of Nkx2-3 is not compensated by the increase of PNAd alone. Both ICAM-1 and VCAM-1 seem unlikely candidates, as we failed to detect an increase in the expression of these addressins.

In human IBD patients increased expression of MAdCAM-1 has been reported [Arihiro *et al* 2002]. Data from B cell lines indicate an upregulated Nkx2-3 activity in IBD patients, which could explain the increased MAdCAM-1 in these disorders. However, our results suggest that the previously proposed direct inductive role of Nkx2-3 on MAdCAM-1 is probably more complex. Also, Crohn's disease and ulcerative colitis are both multifactorial diseases with complex pathologies where increased presence of MAdCAM-1 may also be the consequence, and not the cause, of inflammation.

Together, these results indicate that Nkx2-3 is an important player in both blood and lymphatic vascular differentiation. The similar changes observed in both the spleen and PPs suggests a systemic role for Nkx2-3 in the inhibition of pLN-like vasculature. Importantly, in pLNs where Nkx2-3 is not present, PNAd^{+} HEVs develop, while in tissues where Nkx2-3 is normally expressed in wild type mice endothelial cells do not express PNAd. Both the absence of endothelial MAdCAM-1 and the ectopic presence of PNAd^{+} HEVs may have clinical relevance and should be further investigated. Additional work with this mouse model and/or mice that have an increased Nkx2-3 activity can also help in the better understanding of HEV development and plasticity. The role Nkx2-3 plays in the development and maintenance of secondary and tertiary lymphoid organs through modulating HEV formation emphasizes its central role in developing a proper immune response, suggesting future therapeutic possibilities.

VI. Short summary of results

1. Appearance of LYVE-1^{+} sacs in Nkx2-3$^{-/-}$ spleens

- Nkx2-3$^{-/-}$ spleens contain LYVE-1^{+} sac-like structures that are localized mainly to the peripheral regions of mutant spleens and contain primarily T cells.

- The lymphoid sacs lack direct connection to the blood circulatory system, as transferred cells rarely enter LYVE-1-expressing sacs.

- LYVE-1 is already present in Nkx2-3$^{-/-}$ embryos. Sac-like structures are first seen in neonates, and the peripheral localization develops by the second postnatal week.

- LYVE-1^{+} structures are present also in Nkx2-3 x LTβR double KO mice, and the formation of LYVE-1^{+} sacs is independent from LTβR.

2. Absence of follicular MARCO expression in Nkx2-3$^{-/-}$ spleens

- MARCO, expressed by a subgroup of marginal zone macrophages in the spleen, also displays a fibrillar expression pattern in splenic follicles within FDC-associated conduits. In Nkx2-3$^{-/-}$ mice follicular MARCO expression is absent.

3. PPs in Nkx2-3 deficient mice utilize PNAd as major homing addressin

- mRNAs for several PNAd backbone proteins and modifying enzymes are upregulated in PPs of Nkx2-3$^{-/-}$ mice. This manifests as luminal PNAd expression on PP HEVs. The chemokine expression profile is normal compared to wild type mice.

- Mutant PPs have higher T cell numbers, including an increased frequency of regulatory T cells.

- In PPs of neonatal Nkx2-3$^{-/-}$ mice MAdCAM-1 is present and is gradually replaced by PNAd by the second postnatal week.

- Appearance of PNAd depends on LTβR signaling but does not require mature T or B cells.

- Cell transfer experiments show that in Nkx2-3$^{-/-}$ PPs the majority of homed cells are T cells. Treatment with anti-PNAd mAbs prior to cell transfer efficiently blocks lymphocyte recirculation to mutant PPs.

VII. List of publications

Publications the thesis is based on

Kellermayer Z, Lábadi A, Czömpöly T, Arnold HH, Balogh P. 2011. Absence of Nkx2-3 homeodomain transcription factor induced the formation of LYVE-1-positive endothelial cysts without lymphatic commitment in the spleen. *J Histochem Cytochem* 59:690-700.
IF: 2.725

Czömpöly T**,** Lábadi A, **Kellermayer Z**, Olasz K, Arnold HH, Balogh P. 2011. Transcription factor Nkx2.3 controls the vascular identity and lymphocyte homing in the spleen. *J Immunol.* 186:6981–9.
IF: 5.788 (from this 2.894 is used for this dissertation)

Kellermayer Z, Fisi V, Mihalj M, Berta G, Kóbor J, Balogh P. 2014. Marginal zone macrophage receptor MARCO is trapped in conduits formed by follicular dendritic cells in the spleen. *J Histochem Cytochem* 62:436-449.
*IF: 2.403**

Kellermayer Z, Mihalj M, Lábadi Á, Czömpöly T, Lee M, O'Hara E, Butcher EC, Berta G, Balogh A, Arnold HH, Balogh P. 2014. Absence of Nkx2-3 homeodomain transcription factor reprograms the endothelial addressin preference for lymphocyte homing in Peyer's patches. *J Immunol.* 193:5284-93
*IF: 5.362**

Publications not directly related to the thesis

Mihalj M, **Kellermayer Z**, Balogh P. 2013. Follicles in gut-associated lymphoid tissues create preferential survival niches for follicular Th cells escaping Thy-1-specific depletion in mice. *Int Immunol*. 25:423-35.
IF: 3.181

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