National and Kapodistrian
UNIVERSITY OF ATHENS

"ALEXANDER FLEMING"
Biomedical Sciences Research Center

Elucidation of the mesenchymal LTβR role during tertiary lymphoid organ development and intestinal inflammation

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MSc in Molecular Biomedicine

Master thesis

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Athens 2018

AKNOWLEDGMENTS

I would like to express my gratitude to my supervisor, Prof. George Kollias, who provided me the opportunity to join his laboratory, as a master student. I feel very grateful for the continuous support, the guidance throughout this year and the motivation he offered to me for good and novel research.

Besides my supervisor, I would like to thank my committee members, Dr. Vasiliki Koliaraki and Dr Marietta Armaka. I really appreciate the encouragement and kindness they showed to me as well as their extensive personal and academic guidance in the pursuit of this research project.

Special thanks should be given to my supervisor in the bench Dr. Alejandro Prados. His continuous help, guidance, patience and kindness will be some of the most valuable memories that I will keep for my future research career. Without his precious support it would not be possible to conduct this research.

Of course, I would like to thank separately each member of the lab and histology unit for the perfect cooperation and the friendly environment this whole year.

Last but not least, I would like to thank my parents and my brother for their unconditional love and support during the two years of my master as well as during my whole life.

CONTENTS

ABSTRACT

The intestinal tertiary lymphoid organs (TLOs), commonly known as ILFs, are B-cell clusters located in the lamina propria, important for IgA production. They have been found increased in IBD patients, but their actual role during disease is still unknown. Lymphotoxin beta receptor (LTβR) expressed by mesenchymal cells (MCs) has been reported as necessary for TLO development. Here, we aimed to identify the cellular requirements for ILF formations as well as their functions during disease progression in $TNF^{\Delta ARE}$ mice, a spontaneous murine model of IBD. MC-specific LTβR deficient mice did not show alterations in the ILF number but the ILF-MC network was impaired. This finding revealed the importance of MC-LTβR for ILF organization both in health and disease. Since these mice still developed ILFs, we generated LTβR deficient mice that lack ILFs, to study their importance during inflammation. LTβR deletion in $TNF^{\Delta ARE}$ mice did not affect the disease progression in the terminal ileum, suggesting that ILFs don't participate in the pathogenesis of this model. Interestingly, these mice developed inflammatory colitis characterized by submucosal immune infiltration, indicating an LTβR-dependent anti-inflammatory mechanism that acts specifically in the colon. Collectively, our results suggest that MC-specific LTβR facilitate the proper ILF organization and reveal a protective role for LTβR under colonic inflammatory conditions.

INTRODUCTION

1. The anatomy and physiology of the normal intestine.

The gastrointestinal (GI) tract, the long muscular tube that connects the mouth with the anus, includes the esophagus, the stomach, the small and large intestine. The small intestine (SI) is divided into three distinct parts: the duodenum, the jejunum and the ileum.[1]

The wall of small and large intestine comprises four distinct layers: the **mucosa**, **submucosa, muscularis externa** and **serosa** (Fig.1). The mucosa is the innermost layer and consists of the epithelium which forms finger-shaped protrusions projecting into the gut lumen, known as intestinal villi. In the bottom part of the villi, specialized structures exist -known as intestinal crypts- and their main role is to promote the self-renewal of the intestinal epithelium.[2] Mucosa includes also the **lamina propria** (LP) where many different cell types are located. Immune cells are either circulating or segregated into secondary lymphoid organs (include Peyer Patces and mesenteric lymph nodes) and isolated lymphoid follicles (ILFs). Mesenchymal cells form loose connective tissue while lymphatic and blood vessels form an extensive network. The submucosa, the second layer, comprises mainly mesenchymal cells and neural cells, the muscularis externa includes muscle layers and the serosa, the outer layer of the intestine, consists of loose connective tissue.[1]

