#108429	**Role of Nkx2-3 transcription factor in the regulation of innate lymphocyte distribution in visceral lymphoid organs and the onset of inflammatory bowel diseases**
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1. Introduction

In the first descriptions for determining its putative roles, the lack of Nkx2-3 in mice resulted in the lack MAdCAM-1 addressin from the Peyer's patches' high endothelial venules (HEVs) and led to the aberrant structure of spleen, with some resemblance to the defects caused by the inactivation of lymphotoxin beta receptor (LTβR) [1,2]. These alterations included the lack or organized splenic marginal zone (MZ) and proper segregation of follicles. Our subsequent work has established that the vascular alterations indicate a complex re-programming towards a lymph-node like patterning, including the appearance of peripheral lymph node addressin (PNAd) characteristic for lymph node HEVs as a result of altered gene expression, particularly those of PNAd core proteins and glycosylation enzymes. Moreover, Nkx2-3$^{-/-}$ spleen contains abortive sacs/cysts formed by lymphatic endothelial-like cells displaying LYVE-1 antigen, [3-5]. As Nkx2-3 appeared to exert its morphogenic effect in a tissue-specific manner, we sought to extend the alterations its absence may cause in the spleen and the mucosal lymphoid tissues. Furthermore, as in several GWAS results have indicated, the colonic inflammations are also linked to altered Nkx2-3 expression [6], we aimed to investigate the role of Nkx2-3 in the formation of tertiary lymphoid tissues of the mucosa influenced by innate lymphoid cell type 3 (ILC3), and its effect on the inducibility of oral tolerance.

2. Results

2.1. Role of Nkx2-3 in the follicular transport of MZ-derived scavenger receptor MARCO

To expand the scope of cellular alterations of splenic MZ elicited by the lack of Nkx2-3, several rat monoclonal antibodies (mAbs) against MZ-associated markers were tested. We found that one mAb (clone #IBL-12) against scavenger receptor MARCO showed a strikingly distinct pattern compared to wild-type controls. While the absence of highly IBL-12 positive MZ macrophages was expectable, we also observed the lack of fibrillary MARCO in follicular location. Subsequent studies established that in normal mice the follicular MARCO was deposited onto conduit-like structures associated with follicular dendritic cells (FDCs), while in Nkx2-3-deficient mice the producer MZ macrophages were absent, thus FDCs could not grab soluble MARCO (**Fig. 1**). Our further studies in wild-type and several mutant mice prompted the hypothesis that the MZ-follicular MARCO transport is a tissue-specific phenomenon, and requires the follicular accumulation of B cells under the guidance of CXCL13/CXCR5 chemokine interaction as a crucial inducer for FDC maturation, but it is independent from the follicular shuffling of MZ B cells promoted by S1PR1 [7].

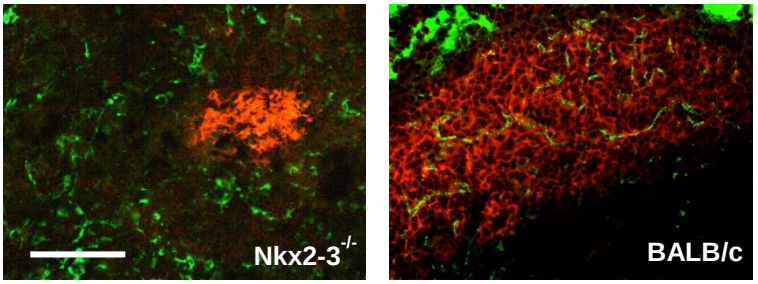

Fig. 1 Effect of Nkx2-3 deficiency on the distribution of MARCO (green - #IBL-12) associated with FDC (red: CD21/35) Scale bar: 100μm

2.2. Effect of Nkx2-3 on the HEV endothelial addressin preference in Peyer's patches

As in the spleen the absence of Nkx2-3 causes the reprogramming towards peripheral lymph node-like vasculature, next we investigated whether similar addressin switch occurs in Peyer's patches. Using qPCR we found enhanced expression of mRNAs for several core proteins, although not as dramatic as in mutant spleens. In addition, PNAd could also be detected by multicolor immunofluorescence (**Fig. 2**). Furthermore, we also found CCL21 and CXCL13 chemokine expression in these PNAd-positive HEVs. By adoptively transferring CFSE-labeled lymphocytes we could demonstrate that these PNAd-positive HEVs in Peyer's patches are fully functional lymphocyte exit ports, where the adhesion and extravasation could partially be blocked by MECA-79 anti-PNAd mAb, whereas anti-MAdCAM-1 mAb MECA-367 had no such effect, unlike in wild-type Peyer's patches.

