# THE ROLE OF TRANSCRIPTION FACTOR NKX2-3 IN THE DISTRTIBUTION OF INNATE LYMPHOID CELLS OF THE GUT-ASSOCIATED LYMPHOID TISSUES AND THE DEVELOPMENT OF INFLAMMATORY BOWEL DISEASES

**Doctoral (PhD) thesis**

Dóra Vojkovics

Doctoral School for Theoretical Medicine
Mentor: Prof. Dr. Péter Balogh
Head of PhD program: Prof. Dr. Péter Németh
Doctoral School Leader: Prof. Dr. Júlia Szekeres-Barthó

University of Pécs
Clinical Centre
Department of Immunology and Biotechnology


Pécs, 2019

# 1. Introduction

## 1.1 Inflammatory bowel diseases

Inflammatory bowel disease (IBD) is one of the most common chronic inflammatory diseases of the gut. We can distinguish two main types of IBD: Crohn's disease (CD) and ulcerative colitis (UC). Several people are affected worldwide and IBD develops mostly in young adults. (*Loftus et al, 2002*) There are several genetic and environmental factors that contribute to the development of these diseases. (*Fisher et al, 2008; Cho et al, 2007*)

In recent years several IBD associated loci were identified with the help of GWAS, meta-analysis and fine-mapping procedures. (*Verstockt et al, 2018; Liu et al, 2015; Zhang et al, 2018*) Despite hundreds of identified genetic alterations –mostly single nucleotide polymorphisms (SNP)– only a few of them proved to have real biological role in the development of IBD. One of those alterations is the SNP of the coding region of the homeodomain containing transcription factor Nkx2-3, that can be associated with the incidence of CD and UC. (*Parkes et al, 2007; Fisher et al, 2008*)

The combination of genetic and environmental factors can alter the composition of the microbiome, decrease epithelial barrier function and induce inflammation due to the pathological activation of the immune system. (*Loftus et al, 2002*)

The individual-specific intestinal microbiome has an important role in ensuring proper nutrient uptake in the intestinal tract and in the development and regulation of the local immune system. (*Bäckhed et al, 2005*) Perturbation of the delicate balance between the host and the microbiome can lead to the development of severe inflammatory processes. (*Elson et al, 2005*)

Epithelial cells ensure limited access of the mucosal immune system to bacterial and other antigens of the digestive tract. In IBD this barrier function is damaged due to the increased permeability of the intestinal wall that can lead to the development of inflammation. (*Wang et al, 2006*)

Immune cells of the GALT can contribute to the maintenance of immunological homeostasis in normal conditions; however, in IBD they can induce inflammation due to the higher influx of bacterial and other antigens through the epithelial barrier with increased permeability. Mucosal accumulation and abnormal activation of these cells can lead to an enhanced production of inflammatory factors.

The regulated migration of immune cells through the vascular-endothelial network is controlled by the α4β7-MAdCAM-1 interaction. In chronic inflammation the increased expression of adhesion molecules, inflammatory chemokines and other soluble factors enhance the migration of lymphoid cells into the intestinal tract. (*Hatoum et al, 2006*)

Transcription factor Nkx2-3 regulates the expression of MAdCAM-1 addressin that ensures gut homing of lymphoid cells through the interaction with leukocyte receptor α4β7. (*Iwata et al, 2004*) In the absence of these endothelial factors the perturbed gut homing of lymphocytes influences the local immune response and the course of inflammatory processes. (*Pabst et al, 2000*) Since Nkx2-3 can regulate the addressin-pattern of the intestinal mucosal vasculature, this transcription factor can be associated with the development of IBD.

## 1.2 Developmental characteristics of gut associated lymphoid tissues

Gut-associated lymphoid tissues (GALT) play a crucial role in the maintenance of local immunological homeostasis and in the regulation of systemic immunological and inflammatory processes. Their structural development and maintenance are regulated through the combined effect of several components. These secondary lymphoid organs serve as a meeting point for immune cells and antigens, therefore they are necessary for the induction of adequate immune responses.

Mesenteric lymph nodes (mLN), Peyer's patches (PP) colonic patches (ColP) and SILTs (solitary intestinal lymphoid tissues) belong to the organized MALT, while the diffuse MALT is composed of the of lamina propria lymphocytes (LPL), intraepithelial lymphocytes (IEL) and other local immune-competent cells.
Programmed development of mLNs, PPs and ColPs starts at the embryonic age, whereas SILT formation is initiated only postnatally upon induction. For their development the interaction of mesenchymal stromal lymphoid tissue organizer (LTo) and hematopoietic lymphoid tissue inducer (LTi) cells is essential. LTi cells express LTαβ2 that functions as a ligand for LTβR. Activation of the NF-κB pathways through the LT-signal transduction is one of the most important steps of lymph node and PP development. Activation of the NF-κB pathway induces the expression of adhesion molecules on the LTo cells and also helps the expression of several cytokines. These factors are inevitable in the attraction of LTi, then mature T and B lymphocytes, thus, they are essential for the development and maintenance of the structural organization (T/B cell zones) of lymph nodes. (*Honda et al, 2001*)
In contrast to lymph nodes PPs located on the antimesenteric side of the gut, do not have an outer capsule and afferent lymphatic vessels, but they have high endothelial venules (HEV) that express MAdCAM-1-that binds to the α4β7 integrin on the surface of LTi cells. Beside LTi and LTo cells lymphoid tissue iniciator (LTin) cells are also necessary for PP development. (*Finke et al, 2001*) The formation and structure of colonic patches (ColP) is almost identical to that of PPs. (*Baptista et al, 2013*)
In contrast to the programmed development of mLNs PPs and ColPs, the formation of SILTs is induced postnatally by the microbial colonization of the intestinal tract. SILTs can be divided into rudimentarily organized cryptopatches (CP) containing mainly LTi cells and isolated lymphoid follicles (ILF). ILFs develop from CPs, have a single B cell follicle and lack a distinct T cell zone and HEVs. The gut microbiome has an important role in ILF maturation. SILTs are widely investigated in animal models; however, only limited data are available on the distribution of SILT structures in humans (*Lügering et al, 2010*)