This complicated structure supports the communication of the intestine with the external environment. Over 100 trillion microorganisms reside in the human intestine and share a mutual beneficial relationship with the host. The **commensal microbiota** participates in the nutrition uptake and the energy balance of the host and in turn, the host provides them with essential factors for their maintenance. Microbiota triggers also the development of intestinal lymphoid organs. Immune tolerance between the host and the microbiota ensures the gut homeostasis.[3]

Various cells types contribute to the achievement of immune tolerance in the intestine. Epithelial cells shape the first barrier by secreting antimicrobial peptides and expressing pattern-recognition receptors (PRR) such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs). Through this, they participate in shaping the composition and localization of intestinal microbiota. Parts of the innate immune system such as tolerogenic dendritic cells (DCs) and macrophages as well as T-regulatory cells (Tregs), through the secretion of TGF-β and IL-10, promote the immune tolerance of the intestinal microbiota.[4] However, disruption of this intestinal homeostasis between microbes and the host can lead to diseases such as colon cancer or inflammatory bowel disease (IBD).

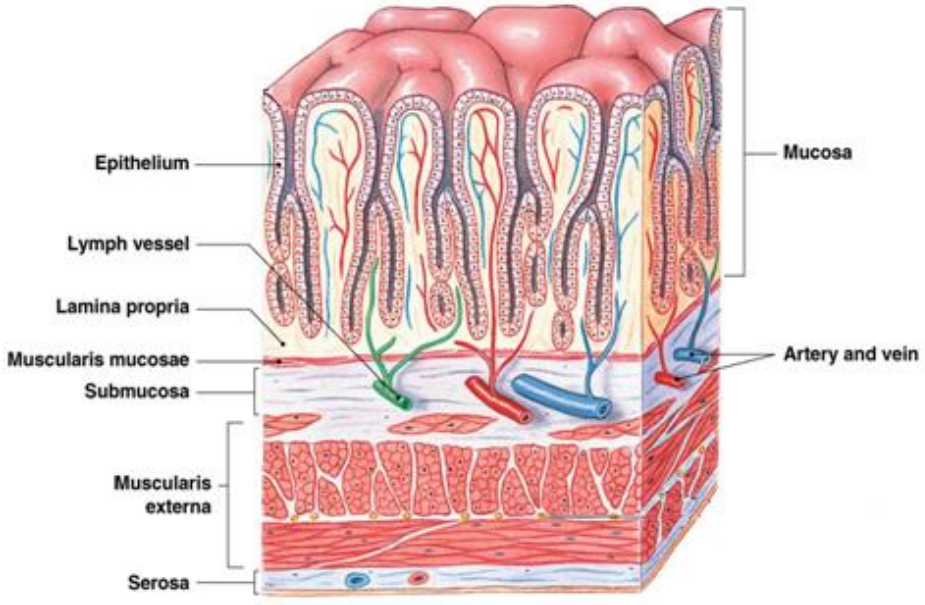

Figure 1. The architecture of the small intestinal wall. The intestinal wall consists of 4 distinct layers: mucosa, submucosa, muscularis externa and serosa. Various cells types such as epithelial, mesencymal, endothelial and immune cells participate in the organization of these layers. *(Pearson Education 2013)*

2. Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a multifactorial, chronic, inflammatory disease of the intestine with increasing incidence worldwide (0,04-24,3/100.000)[5]. IBD onset may occur at any age, but most patients are diagnosed between 25 and 35 years old. **Ulcerative colitis (UC) and Crohn's disease (CD**) are the two main distinct

pathological disorders of IBD that share common symptoms such as diarrhea, hematochezia, and abdominal pain.[6]

The etiology of IBD is still unclear. However, genetic, microbial and environmental factors have been associated with IBD initiation.[7] Until now genome-wide association studies (GWAS) have revealed many susceptibility loci for IBD in genes mainly related to autophagy, autoimmunity, innate immunity, and phagocytosis.[8] In addition, many environmental factors such as smoking and antibiotics have been associated with IBD. Dysbiosis has been linked with the IBD pathogenesis, but it is not clear whether it's a cause or a consequence of the pathology. Enhanced immune auto reactivity against microbiota antigens has been reported both in CD and UC patients with the identification of intestinal antimicrobial antibodies in their serum.[9,10] Also, increased number of specific microbial families, mainly from phyla Bacteroidetes and Proteobacteria, have been found to colonize the intestine of IBD patients.[11] These factors interact with the immune system and seem to contribute to the disease susceptibility.