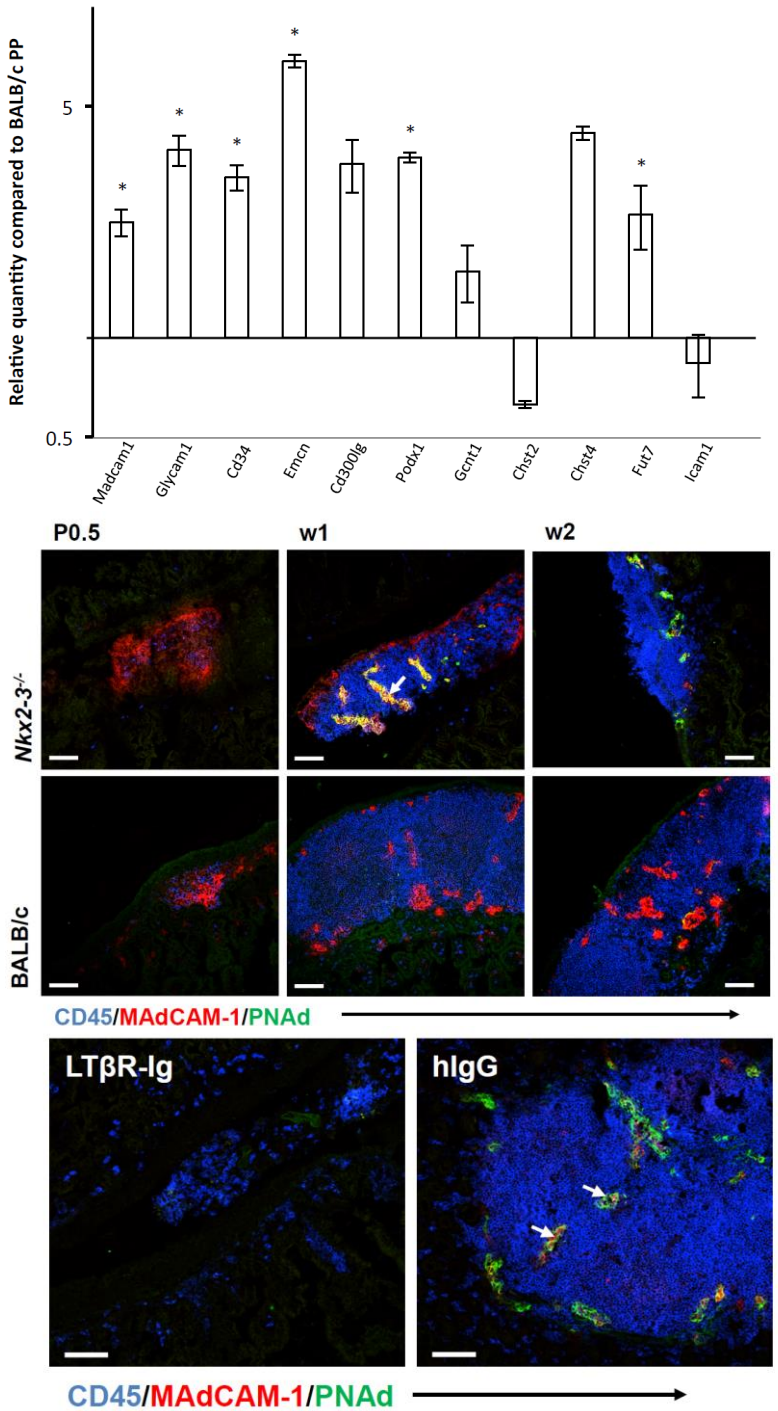

Fig. 2 Altered mRNA expression for PNAd core proteins and glycosylation enzymes in Nkx2-3$^{-/-}$ Peyer's patches measured by qPCR (top). Middle: Replacement of MAdCAM-1 with PNAd in the postnatal maturation of Peyer's patches induced by the absence of Nkx2-3. Markers are as indicated. Bottom: Expression of PNAd in Nkx2-3-deficient Peyer's patches is sensitive to treatment with LTβR fusion protein (left) compared to human IgG-treated control (right; scale bar: 100μm)

To determine whether the development of PNAd-positive HEVs requires mature T and B cells we bred (Nkx2-3xRag2) double mutants, and found that although with significantly diminished size, immature Peyer's patches containing PNAd-positive HEVs are detectable. On the other hand, postnatal treatment of Nkx2-3-deficient mice with soluble lymphotoxin beta-receptor decoy receptor-Ig fusion protein (LTβR-Ig) we found that the acquisition of PNAd^{+} features requires LTβR-mediated signaling. We concluded that the absence of Nkx2-3 reprograms the endothelial addressin preference towards

dominant display of PNAd, which requires LTβR activity stimulated by non-T/B cells, but the enhancement of expression requires mature lymphocytes [8].

2.3. Expression of Nkx2-3 in human spleen and colon – similarities and differences with murine tissues

To correlate our findings in mouse spleen and gut on the effect of Nkx2-3 deficiency with human tissues, we used dual immunohistochemistry of spleen and colon samples from human biopsies, and correlated these findings with the tissue alterations observed in Nkx2-3$^{-/-}$ mice (spleen) or using an Nkx2-3^{LacZ} reporter mouse (colon; [2]).