### 1.3 Importance of ILC cells and the features of their subgroups

The development of innate lymphoid cells (ILC) from hematopoietic stem cells depends on several transcription factors. Their morphology is similar to lymphoid cells and as an effect of selectively expressed transcription factors they can produce T-helper like cytokines, however they lack antigen specific receptors generated through gene rearrangement.
ILCs fulfil several functions in different immunological processes, from lymphoid tissue development through maintenance of mucosal homeostasis to defence against pathogens and regulation of inflammatory processes. (*Spits et al, 2016*) Upon their transcription factor profile and cytokine producing capability they can be divided into three main groups: ILC1, ILC2 and ILC3, including LTi cells essential for the development of lymphoid organs. ILC3 cells play a dual role in inflammatory processes, they can produce IL-22 and IL-17 thus they have anti-inflammatory and pro-inflammatory qualities at the same time. (*Kellermayer et al, 2017*)
Although the research of ILC cells has recently undergone through a remarkable progress, the distinct development of individual subgroups, their tissue-specific functions, and many parts of their homeostasis have not been resolved yet. (*Spits et al, 2012*)

### 1.4 The transcription factor Nkx2-3

The homeodomain transcription factor Nkx2-3 has an important regulatory role in the development of spleen and PPs, but has no effect on lymph node formation. Nkx2-3 is required for the expression of MAdCAM-1 on endothelial cells that is essential for the

homing of lymphocytes to the mucosal tissues, through binding α4β7 and L-selectin leukocyte receptors. (*Mebius et al, 1996*)
In the absence of Nkx2-3 factor, the spleen is atrophic and structurally abnormal, there are fewer and smaller PPs with a perturbed vascular-endothelial addressin pattern, colonic crypts are enlarged and structurally altered, villi formation is abnormal and lymphocyte homing is also perturbed. (*Pabst et al, 1999*)
In Nkx2-3$^{-/-}$ mice MAdCAM-1 is replaced by PNAd addressin on mucosal HEVs of PPs (*Kellermayer et al, 2014*) but there is no structural alterations in pLNs. (*Pabst et al, 2000*) Hence the Nkx2-3 factor contributes to the development of the vascular network of the GALT and lymphocyte homing through the regulation of MAdCAM-1 expression in a tissue specific manner.
This restricted expression of Nkx2-3 -especially in the intestinal tract- can also be observed in humans. In connection with this tissue specific expression, the altered expression of Nkx2-3 factor has been associated with both forms of IBD. (*Yu et al, 2012*) The role of human Nkx2-3 factor includes the maintenance of colorectal myofibroblast identity, which probably contributes to the regulation of the colonic stem cell population. (*Hsia et al, 2016*)
Despite of these observations, the exact expression of Nkx2-3 factor has not been defined yet either in mice or in humans, thus further investigation is necessary for the identification of the exact cell types expressing Nkx2-3.

## 2. Objective

Our research group has investigated the role of Nkx2-3 transcription factor in the structural development of spleen and Peyer's patches, with a focus on vascular network, lymphocyte recirculation and homing processes.

As our earlier results have established, Nkx2-3 plays an important role in the regulation of the expression of mucosal addressin MAdCAM-1. In the absence of Nkx2-3 the endothelial expression of MAdCAM-1 is preserved in embryonic age and disappears gradually during the first postnatal months, in contrast to MAdCAM-1 deficient mice, where the general absence of MAdCAM-1 can be observed.

Our aim was to investigate the role of transcription factor Nkx2-3 in the distribution of lamina propria ILC3 cells, SILT formation and the development of inflammatory bowel diseases.

In my PhD work I performed experiments with Nkx2-3 and MAdCAM-1 deficient mice to answer the following questions:

- **How does the distribution of lamina propria ILC3 cells change in the absence of Nkx2-3 and MAdCAM-1 in postnatal and adult age?**

- **How does the absence of Nkx2-3 and MAdCAM-1 influence the development and distribution of SILT in postnatal and adult age?**

- **What kind of alterations occur in adult Nkx2-3 and MAdCAM-1 deficient mice in the course of DSS induced colitis and the distribution of lamina propria ILC3s and SILTs?**

- **What could influence the altered course of DSS induced colitis in the absence of Nkx2-3?**

## 3. Materials and methods

### Experimental animals

Nkx2-3$^{-/-}$ mice on a 129SvxB6 mixed background were back-crossed to BALB/cJ mice through 14 generations. Genotyping of mice was done with conventional duplex PCR, with the amplification of Nkx2.3 and neomycin-phosphotransferase sequences. (*Pabst et al, 1999*) MAdCAM-1$^{-/-}$ (Madcam1$^{tm1.2Nwag}$) mouse strain on C57BL/6 background was generated by Angela Schippers and her coworkers. (*Schippers et al, 2009*) BALB/cJ and C57BL/6J mice were purchased from the Jackson's Laboratory. (Bar Harbor, USA). During our experiments we used mCD19CherryLuciferase (CD19CL) transgenic mice (*Scotto et al, 2012*) and samples from LacZ-Nkx2.3$^{+/-}$ reporter mice. (*Wang et al, 2000*) All procedures involving live animals were conducted in accordance with the guidelines of the Ethics Comittee on Animal Experimentation of the University of Pécs.

### DSS treatment

To induce colitis 8-10 weeks old mice were given tap water containing 2,5% DSS (AppliChem GmbH) for seven days. Mice were sacrificed on the 7$^{th}$ day to investigate acute colitis, and on the 14$^{th}$ day to investigate subacute colitis. Littermates were used as controls, or the bedding was changed regularly within the cages.

### Lamina propria lymphocyte isolation and flow cytometry

Lamina propria lymphocytes were isolated according to an earlier protocol. (*Sawa et al, 2011*) Small intestine and colon were processed separately, in case of one and two weeks old mice minimum 3 samples were pooled. Samples were treated with EDTA then washed vigorously and dissociated with the help of collagenase D and DNase I. Mononuclear cells were separated with density gradient centrifugation.

Cell surface labelling was conducted on the separated cells with different labelled and unlabelled antibodies where the unlabeled antibodies were detected with fluorochrome-conjugated secondary antibodies. Intracellular labelling was done after permeabilization according to the manufacturer's protocol. The measurements were performed with BD FACSCanto II or BD FACSCalibur flow cytometers. Within the lymphoid gate minimum five thousand CD45$^{+}$CD3$^{-}$CD19$^{-}$ cells were collected and analyzed with the FCS Express software.