2.1 Cell types participating in IBD

Various cells types, through secreted factors or internal signals participate in the pathogenesis of the disease.

Intestinal epithelial cells (IECs) are major cells participating in the initiation and progression of IBD that forms the intestinal mucosal barrier by secreting mucus and antimicrobial peptides. Any disruption of this barrier may be responsible for the entrance of pro-inflammatory microbes or immunogenic environmental antigens in the gut lumen and the initiation of immune responses[12]. IECs sense the environmental changes by expression of PPRs receptors. Alterations in the expression of specific TLRs, such as over-expression of TLR2 and TLR4 in IBD patients[13], may affect the ability of IECs to distinguish commensals from pathogenic microbes or amplify immune responses. Besides the mucosal barrier that IECs constitute, emerging evidence propose that they can also act as non-professional

antigen presenting cells (APCs), served by their expression of MHC class II molecules[14,15]. In vitro experiments revealed that IECs from IBD patients exhibit impaired activation of $CD8^+$ regulatory T cells suggesting a potential mechanism through which IECs directly affect adaptive immune system in IBD pathogenesis.[16]

Another very important stromal cell type involved in IBD is the **intestinal mesenchymal cell (IMC)**. Intestinal myofibroblasts and fibroblasts seem to promote chronic intestinal inflammation by extracellular matrix (ECM) deposition, increased production of proinflammatory cytokines and disruption in the balance of inflammatory cells in the mucosa[17]. Indeed, IBD-derived fibroblasts in response to PDGF, bFGF, and TGFβ1 produce increased collagen[18] while they migrate less and show an altered phenotype compared to normal intestinal fibroblasts.[19] Additionally, epithelial to mesenchymal transition (EMT) has also been associated with persistent fistulae in Crohn's disease[20] and increased intestinal fibrosis during IBD.[21]

Besides stromal cells, other cell types, part of the immune system, cooperate and perpetuate the inflammation in the intestinal environment during IBD progression (Fig.2). Neutrophils, macrophages and DCs are key innate immune players in the intestine of IBD patients.[22,23,2425] CD is characterized as a mixed Th1/Th17 inflammatory condition.[26,27,28,29] Th1 produced cytokines such as IL-6, TNF and IFN-γ are elevated in CD patients.[30,31,32] Th17 cells exert a pro-inflammatory role via secretion of IL-17A, IL-17F and IL-21[33] while they produce IL-22, an important anti-inflammatory cytokine acting on intestinal epithelial cells.[34] It has been suggested that Th17 cell responses mediate tissue destruction under intestinal inflammatory conditions.[35] Humoral immunity has also been implicated in the IBD pathogenesis. Interestingly, serum antibodies against specific microbial antigens as well as antibodies against the tropomyosin 5 and 1 isoforms [36] and anti-neutrophil cytoplasmic antibodies are detectable in sera of many UC and CD patients.[37]

Given the significant role of immune system in the IBD pathogenesis, increased lymphoid structures are developed in colon and SI of IBD patients.[38,39] These structures that arise ectopically in the inflamed mucosa are defined as tertiary lymphoid organs (TLOs).[26]