In the human spleen we found that the expression of Nkx2-3 protein was restricted to the nuclei of cells with a paired cord-like arrangement suggesting vascular reactivity. To identify these vessel-like formations, we used reference markers against endothelial cells. We found that the red pulp vessels with nuclear Nkx2-3 protein expression had aSMA^{-}/CD34$^{+/-}$/vWF^{+}/CD31^{+} phenotype (**Fig. 3**). We suggest these vessels to be the human homologues of those segments in the murine spleen that display IBL-9/2 marker, and are substantially reduced in Nkx2-3 deficient mice [3, 9]. In addition, the ectopic PNAd-positive vessels formed in the absence of Nkx2-3 in mice also expressed DNA-binding protein *Dach1* (Dachshund), a chromatin-associated DNA binding protein, present in MAdCAM-1 positive immature endothelial cells in normal peripheral lymph nodes, again indicating a shift in vascular patterning elicited by loss of Nkx2-3 [10].

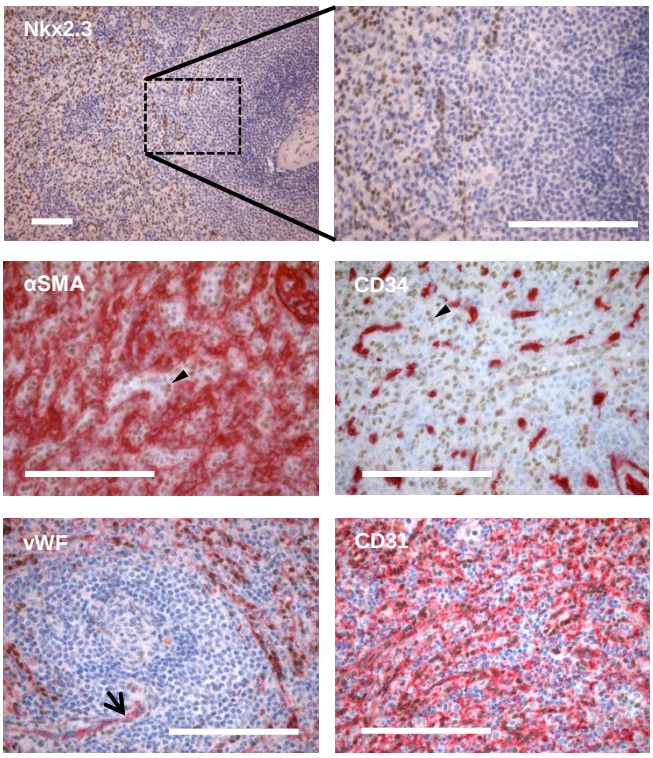

Fig. 3 Phenotype of human splenic Nkx2-3-positive (brown, nuclear, arrowheads) vascular segments using reference markers as indicated (red). The field in the dashed rectangle corresponds to the inset with higher power magnification. Arrow points to a terminal arteriole in the white pulp. Scale bar: 100 μm

In contrast to the spleen with well-defined endothelial markers, the human colonic Nkx2-3-positive cells could not be assigned to any lineage. We found that although some Nkx2-3-positive cells had fibroblastic (αSMA, MSA) or endothelial (CD34, vWF) marker expression, the majority of Nkx2-3-positive cells could not be defined using these markers [11]. On the other hand, in mice we found co-expression of VAP-1 and PDGF-R1 in the Nkx2-3^{LacZ} reporter using X-gal staining, whereas the endothelial marker IBL-20 or epithelium-specific EpCAM-1 did not label these cells [Kellermayer et al., under revision at J Immunol].

2.4. Impaired maturation of solitary intestinal lymphoid tissues (SILT) in Nkx2-3 deficiency

As the formation of Peyer's patches as programmed secondary lymphoid tissue of the mucosa is affected by the absence of Nkx2-3, next we investigated whether SILT as tertiary formation is

affected, by analyzing postnatal SILT development, and as pathological model, using DSS-induced colitis, associated with lymphoid neogenesis. To determine the impact of MAdCAM-1 deficiency as a phenotypic consequence of Nkx2-3 inactivation to other non-MAdCAM-1-dependent effects, we also used MAdCAM-1-deficient mice with normal Nkx2-3 production. We analyzed the composition of SILT spectrum, the distribution of colonic ILC3/LTi (lymphoid tissue inducer) cells responsible for lymphoid neogenesis, the course of DSS-colitis, lymphocyte migratory characteristics monitored by Kikume photoconversion and qPCR analysis of immunomodulatory cytokines (including IL-22, IFNγ, TGFβ) and their antibody-mediated modulation in vivo. We also investigated the epithelial regeneration and potential IL-22 downstream mediators Reg3β and Reg3γ.