### Histology

To investigate the composition of SILT structures small intestinal and colonic Swiss-rolls were prepared then 8μm sections were cut at 4-6 planes. The sections were fixed in acetone then multiple immunofluorescence was carried out with labelled and unlabeled antibodies. Unlabeled primary antibodies were detected with labelled secondary antibodies. Sections were covered with glycerine then examined under Olympus BX61 fluorescence microscope. Representative images were prepared with the help of Olympus Fluo-View FV-1000 laser scanning confocal imaging system. For histological evaluation of DSS treated mice colon Swiss-roll cryostat sections were stained with hematoxylin-eozin and evaluated according to a standard procedure at the Institute of Pathology, University of Pécs. (*Barthel et al, 2003*)

### B cell colony analysis with whole colon bioluminescence

To examine the global distribution of B cells in the intestines Nkx2-3$^{-/-}$ mice crossed with mCD19CherryLuciferase (CD19CL) transgenic mice were used. Mice were anaesthetised then injected with luciferin-D. 10 minutes later mice were sacrificed and their

colons were analyzed using the IVIS Lumina II imaging system. Data were evaluated with the Living Image software, regions of interest (ROI) were determined according to their individual threshold.

**Immunisation and ELISA**

To induce anti-ovalbumin response mice were immunized with 50µl of 50mg/ml OVA mixed with Freund's adjuvant. Ovalbumin was injected subcutaneously into the left footpad on the $0^{th}$ and $7^{th}$ days. Mice were sacrificed on the $21^{st}$ days to collect their sera. Anti-OVA IgG response was measured with ELISA test using PO-conjugated anti-mouse IgG. Peroxidase activity was detected in citrate-phosphate buffer with ortho-phenilenediamine and $H_2O_2$. Samples were measured in duplicates at 492 nm.

**RNA isolation, cDNA synthesis, RT-PCR**

Whole RNA was isolated with the NucleoSpin RNA (macherey-Nagel GmbH) RNA isolating kit. cDNA was synthetised with the help of the High Capacity cDNA RT Kit. PCR reaction was run in duplicates in the ABI-PRISM 7500 machine with SYBR green primers (*Czömpöly et al, 2011*), or TaqMan probes. Results are shown as percentage of β-actin, or mGAPDH housekeeping genes.

**Serum IL-22 measurement**

Serum IL-22 was measured with the Mouse/Rat IL-22 Quantikine ELISA kit (R&D Systems) according to manufacturer's protocol. Concentrations were defined according to a standard curve.

**Anti-IL-22 treatment**

Nkx2-$3^{-/-}$ mice drank 2,5% DSS containing tap water for 7 days. On the $2^{nd}$, $3^{rd}$, $4^{th}$ and $6^{th}$ days mice were injected intraperitoneally with 150µg anti-IL22 monoclonal antibody, or isotype control. Mice were processed on the $7^{th}$ day and their colons were analyzed with histology or flow cytometry.

**Generation of bone marrow chimeras**

Four weeks old eGFP-tgBALB/c mice were irradiated with 2 x 5,5 Gy lethal dose from a $Co^{60}$ source, in 6 hours intervalls in the Institute of Oncotherapy, University of Pécs. Three hours after the second irradiation mice were injected with 5 x $10^6$ bone marrow cells from BALB/c or Nkx2-$3^{-/-}$ mice into their tail veins. Ratio of chimerism was determined upon PBMC eGFP/CD45 expression of PBMC samples.

**Combined LacZ/β-galactosidase enzyme and immunohistochemistry**

Colonic Swiss roll cryostat sections of LacZ-Nkx2-3 reporter (*Wang et al, 2000*) and BALB/c mice were labelled with different rat mABs against epithelial, fibroblastic or endothelial markers after acetone fixation. Antibodies were detected with the ImmPRESS-HRP goat anti-rat IgG kit. After washing, samples were incubated overnight with an X-gal containing substrate solution at 37°C.

**Statistical analysis**

Our data were analyzed with the IBM® SPSS® Statistical Software (version 22). Groups with not normal distribution were analyzed with Mann-Whitney test. Data are presented as mean±SEM. Statistical significance was considered as $p < 0.05$.

# 4. Results

## 4.1 Postnatal distribution of lamina propria ILC3 in the absence of Nkx2-3

The absence of transcription factor Nkx2-3 blocks the endothelial expression of MAdCAM-1 addressin, however, in contrast to MAdCAM-1$^{-/-}$ mice in which there is a general absence of MAdCAM-1, in Nkx2-3$^{-/-}$ mice the endothelial MAdCAM-1 expression disappears gradually during the first few postnatal weeks. In our work we wished to determine the effect of these two different MAdCAM-1 deficiencies on the postnatal distribution of intestinal ILC3. From lamina propria lymphocytes of 1, 2 and 4 weeks old mice we identified ILC3 cells as CD45$^{+}$CD3$^{-}$CD19$^{-}$CD90$^{+}$RORγt$^{+}$, using flow cytometry.

The absolute number of ILC3 cells was higher in the small intestine, than in the colon in each investigated mouse strains at each time points.

The highest small intestinal ILC3 (siILC3) number was measured on the 1$^{st}$ postnatal week. On the 2$^{nd}$ postnatal week siILC3 numbers decreased considerably in each mouse strain. By the 4$^{th}$ week with the exception of C57BL/6 mice, whose siILC3 number slightly increased siILC3 numbers decreased further in each strain. At this time point the siILC3 number was significantly lower in Nkx2-3$^{-/-}$ mice according to their Nkx2-3$^{+/-}$ control group.

Interestingly the lowest ILC3 absolute numbers were observed in MAdCAM-1 deficient mice in each investigated time points, furthermore this low number decreased continuously, both in the small intestine and colon.

On the first week the colonic ILC3 (cILC3) number in MAdCAM-1$^{-/-}$ mice was only one third of cILC3 numbers of the other mouse strains. By the 4$^{th}$ week cILC3 numbers dropped significantly in Nkx2-3 deficient and heterozygous mice. In contrast to the small intestine where the ILC3 number was significantly lower in the absence of Nkx2-3, in the colon we detected higher ILC3 number, however in MAdCAM-1$^{-/-}$ mice the cILC3 number decreased to a very low level by this time point.