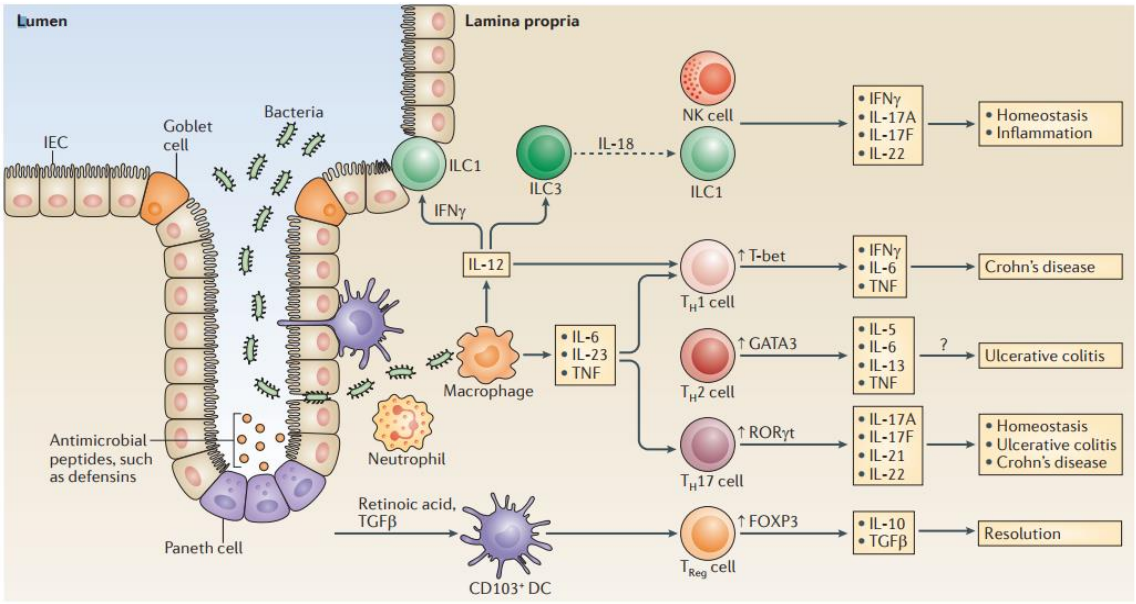

Figure 2. Cytokines and immune cells involved in IBD pathogenesis. Various immune cell types produce pro-inflammatory and anti-inflammatory cytokines in the LP of IBD patients. These cells include members of the innate immune system such as DCs, neutrophils, macrophages, NK cells, ILCs and leykocytes such as effector T cells (T helper 1 (TH1), TH2 and TH17) and Tregs. The key transcription factors and cytokines produced by T helper cell subsets in IBD-affected mucosa are shown.[29]

2.2 Tertiary lymphoid organs

The initiation of adaptive immune responses requires the activation of immune cells in specialized immunological filters known as, secondary lymphoid organs (SLOs) which include the spleen, lymph nodes (LNs) and Peyer's patches (PPs). However, in response to diet and host microbiota or under chronic inflammatory conditions, hematopoietic and mesenchymal cells interact to build specialized lymphoid structures, defined as **tertiary lymphoid organs (TLOs)**.[40] TLOs can be found in almost every inflamed tissue. Both lymphatic vessels (LVs)[41] and high endothelial venules (HEVs)[42] have been described inside TLOs. They are compartmentalized into a distinct B-cell area surrounded by a few T cells and DCs. The development and

function of TLOs are supported, by a mesenchymal network, consisting of LTβR expressing cells that promote the TLO's development.