We found that the postnatal SILT formation was blocked in Nkx2-3 deficient mice, although not as severely as in mice lacking MAdCAM-1. As a result, more SILT components remained in the cryptopatch stage ($CD45^+/Thy\text{-}1^+/B220^-$) or immature isolated lymphoid follicle ($CD45^+/Thy\text{-}1^-/B220^+$ lacking germinal center) than in wild-type control. This blocked maturation of follicles was also verified using B-cell linked luciferase bioluminescence with Luc gene driven by CD19 promoter on $Nkx2\text{-}3^{-/-}$ background, revealing smaller and fewer B-cell clusters. In a reverse manner, however, in Nkx2-3 deficient colons significantly more ILC3/LTi cells (defined as $CD45^+/CD3^-/CD19^-/CD90^+/ROR\gamma t^+$) were found (**Fig. 4A**).

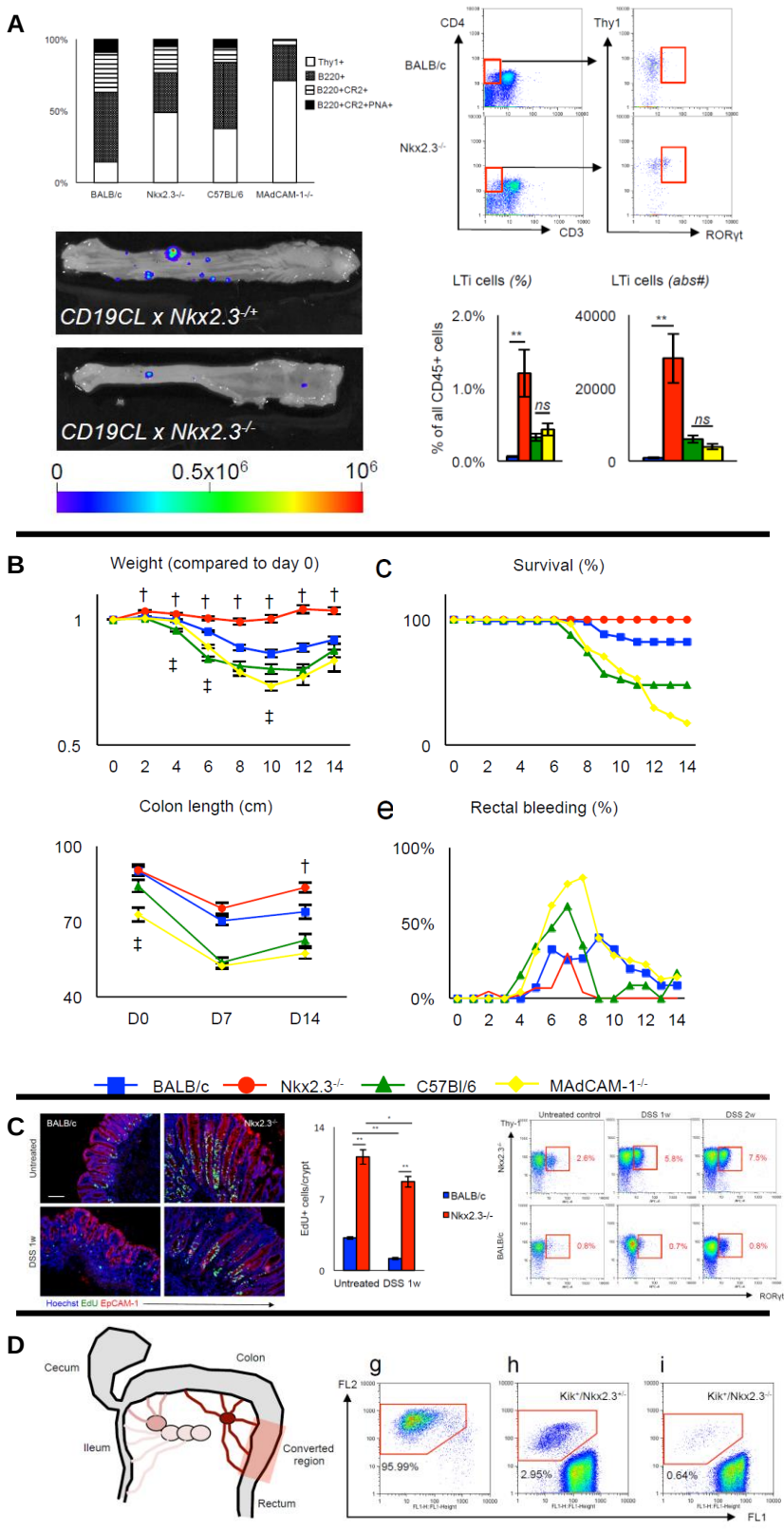

Fig. 4 Differential course of SILT formation, ILC3 distribution and onset of DSS-induced colitis in mice deficient for Nkx2-3. Details in the text.