These results suggest that different MAdCAM-1 deficiencies distinctly influence the distribution of ILC3s in different intestinal regions. Furthermore the most severe alterations were observed in the general absence of MAdCAM-1 in comparison the relevant control group.

## 4.2 Kinetics of postnatal SILT maturation in the absence of Nkx2-3

Based on the earlier work of our research group, in the absence of Nkx2-3 the perturbed lymphocyte distribution is associated with an altered PP vasculature, due to the gradual replacement of MAdCAM-1 with PNAd addressin. (*Kellermayer et al, 2014*) Hence in our recent work we wished to examine how this altered addressin pattern influences SILT formation. We compared different maturational stages of colonic SILT in Nkx2-3, MAdCAM-1 deficient and control mice. To ensure the same environmental conditions in case of Nkx2-3$^{-/-}$ mice we used Nkx2-3 heterozygotes as controls. The different SILT structures were detected with immunofluorescence.

On the first postnatal week only CP and imILFs were present in the colon of each mouse strain and their distribution in Nkx2-3$^{-/-}$ and MAdCAM-1$^{-/-}$ mice were similar to that of their relevant controls. Interestingly in C57BL/6 and MAdCAM-1$^{-/-}$ mice we observed a higher CP ratio.

On the second week with the exception of MAdCAM-1$^{-/-}$ mice matILFs appeared in each group however their ration was significantly lower in the absence of Nkx2-3.

By the fourth postnatal week matILFs appeared in MAdCAM-1$^{-/-}$ mice too, but their ratio was significantly lower compared to the control C57BL/6 group. We also observed lower matILF ratio in Nkx2-3 deficient mice compared to Nkx2-3 heterozygotes, although this difference was statistically not significant.

Based on these results we can conclude that different MAdCAM-1 deficiencies (transcriptional in Nkx2-3$^{-/-}$, genomic in MAdCAM-1$^{-/-}$ mice) influence colonic SILT maturation in different ways. In the absence of Nkx2-3 SILT maturation was only slightly altered compared to the control group, while the global absence of MAdCAM-1 caused much more significant alterations. Although LTi-containing CP formations appeared in MAdCAM-1$^{-/-}$ mice, further SILT maturation showed a significant delay. Nevertheless the general absence of MAdCAM-1 addressin cannot block colonic SILT formation and development. The most striking alteration was observed on the second week when matILFs have already appeared in Nkx2-3$^{-/-}$ mice, (but at a lower ratio compared to the relevant controls), while in MAdCAM-1$^{-/-}$ mice matILF were observed later by the 4$^{th}$ postnatal week at a significantly decreased ratio.

### 4.3 Relationship of postnatal ILC3 distribution and vascular addressin expression in the gut

We investigated the correlation between the perturbed ILC3 distribution and the altered expression of MAdCAM-1 and PNAd addressins. For this purpose we examined MAdCAM-1 expression in Nkx2-3$^{-/-}$ mice and PNAd expression of MAdCAM-1$^{-/-}$ mice using quantitative immunofluorescence and ImageJ analysis.

In the small intestine of Nkx2-3$^{-/-}$ mice we observed an increased level of MAdCAM-1 expression on the first postnatal week, although by the fourth week MAdCAM-1 decreased to a significantly lower level compared to the Nkx2-3$^{+/-}$ control group. In contrast, colonic MAdCAM-1 expression was lower at each investigated time points in the absence of Nkx2-3. From these results we suggest that the gradual disappearance of MAdCAM-1 expression is kinetically different in the two intestinal regions, furthermore the gradual change in MAdCAM-1 expression did not show any correlation with the perturbed local distribution of ILC3 cells.

In MAdCAM-1$^{-/-}$ mice the level of small intestinal PNAd expression remained under that of the relevant C57BL/6 control group during the first two weeks after birth, however by the fourth week the PNAd expression exceeded that of the control group. The number of small intestinal ILC3 cells remained relatively stable in C57BL/6 mice, but the level of PNAd increased continuously. In contrast, in MAdCAM-1$^{-/-}$ mice the number of siILC3 decreased significantly over time parallel to the continuous increase of PNAd level. The colonic expression of PNAd in MAdCAM-1$^{-/-}$ mice showed a significant increase by the second week compared to the control group and increased further till the fourth week. In contrast cILC3 number showed a continuous decrease in MAdCAM-1 deficient and wild type mice, moreover in the absence of MAdCAM-1 ILC3 cells were present in a barely detectable amount.

### 4.4 Altered PNAd core protein and modifier enzyme mRNA expression in the absence of Nkx2-3 and MAdCAM-1

The MAdCAM-1 addressin ensures lymphocyte homing to the PPs and the intestinal tract. In the absence of Nkx2-3 endothelial cells do not express MAdCAM-1, but the mRNA level of several PNAd core proteins and modifying enzymes increase in PPs. (*Kellermayer et al, 2014*) In our work we investigated similar alterations in MAdCAM-1$^{-/-}$ mice. In the absence of MAdCAM-1 the endomucin and podocalyxin-like PNAd core mRNA increased significantly compared to the wild type control group. The CD34 mRNA level increased too, although not significantly, while the expression level of Glycam1 and nepmucin did not show any changes.

From the modifying enzymes necessary for the formation of the PNAd glycoepitope – recognized by the MECA-79 antibody– the betaGal beta-1,3-N-acetyl glucosaminyl

transferase 3 sulfotransferase (B3gnt3) and alpha-(1,3)-fukosyltransferase (Fut7) mRNA levels did not show significant changes, however the N-acetilglucosamine 6-O sulfotransferase (Chst4) mRNA level increased significantly in the PP of MAdCAM-1 deficient mice. These alterations differ from the earlier observations in Nkx2-3 deficient mice, where the most striking increase was in the mRNA expression of Glycam1 and Chst4. (*Kellermayer et al, 2014*)

### 4.5 Preserved lymph node architecture and normal T cell-dependent antibody response in the absence of MAdCAM-1

Peripheral lymph node (pLN) HEVs express MAdCAM-1 addressin during their embryonic and early postnatal maturation; however, the significance of lymphocyte colonization through this molecule in the postnatal functionality of lymph nodes has remained undetermined. (*Mebius et al, 2003*) Therefore we investigated the effect of different levels of Nkx2-3 and MAdCAM-1 absence on pLN formation and structural maturation. During immunohistological examination neither lymphoid compartmentalisation nor the follicular stromal organisation showed any alterations.