Using DSS-induced colitis model we found that the Nkx2-3 deficient mice were protected from the disease, with significantly less severe physical and histological parameters associated with colitis (**Fig. 4B**). In addition, both untreated and DSS-treated Nkx2-3 deficient mice showed enhanced colonic epithelial cell proliferation by EdU assay, and upon DSS treatment, further increase of ILC3/LTi cells was observed (**Fig. 4C**). In

addition, Nkx2-3 deficiency also leads to reduced mucosal departure/egress of lymphocytes into the draining mesenteric lymph node, as evidenced by Kikume green→red photoconversion. In this experiment surgically exposed segments of colon of Nkx2-3$^{-/-}$:KikG mice were illuminated with λ=405 nm LED, inducing green→red shift detectable by flow cytometer in the FL1:FL2 channels (**Fig. 4D**).

In untreated Nkx2-3 deficient mice we found an increased IFNγ mRNA level which further increased during DSS treatment, whereas IL-17a mRNA had a higher starting level, but showed only a modest increase, while for IL-22 mRNA we found a lower starting level, but higher increase upon treatment, compared to wild-type controls. Administration of IL-22-blocking mAb (provided by Genentech Inc., USA) in DSS-treated Nkx2-3 deficient mice did not augment the severity of inflammation. Furthermore, mice lacking MAdCAM-1 showed a significantly increased sensitivity towards DSS-induced colitis, leading to the conclusion that the blunted inflammatory response in Nkx2-3 deficient mice is independent from the increased production of IL-22, and it is also unrelated to the absence of MAdCAM-1 from the mucosal HEV endothelium [Kellermayer et al., under revision at J Immunol].

2.5. Role of Nkx2-3 in the postnatal distribution of intestinal ILC type 3 cells

As Nkx2-3 was found to alter the presence of colonic ILC3/LTi cells and composition of SILT spectrum, and also has important role in the Peyer's patches organogenesis in the small intestine, respectively, next we investigated whether the global distribution of ILC3/LTi cells in the gut during the early postnatal period is affected by Nkx2-3. Using multicolor flow cytometry, we found that ILC3 cells (gated as CD45$^+$/CD3$^-$/CD19$^-$/CD90$^+$/RORγt$^+$) show different postnatal distribution kinetics in Nkx2-3 deficient between the small intestine and the colon. Furthermore, this difference compared to wild-type mice is unrelated to the absence of MAdCAM-1 (**Fig. 5**) [Vojkovics et al., submitted to Front Immunol].

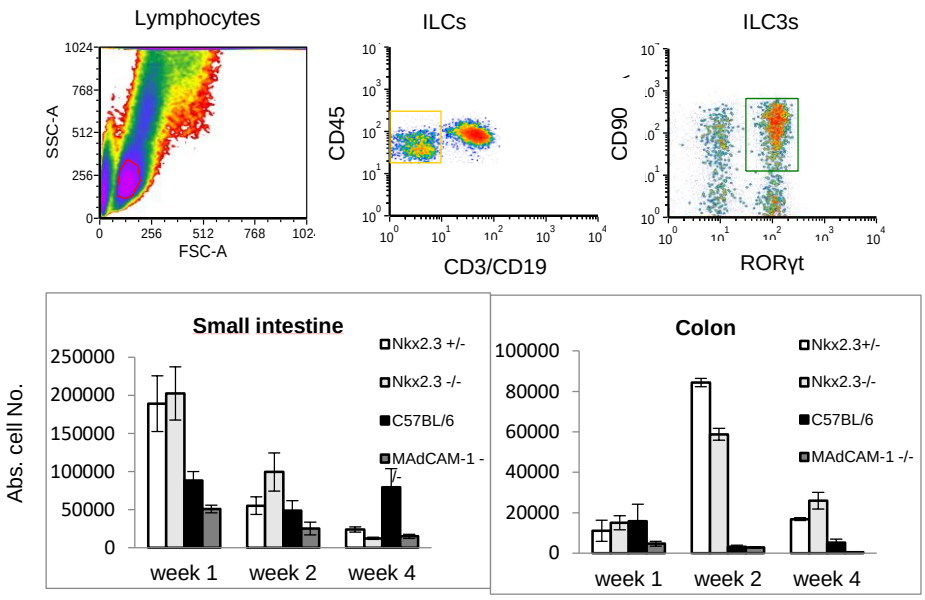

Fig. 5 Identification of intestinal ILC3/LTi cells and their postnatal distribution in small inestine and colon

2.6. Preserved oral tolerance in Nkx2-3 deficiency independent from MAdCAM-1 deficiency

An important aspect of the maintenance of mucosal integrity is the ability to establish oral tolerance, therefore we also investigated whether the absence of Nkx2-3 affects the induction of oral tolerance. Oral tolerance was induced in young adult BALB/c, Nkx2-3$^{-/-}$, C57BL/6 and MAdCAM-1$^{-/-}$ mice by giving 5mg/ml ovalbumin (OVA) in drinking water for 7 days. Mice were then injected intraperitoneally with OVA:complete Freund's adjuvant on day 7 and OVA:incomplete Freund's

adjuvant on day 14. On day 21 Treg frequencies (defined as $CD3^+/CD4^+FoxP3^+$) were measured with flow cytometry from mesenteric lymph nodes and gut lamina propria samples, while serum anti-OVA IgG levels were measured with ELISA. To induce colitis mice received 2.5% DSS in drinking water for 7 days. Weights were measured daily. RNA was isolated from colonic samples and qPCR was performed to measure anti-inflammatory IL-10 and TGFβ mRNA levels at various time points.

We found that tolerization with OVA blocked anti-OVA antibody production in Nkx2-$3^{-/-}$ mice. In contrast, feeding MAdCAM-$1^{-/-}$ mice with OVA did not prevent anti-OVA antibody production. Colonic Tregs were significantly increased at day 7 in the absence of Nkx2-3 compared to BALB/c mice. mRNA for IL-10 was significantly higher at D0 and D7 in Nkx2-$3^{-/-}$ mice, while TGFβ was lower at D0 compared to BALB/c mice. We also found higher Treg numbers in MAdCAM-$1^{-/-}$ mice at D7; however, this was not coupled with an increase in mRNA for IL-10 or TGFβ [Kellermayer et al., under revision at J Immunol].

2.7. Ectopic expression of Nkx2-3: from mucosal lymphoid organogenesis to marginal zone lymphoma

Although Nkx2-3 expression has only been described in non-hematopoietic cells in every lymphoid tissue studied, an unexpected finding has led to the discovery of Nkx2-3 as a potential risk factor in certain types of non-Hodgkin B-cell lymphomas. Search for novel translocation patterns in human samples repeatedly revealed a novel t(10;14)(q24;q32) translocation involving IgH and Nkx2-3 genes, resulting in marginal zone B-cell lymphoma. Cloning this translocation variant and creating Tg mice with this mutation has also led to the development of low-grade marginal zone B-cell lymphoma in the mutants between the ages of 12-18 months. Interestingly, the development of lymphoma was preceded by a gradual dissolution of normal splenic architecture, particularly affecting the MZ and follicular stromal organization (**Fig. 6**).

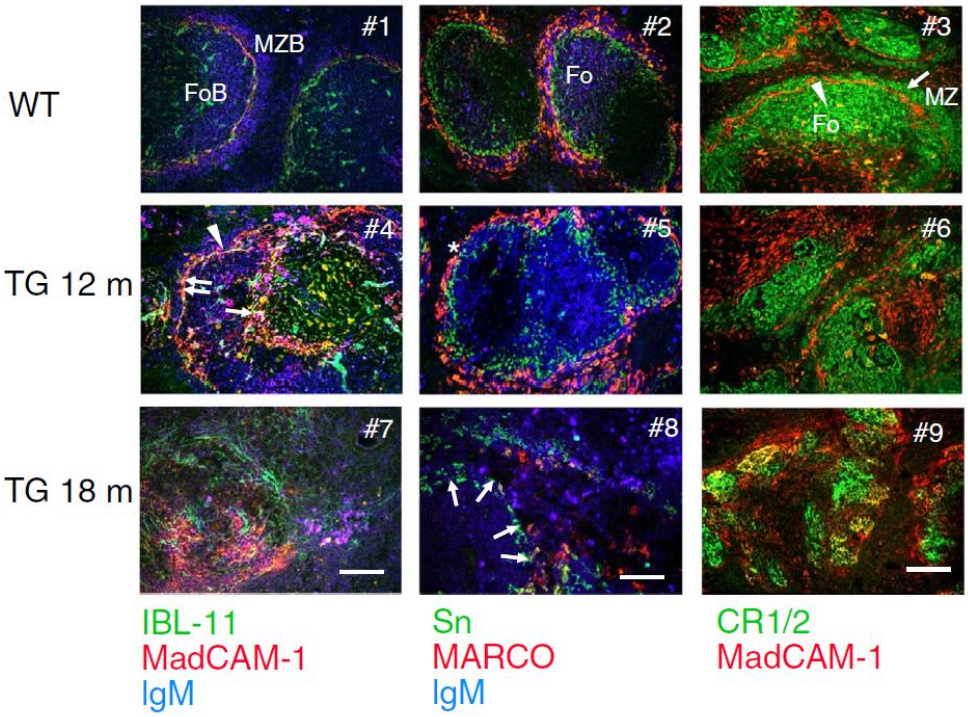

Fig. 6 Dissolution of splenic architecture preceding the appearance of MZB cell lymphoma

Subsequent studies have established that this condition results in increased B-cell adhesion accompanied to strengthened BcR stimulation and enhanced signaling involving NF-κB and PI3K-AKT pathways [12]. Thus this condition adds to the growing list of the involvement of Nkx family members in the emergence of different malignancies (reviewed in [11]).