Next we examined serological evidences for the alterations of local T-dependent immune response in Nkx2-3 and MAdCAM-1 deficient mice after ovalbumin immunisation using indirect ELISA test. We found that the absence of MAdCAM-1 did not cause significantly lower antibody levels either in MAdCAM-$1^{-/-}$ or in Nkx2-$3^{-/-}$ mice compared to their relevant control groups.

These results suggest that beside normal pLN structure, the T-dependent antibody response is also preserved despite the absence of MAdCAM-1.

### 4.6 SILT maturation in adult age is partially blocked in the absence of Nkx2-3

We investigated SILT composition in young adult mice lacking Nkx2-3, with immunofluorescent labelling of colon sections. In Nkx2-$3^{-/-}$ mice we observed the absence of vascular MAdCAM-1 expression and luminal appearance of PNAd in ColPs similarly to PPs. In BALB/c mice PNAd expression was only detectable on the abluminal side of HEVs.

Next we examined whole colonic distribution of B cell clusters in Nkx2-$3^{-/-}$ x mCD19Luc$^{+}$ mice. In this ex vivo experiment we observed significantly fewer B-cell clusters; however their area were significantly larger than that of their heterozygous (Nkx2-$3^{+/-}$ x mCD19Luc$^{+}$) controls.

In the absence of Nkx2.3 we found normally structured SILT in the colon however their immature forms were present at a higher ratio, than in the control group. Thus in the absence of Nkx2-3 CP development is normal, but their further maturation is partially blocked.

To determine whether the perturbation of SILT maturation is due to the absence of endothelial MAdCAM-1 (consequently to Nkx2-3 deficiency), we also investigated SILT maturation in MAdCAM-$1^{-/-}$ mice with preserved Nkx2-3 activity. In the absence of MAdCAM-1 luminal expression of PNAd was also observed in ColP HEVs. In MAdCAM-$1^{-/-}$ mice there were no structural alteration of SILT, but we found a more severe blockade of CP maturation to imILF and matILF.

According to flow cytometric measurements the number of SILT-associated B cells was the lowest in MAdCAM-1 deficient mice representing an altered lymphocyte distribution and a more pronounced blockade of ILF maturation.

### 4.7 Distribution of colonic ILC3 in adult age in the absence of Nkx2-3 and MAdCAM-1 in normal and inflammatory conditions

In the absence of Nkx2-3 the number of ROR$\gamma$t$^{+}$ colonic ILC3 cells was higher in each investigated time points (before DSS treatment, in acute and subacute colitis) compared to

BALB/c mice. The distribution of ILC3 cells was investigated in MAdCAM-1$^{-/-}$ mice too to determine whether the endothelial absence of MAdCAM-1 is responsible for their altered ratio. During this experiment we found that upon DSS treatment ILC3 numbers increased and by the 14$^{th}$ day their number became significantly higher in the absence of MAdCAM-1 than in the wild type control group.

**4.8 Nkx2-3 deficient mice are protected against DSS induced colitis**

As earlier observations have indicated, colitis can induce the maturation of CPs into matILFs, (*Lochner et al, 2011; Olivier et al, 2016*) therefore next we investigated colonic distribution of SILT in a mouse model of DSS induced colitis. Intestinal samples were examined on the 7th (acute colitis) and the 14$^{th}$ (subacute colitis) days. Interestingly Nkx2-3 deficient mice demonstrated protection against DSS induced colitis according to several physiological parameters. In contrast to the control group, in Nkx2-3$^{-/-}$ mice we observed only a minimal weight loss, rectal bleeding occured only in some animals, the shortening of the colon was minimal and their survival rate was 100% during DSS treatment. According to the histological analysis of hematoxylin-eosin stained colon sections, mice lacking Nkx2-3 had a lower pathological score compared to wild type (BALB/c) mice.

To find out whether the endothelial loss of MAdCAM-1 alone caused protection against DSS induced colitis in Nkx2-3$^{-/-}$ mice, these experiments were performed on MAdCAM-1$^{-/-}$ mice too. Surprisingly, in these mutants an even more severe inflammation developed upon DSS treatment.

In wild type mice the ratio of mature SILT structures increased upon DSS treatment, in contrast to mice lacking Nkx2-3 demonstrating a delay in SILT maturation. In MAdCAM-1$^{-/-}$ mice we found a more pronounced blockade in SILT maturation, since the ratio of SILTs containing B cells increased only by the 14$^{th}$ day of the treatment.

The differences of kinetics of B-cell growth were also confirmed by flow cytometry. In the colon of Nkx2-3 and MAdCAM-1 deficient mice the number of B cells was lower than in the control groups throughout the whole treatment.

The different types of MAdCAM-1 deficiencies in Nkx2-3$^{-/-}$ and MAdCAM-1$^{-/-}$ mice and their altered vascular addressin expression of colonic vessels could explain the delay in SILT maturation; however only Nkx2-3$^{-/-}$ mice were protected against DSS induced colitis, thus this protection is likely to have other components than the absence of endothelial MAdCAM-1.

**4.9 Protection against intestinal inflammation in the absence of Nkx2-3 is IL-22 independent**

Previous research has shown that IL-22 produced by ILC3s can induce Reg proteins and mucins, important in mucosal regeneration, thus IL-22 can play a protective role in colitis. (*Zhengés et al 2008; Pichert et al, 2009*) Our research group detected increased mRNA level of IL-22, RegIIIβ and RegIIIγ in acute colitis in the colon of Nkx2-3$^{-/-}$ mice. However we did not find significant changes in IL-22 mRNA expression in MAdCAM-1$^{-/-}$ mice during the acute phase of colitis. By the 14$^{th}$ day of treatment cycle, IL-22 level was significantly lower in Nkx2-3$^{-/-}$ and MAdCAM-1$^{-/-}$ colons compared to their relevant control groups.

Next, we investigated whether elevation of IL-22 levels in Nkx2-3 deficient mice may cause protection against colitis. We could not find any differences in serum IL-22 levels of untreated Nkx2-3 and wild type mice; however the serum level of IL-22 level was significantly lower in the absence of Nkx2-3 during DSS treatment. Upon these results we conclude that Nkx2-3 deficient mice were protected against colitis despite their decreased IL-22 levels. On the other hand, BALB/c mice developed severe colitis despite their increased serum IL-22 level.