2.8. Analysis of Nkx23-3-related mRNA profile in experimental colitis and correlation with the human

To determine how the absence of Nkx2-3 affects the global gene expression pattern in non-hematopoietic cells upon DSS treatment, we have established a mesenchymal stroma cell sorting protocol using colonic samples by removing CD45^{+}/EpCAM-1^{+} cells. The analysis of samples (approximately 14K genes) is currently underway, our preliminary findings have identified several genes with altered expression between Nkx2-3 deficient and wild-type mice following DSS-induced colitis (**Fig. 7**).

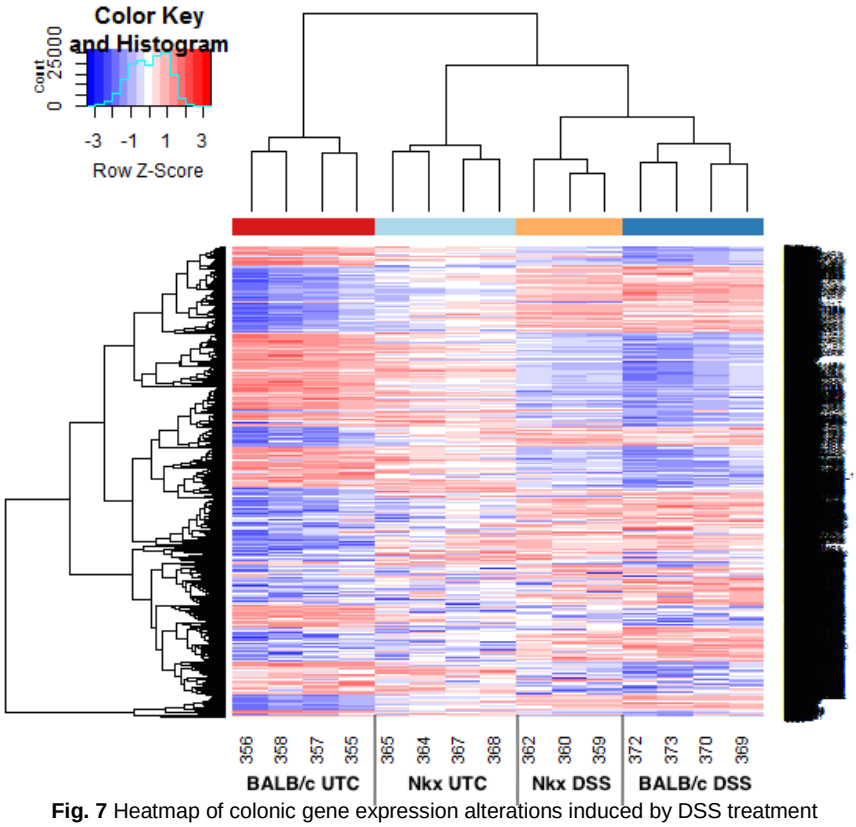

Fig. 7 Heatmap of colonic gene expression alterations induced by DSS treatment between wild-type and Nkx2-3 deficient mice (UTC: untreated control, DSS: colitis)

To search for human parallel gene expression alterations, we collected biopsies from a representative cohort of 10 adult inflammatory bowel disease patients (Crohn's disease a ulcerative colitis, from inflamed and non-inflamed segments in parallel), and analyzed using a rat model of colitis. It was found that several genes involved in the induction and/or maintenance of mesenchymal phenotype were upregulated (SNAI1, ZEB2, VIM, MMP9, and HIF1α) whereas the mRNA for epithelial marker E-cadherin (CDH1) was downregulated [13]. Currently we are using this pool of samples to correlate the Nkx2-3-related gene expression, with particular reference to the genetic signature of those intestinal Nkx2-3-positive pericryptal myofibroblastic stromal cells that may regulate the colorectal epithelial stem cell niche [14].

3. References

1. Pabst O, Zweigerdt R, Arnold HH. Targeted disruption of the homeobox transcription factor Nkx2-3 in mice results in postnatal lethality and abnormal development of small intestine and spleen. Development. 1999 May;126(10):2215-25.

2. Wang CC, Biben C, Robb L, Nassir F, Barnett L, Davidson NO, Koentgen F, Tarlinton D, Harvey RP. Homeodomain factor Nkx2-3 controls regional expression of leukocyte homing coreceptor MAdCAM-1 in specialized endothelial cells of the viscera. Dev Biol. 2000 Aug 15;224(2):152-67.

3. Balogh P, Balázs M, Czömpöly T, Weih DS, Arnold HH, Weih F. Distinct roles of lymphotoxin-beta signaling and the homeodomain transcription factor Nkx2.3 in the ontogeny of endothelial compartments in spleen. Cell Tissue Res. 2007 Jun;328(3):473-86.

4. Czömpöly T, Lábadi A, Kellermayer Z, Olasz K, Arnold HH, Balogh P. Transcription factor Nkx2-3 controls the vascular identity and lymphocyte homing in the spleen. J Immunol. 2011 Jun 15;186(12):6981-9.

5. Kellermayer Z, Lábadi A, Czömpöly T, Arnold HH, Balogh P. Absence of Nkx2-3 homeodomain transcription factor induces the formation of LYVE-1-positive endothelial cysts without lymphatic commitment in the spleen. J Histochem Cytochem. 2011 Jul;59(7):690-700.

6. Bonder MJ, Luijk R, Zhernakova DV, Moed M, Deelen P, Vermaat M, van Iterson M, van Dijk F, van Galen M, Bot J, Slieker RC, Jhamai PM, Verbiest M, Suchiman HE, Verkerk M, van der Breggen R, van Rooij J, Lakenberg N, Arindrarto W, Kielbasa SM, Jonkers I, van 't Hof P, Nooren I, Beekman M, Deelen J, van Heemst D, Zhernakova A, Tigchelaar EF, Swertz MA, Hofman A, Uitterlinden AG, Pool R, van Dongen J, Hottenga JJ, Stehouwer CD, van der Kallen CJ, Schalkwijk CG, van den Berg LH, van Zwet EW, Mei H, Li Y, Lemire M, Hudson TJ; BIOS Consortium, Slagboom PE, Wijmenga C, Veldink JH, van Greevenbroek MM, van Duijn CM, Boomsma DI, Isaacs A, Jansen R, van Meurs JB, 't Hoen PA, Franke L, Heijmans BT. Disease variants
alter transcription factor levels and methylation of their binding sites. Nat Genet. 2017 Jan;49(1):131-138.

7. Kellermayer Z, Fisi V, Mihalj M, Berta G, Kóbor J, Balogh P. Marginal Zone Macrophage Receptor MARCO Is Trapped in Conduits Formed by Follicular Dendritic Cells in the Spleen. J Histochem Cytochem. 2014 Jun;62(6):436-449.

8. Kellermayer Z, Mihalj M, Lábadi Á, Czömpöly T, Lee M, O'Hara E, Butcher EC, Berta G, Balogh A, Arnold HH, Balogh P. Absence of Nkx2-3 homeodomain transcription factor reprograms the endothelial addressin preference for lymphocyte homing in Peyer's patches. J Immunol. 2014 Nov 15;193(10):5284-93.

9. Balázs M, Horváth G, Grama L, Balogh P. Phenotypic identification and development of distinct microvascular compartments in the postnatal mouse spleen.Cell Immunol. 2001 Sep 15;212(2):126-37.

10. Kellermayer Z, Hayasaka H, Kajtár B, Simon D, Robles EF, Martinez-Climent JA, Balogh P. Divergence of Vascular Specification in Visceral Lymphoid Organs-Genetic Determinants and Differentiation Checkpoints. Int Rev Immunol. 2016 Nov;35(6):489-502.

11. Vojkovics D, Kellermayer Z, Kajtár B, Roncador G, Vincze Á, Balogh P. Nkx2-3 - A Slippery Slope From Development Through Inflammation Toward Hematopoietic Malignancies. Biomark Insights. 2018 Feb 6;13:1177271918757480.

12. Robles EF, Mena-Varas M, Barrio L, Merino-Cortes SV, Balogh P, Du MQ, Akasaka T, Parker A, Roa S, Panizo C, Martin-Guerrero I, Siebert R, Segura V, Agirre X, Macri-Pellizeri L, Aldaz B, Vilas-Zornoza A, Zhang S, Moody S, Calasanz MJ, Tousseyn T, Broccardo C, Brousset P, Campos-Sanchez E, Cobaleda C, Sanchez-Garcia I, Fernandez-Luna JL, Garcia-Muñoz R, Pena E, Bellosillo B, Salar A, Baptista MJ, Hernandez-Rivas JM, Gonzalez M, Terol MJ, Climent J, Ferrandez A, Sagaert X, Melnick AM, Prosper F, Oscier DG, Carrasco YR, Dyer MJ, Martinez-Climent JA. Homeobox NKX2-3 promotes marginal-zone lymphomagenesis by activating B-cell receptor signalling and shaping lymphocyte dynamics. Nat Commun. 2016 Jun 14;7:11889.

13. Boros É, Kellermayer Z, Balogh P, Strifler G, Vörös A, Sarlós P, Vincze Á, Varga C, Nagy I. Elevated Expression of AXL May Contribute to the Epithelial-to-Mesenchymal Transition in Inflammatory Bowel Disease Patients. Mediators Inflamm. 2018 Jul 22;2018:3241406.

14. Hsia LT, Ashley N, Ouaret D, Wang LM, Wilding J, Bodmer WF. Myofibroblasts are distinguished from activated skin fibroblasts by the expression of AOC3 and other associated markers. Proc Natl Acad Sci U S A. 2016 Apr 12;113(15):E2162-71.