To further investigate the role of IL-22, Nkx2-3$^{-/-}$ mice were treated with antagonistic anti-IL-22 antibody or isotype control during DSS treatment. Interestingly Nkx2-3 deficient mice still did not show any severe symptoms of colitis either macroscopically or histologically, moreover there were no significant alterations in SILT maturation and ILC3 distribution.
Despite all these conditions, the RegIIIβ and RegIIIγ mRNA levels decreased considerably in anti-IL-22 treated mice, which confirmed the biological efficacy of the treatment. According to our results we concluded that the inhibition of IL-22 did not induced colitis in the absence of Nkx2-3, but caused a decrease in the level of Reg proteins important for mucosal healing, again without causing colitis.

### 4.10 Protection against colitis in mice lacking Nkx2-3 is mediated by non-hematopoietic cells

In our next experiment we wished to specify whether the hematopoietic or non-hematopoietic cells of the intestines cause protection against inflammation in the absence of Nkx2-3. For this purpose bone marrow chimeras were generated as described in the materials and methods section. Five weeks after transplantation mice with a minimum of 90% chimerism were treated with drinking water containing 2.5% DSS then sacrificed on the 7th day. We did not see difference in weight loss and the severity of colitis, both groups (eGFP-Tg-BALB/c recipient mice transplanted with either Nkx2-3-/- or BALB/c bone marrow) were sensitive to colitis induction. From these results we concluded that the inhibited development of colitis (as observed in mice lacking Nkx2-3) is related to its presence in the stromal cells, independently from the hematopoietic cells (irrespectively of their Nkx2-3 genotype).
To identify non-hematopoietic intestinal stromal cells that express Nkx2-3 protein we used LacZ-Nkx2-3+/- reporter mice. With the combination of immunohistochemistry and X-gal staining we determined that neither epithelial nor endothelial cells, but myofibroblast-like VAP-1 positive cells in the tunica-muscularis-mucosae layer of the intestinal wall express Nkx2-3. This observation is also consistent with the recently published human results. (*Hsia et al, 2016*)

## 5. Discussion

IBD is one of the most common chronic inflammatory diseases of the intestines. Besides abnormal immunological responsiveness to bacteria and bacterial dysbiosis, many environmental and genetic factors may contribute to its development. One of these is the Nkx2-3 homeodomain transcription factor, which can play a role in predisposing both Crohn's disease and ulcerative colitis.

ILC3 cells regulate a number of immunological process, they are involved in mucosal lymphoid tissue formation and epithelial regeneration in IBD. These diseases are characterised by the ectopic neogenesis of lymphoid tissues, in which ILC3 cells play an important role. (*Geremia et al, 2017*). In order to exert their effects locally, ILC3 cells must migrate to the intestinal tract. For their homing to the gut they have to bind to the MAdCAM-1 addressin on mucosal HEVs and lamina propria vessels through their α4β7 integrin. Thus, through the regulation of MAdCAM-1 expression, the Nkx2-3 factor may influence the distribution of ILC3 cells and have a local role in the development of gut associated lymphoid tissues and the pathogenesis of IBD.

In our work we examined Nkx2-3 deficient mice, in which the endothelial MAdCAM-1 expression gradually disappears during the first postnatal month and replaced by PNAd, while non-endothelial MAdCAM-1 expression (eg on the surface of FDC) is retained, similarly to peripheral lymph nodes. (*Mebius et al, 1996; Hamada et al, 2002*) In contrast to Nkx2-3 deficient mice, in MAdCAM-1 knockout mice MAdCAM-1 is completely missing (*Schippers et al, 2009*) and have elevated PNAd expression level, thus the study of these two mouse models may help to understand the role of MAdCAM-1 addressin and the process of intestinal lymphoid colonization in IBD.

The postnatal distribution of ILC3 cells was studied in various intestinal regions. According to our results, the small intestine contained more ILC3 cells than the colon. On the first postnatal week there was no difference in the distribution of ILC3s in Nkx2-3$^{-/-}$ and Nkx2-3$^{+/-}$ mice, suggesting that the mucosal colonization of ILC3 cells was effective in the small intestine.

In contrast to the first week, later in the postnatal period, lower absolute ILC3 numbers were measured in the small intestine of Nkx2-3$^{-/-}$ mice, and the number of mature ILFs was also significantly lower, which can be correlated with the late-onset gradual disappearance of the MAdCAM-1 addressin.

The distribution of colonic ILC3 during the postnatal period showed significant differences compared to the small intestine. This could be explained with the presence of different factors in the small intestine ensuring the formation of ILFs or an alternative addressin(s) that can partially compensate the absence of MAdCAM-1. (*Knoop et al, 2011*)

The differential distribution of colonic ILFs could be influenced by several factors. One of those is the delayed ILF maturation in mice on C57BL/6 background that was confirmed by our observation as a shift in CP/ILF ratio compared to mice with BALB/c background. Similarly to the earlier observations in the small intestine, colonic imILF and matILF maturation is also delayed compared to the development of CPs. (*Ibiza et al, 2016*) Interestingly, the CP/ILF ratio was similar in MAdCAM-1$^{-/-}$ and C57BL/6 strains, that may support the MAdCAM-1 independence of CP-ILF transformation. (*Garcia-Barcelo et al, 2007*) In contrast, the further ILF maturation (presumably in its B-cell dependent phase) is highly blocked in the absence of MAdCAM-1. Upon these results we can conclude that in the absence of MAdCAM-1 even the increased expression of the PNAd addressin was unable to substitute MAdCAM-1, but in Nkx2-3$^{-/-}$ mice inhibition of ILF maturation is less severe due to the early postnatal partial preservation of MAdCAM-1 expression.

In our work we examined the effect of different MAdCAM-1 deficiencies on the immune response in peripheral lymph nodes (pLN), because the α4β7 integrin – MAdCAM-1 interaction has an important role in the embryonic development of PP, and pLN. (*Mebius et al, 1996*) According to our observation, although the development of PP and maturation of ILF are partially blocked, pLN did not show any structural or functional alterations in the absence of MAdCAM-1. Upon these findings, we conclude that in pLN ILC3/LTi cells migrate to the lymphoid anlagen through another endothelial ligand. Partial retention of ILC3 migration to mucosal surfaces (to maintain intestinal lymphoid neogenesis) and migration of LTi cells into the lymph node anlagen in the embryonic age in the absence of MAdCAM-1 also shows the plasticity of the endothelial addressin profile. This plasticity questions the effectiveness of anti-adhesive therapeutical interventions in IBD. It is still questionable how ILC cells and other leukocytes migrate to the developing lymph nodes and mucosal areas such as the intestinal tract in the absence of Nkx2-3 or MAdCAM-1, in case of an altered vascular addressin pattern. It is possible that other currently unknown addressins have role also in this process, which can be possible targets in the treatment of IBD.

In the first four weeks of the postnatal period, delayed SILT maturation was observed in mice with C57BL/6 background compared to BALB / c mice, but this difference could not be observed in young adult mice. Endothelial MAdCAM-1 expression of Nkx2-3$^{-/-}$ mice disappeared by this time point. As a result of DSS treatment, the proportion of mature SILT structures increased in Nkx2-3 deficient mice, but colitis did not develop.
In contrast, DSS treatment in MAdCAM-1$^{-/-}$ mice resulted in a very severe colitis, whereas maturation of CPs to ILF was observed at a very low rate. Differences observed between the two strains led to the conclusion that the lack of endothelial MAdCAM-1 alone does not lead to the perturbed SILT maturation and protection against colitis in adult Nkx2-3 deficient mice.

Significant differences were also observed between the two knockout mouse strains in the pattern of mRNA expression of PNAd core proteins and glycosylation enzymes that also highlited the plasticity of mucosal endothelial cells. Thus, these results indicate that in these mouse strains with distinct levels of MAdCAM-1 deficiencies, compensation via increased PNAd expression levels is achieved by different mRNA expression variations. This altered PNAd core protein and glycosylation enzyme mRNA expression allows the generation of the sulfated carbohydrate-dependent epitope of MECA-79 in both strains. In spite of the observed changes the results of previous studies (*Hamada et al, 2002*) showed that the appearance of CPs and imILFs is independent of the interaction of MAdCAM-1/α4β7. However it is questionable, whether, similarly to the Nkx2-3$^{-/-}$ PPs (*Kellermayer et al, 2014*), in MAdCAM-1$^{-/-}$ mice the mucosal homing mechanism has switched to a L-selectin/PNAd-dependent process. To answer this question, a competitive lymphocyte transfer experiment with MEL-14 antibody could be applied.

In addition to the protection against colitis, we observed the accumulation of RORγt$^{+}$ ILC3 cells in the colon of Nkx2-3$^{-/-}$ mice, which -according to previous studies- are capable of producing protective IL-22 in DSS-induced colitis. (*Ibiza et al, 2016*) In spite of this, following antagonistic anti-IL-22 mAb treatment Nkx2-3$^{-/-}$ mice did not develop inflammation, while the increased IL-22 level did not protect BALB/c mice from the development of colitis, therefore the protection against DSS-induced colitis is not an IL-22-mediated process. As Nkx2-3 factor also plays a role in the regulation of the neural system, so we can also assume that in the absence of this factor alterations of the enteric nervous network can contribute to the protection against colitis. (*Garcia-Barcelo et al, 2007*)

The effect of DSS treatment was also investigated on bone marrow chimeras. In this experiment, we found that there was no difference in the severity of DSS-induced colitis after the reconstruction of irradiated wild-type recipients with either Nkx2-3$^{-/-}$ or BALB/c bone

marrow, therefore replacing normal hematopoietic cells with Nkx2-3 deficient cells in wild-type recipients did not prevent the development of colitis. In spite of our repeated attempts, the inverse experiment (reconstruction of irradiated Nkx2-3 mice with wild-type bone marrow) was unsuccessful, presumably due to the structurally defective red pulp of Nkx2-3 mice, which may play an important complementary role in post-irradiation hematopoietic regeneration.

Our research on the identification of stromal cells expressing Nkx2-3 confirmed the expression of the Nkx2-3 factor in VAP-$1^{+}$ myofibroblasts. All these results suggest that the distribution of ILC3 cells and the protection against colitis due to the absence of Nkx2-3 are influenced by non-haematopoietic components, thus the role of Nkx2-3 in intestinal inflammation manifests primarily in stromal cells. Our results also suggest that in adult Nkx2-$3^{-/-}$ and MAdCAM-$1^{-/-}$ mice, the higher proportion of immature SILT structures is caused by the lack of endothelial MAdCAM-1, but no inflammation has occurred in Nkx2-3 mice during DSS treatment. Thus the protection against colitis is independent from the lack of endothelial MAdCAM-1. It appears that in the absence of Nkx2-3, Nkx2-3 deficient stromal cells might provide a protective microenvironment exerting IL-22 independent induction of mucosa after epithelial injury. However, it is still questionable what function and phenotype those cells have that are involved in this process. These cells and their signalling pathways regulated by Nkx2-3 might be potential therapeutic targets in the future. The Nkx2-3 factor is expressed in the muscle layer under lamina propria both in humans and mice (*Wang et al, 2000*), therefore the increased epithelial regeneration in mice lacking Nkx2-3 is presumably due to the effect on myofibroblasts, which may influence the homeostasis of intestinal stem cells via Wnt signalling. (*Perochon et al, 2018; Yu et al, 2010* The cell line-specific deletion of Nkx2-3 could answer this question, but mice with the appropriately floxed Nkx2-3 genotype is not yet available.

As an alternative method, analysis of isolated intestinal stromal cells could provide further information relevant to the protection against colitis in the absence of Nkx2-3 in the future. In addition, further investigation of lymphoid and epithelial components that may be under the control of Nkx2-$3^{+}$ stromal cells may highlight their interactions. By discovering these cells and mechanisms, new therapeutic targets can be identified not only for patients with IBD, but also for the treatment of other inflammatory diseases.

## 6. Summary of new results

Our aim was to investigate the role of transcription factor Nkx2-3 in the distribution of lamina propria ILC3 cells, SILT formation and the development of inflammatory bowel diseases.

In my PhD work I performed experiments with Nkx2-3 (absence of endothelial MAdCAM-1) and MAdCAM-1 deficient (general absence of MAdCAM-1) mice and answered the following questions:

- **How does the distribution of lamina propria ILC3 cells change in the absence of Nkx2-3 and MAdCAM-1 in postnatal and adult age?**
    - Colonization of ILC3 cells occured in each investigated intestinal regions of Nkx2-3 $^{-/-}$ and MAdCAM-1$^{-/-}$ mice.
    - The absolute number of ILC3 was higher in the small intestine than in the colon in each investigated mouse strain.
    - In the absence of Nkx2-3, the number of siILC3 cells decreased after the first postnatal week due to the gradual loss of endothelial MAdCAM-1, whereas the absolute number of ILC3 in MAdCAM-1$^{-/-}$ mice was the lowest at each investigated time point; furthermore, this low cell count also showed a gradual decline. This decrease in the small intestine was not as pronounced as in the colon.
    - In adult age, the number of RORγt$^{+}$ ILC3 cells in the colon was higher in Nkx2-3$^{-/-}$ mice than in their wild type controls.

- **How does the absence of Nkx2-3 and MAdCAM-1 influence the development and distribution of SILT in postnatal and adult age?**
    - In MAdCAM-1$^{-/-}$ and wild type mice with C57BL/6 background the maturation of colonic CP to imILF and matILF was delayed compared to CP formation.
    - Altered PNAd mRNA expression allows the expression of the MECA-79 sulfated carbohydrate-dependent epitope in both strains.
    - In these mouse strains with distinct levels of MAdCAM-1 deficiencies, compensation with increased PNAd expression levels is achieved by different mRNA expression patterns for core proteins and glycosylation enzymes.
    - Despite the increased expression of PNAd addressin, ILF maturation is highly blocked in the absence of MAdCAM-1.
    - In Nkx2-3$^{-/-}$ mice, inhibition of ILF maturation is less severe as a result of the partially retained MAdCAM-1 expression.
    - Higher proportion of immature SILT structures in Nkx2-3$^{-/-}$ and MAdCAM-1$^{-/-}$ mice is caused by the lack of endothelial MAdCAM-1.
    - In MAdCAM-1$^{-/-}$ mice, the structure and function of pLNs was retained, thus ILC3/LTi cells migrate to the lymphoid anlagen through another endothelial ligand.

- **What kind of alterations occur in adult Nkx2-3 and MAdCAM-1 deficient mice in DSS induced colitis, in the distribution of lamina propria ILC3s and SILTs?**
    - Following DSS treatment of Nkx2-3$^{-/-}$ mice the number of mature ILF structures increased, but the mice did not develop colitis.
    - In MAdCAM-1$^{-/-}$ mice, DSS treatment resulted in a low number of CP-ILF transformations, but the ensuing colitis was very severe, hence in Nkx2-3

deficient mice the lack of endothelial MAdCAM-1 did not cause perturbation in SILT development and protection against colitis.
- Beside the protection against colitis we observed the accumulation of RORγt$^{+}$ colonic ILC3 cells in the absence of Nkx2-3.

- **What could influence the altered course of DSS induced colitis in the absence of Nkx2-3?**
  - During DSS treatment only Nkx2-3 mice did not develop inflammation, thus protection against colitis is independent of MAdCAM-1 addressin.
  - In Nkx2-3$^{-/-}$ mice the anti-IL-22 antibody treatment did not exacerbate inflammation, so the protection against colitis is not an IL-22 mediated process.
  - In wild-type mice the replacement of normal hematopoietic cells with Nkx2-3 deficient cells did not prevent the induction of colitis, so the role of the Nkx2-3 factor is associated with non-hematopoietic stromal cells.
  - With the combination of immunohistochemistry and X-gal staining we showed the expression of Nkx2-3 in VAP-1$^{+}$ myofibroblasts in the tunica-muscularis mucosal layer of the intestinal wall in LacZ-Nkx2-3$^{+/-}$ reporter mice.

## 7. List of publications

### Publications the thesis is based on

**Vojkovics D**, Kellermayer Z, Gábris F, et al. Differential effects of the absence of Nkx2-3 and MAdCAM-1 on the distribution of intestinal type 3 innate lymphoid cells and postnatal SILT formation in mice. *Front Immunol.* 2019 Mar 5;10:366.
**IF: 5,511**

Kellermayer Z, **Vojkovics D**, Dakah TA, et al. IL-22-independent protection from colitis in the absence of Nkx2-3 transcription factor in mice. *J Immunol*. 2019; pii: ji1801117.
**IF: 4,539**

**Vojkovics D**, Kellermayer Z, Balogh P., et al. Nkx2-3 – a slippery slope from development through inflammation toward hematopoietic malignancies. *Biom. Ins*. 2018; 13: 1–6.

Kellermayer Z, **Vojkovics D**, Balogh P. Innate lymphoid cells and their stromal microenvironments. *Immunol Lett*. 2017; Sep; Volume 189:3-9.
**IF: 2,438**

### Publications not directly related to the thesis

**Vojkovics D**, Kellermayer Z, Balogh P. et al. Isolation and characterization of a murine spontaneous high-grade follicular lymphoma with restricted in vivo spreading--a model for lymphatic metastasis via the mesentery. *Pathol Oncol Res*. 2016; Apr; 22 (2):421-30. doi: 10.1007/s12253-015-0025-6.
**IF: 1,935**

**Total IF: 14,423**

## Acknowledgement

I would like to express my gratitude to all of those people, who helped me in one way or another to complete my PhD work.

First of all I would like to thank my supervisor Prof. Dr. Péter Balogh his support, guidance and practical advices throughout the years of my student research and PhD work.

I am especially grateful to my colleague Dr. Zoltán Kellermayer for helping me to learn laboratory techniques and selflessly assisting me in performing my experiments.

I am also grateful to the staff of the Department of Immunology and Biotechnology for their help and support.

I would like to thank Dr. Gergely Berta for his help in the preparation of confocal microscopic images.

I would like to thank Dr. Kornélia Farkas for helping me in the statistical evaluation of my results.

I am grateful to my family and friends for their support and encouragement throughout my work.

My work was supported by the following grants:
OTKA-108429
EFOP-3.6.1.-16-2016-00004
FIKP-20765-3/2018/FEKUTSTRAT