Although TLOs share some common characteristics with SLOs, they are considered as distinct structures. Their main differences are that TLOs are developed **postnatally** in response to external stimulus, and their formation is not genetically programmed. TLOs have a similar role with SLOs in initiating immune responses. Germinal centers are formed inside the TLOs where class switching, affinity maturation and somatic hypermutation occur.[40]

Regarding the role of TLOs in disease, it still remains unclear whether they act in a beneficial or detrimental manner. Increased TLOs numbers have been detected in autoimmunity, infection and cancer.[43] It is believed that the increased demand for localized immune responses drive the development of TLOs. Evidences from patients show that increased TLO numbers are associated with RA,[44] human autoimmune thyroiditis,[45] systemic lupus erythematosus (SLE)[46] and multiple sclerosis (MS)[47] progression while their presence in cancer tissues may be beneficial.[48] Additionally, TLOs seem to have a protective role against atherosclerosis.[49] A potential therapeutic target would be the modulation or inhibition of TLOs function, depending on the disease.

2.2.1 Cryptoparches and Isolated lymphoid follicles.

Gut associated lymphoid tissue except from mesenteric LNs and PPs includes also tertiary lymphoid structures in SI and colon defined as **cryptopatches (CPs) and isolated lymphoid follicles (ILFs)**. CPs have an average diameter of 50μm, are located close to the intestinal crypts and comprise hematopoietic $ckit^+$ $IL7R\alpha^+$ $ROR\gamma t^+$ LTi-like cell population, along with a small proportion of $CD11c^+$ DCs.[51,52] Specific molecular events, such as the LTβR signaling induce the maturation of CPs that give rise to ILFs.[53] ILFs are bigger than CPs, with an average diameter of 150μm. 100-200 ILFs are found to the murine intestine.[54] ILFs vary from small B cells segregations (immature) until organized B-cell follicles with GCs (mature). ILFs are surrounded by a specialized type of epithelium defined as follicle-associated epithelium (FAE) that

contain M cells, a cell type that participates in the uptake of antigens from the gut lumen.[53] Mature ILFs contain also few T cells, dispersed throughout the structure as well as $CD11c^+$ DCs (Fig.3). Besides the immune cell compartment of CPs and ILFs, ILFs contain $VCAM^+$ $ICAM^+$ mesenchymal cells that support the development and their own function. However, their role hasn't been well defined. The role of CPs/ILFs remains hazy. They have been implicated in the local thymus-independent generation of intraepithelial T cells and the generation of intestinal IgA responses. During intestinal inflammatory conditions such as in IBD patients, an increased number of ILFs has been observed in colon and SI.[38,39]

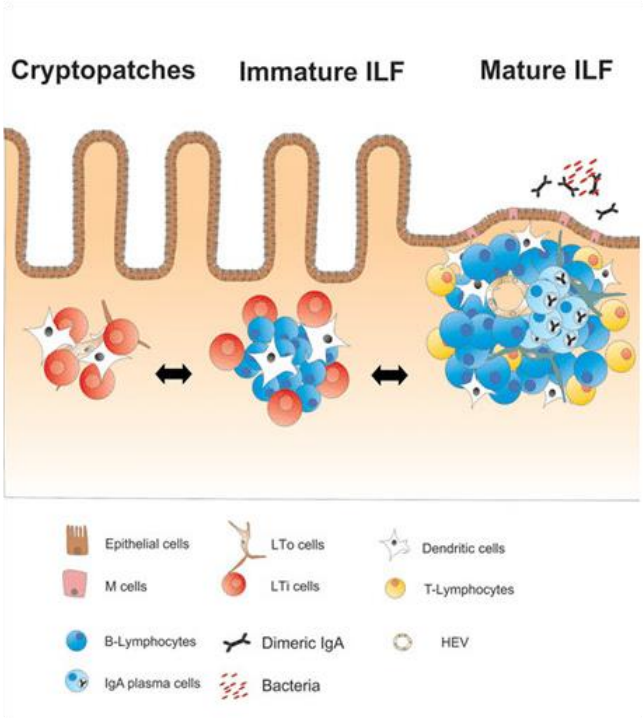

Figure 3. The structure and anatomy of CP and ILFs in the small intestine. CP, the precursors of ILFs start to develop into immature ILF by recruiting B cells. Mature ILF contain one big B cell follicle, germinal centers, vascular structures, and a follicle associated epithelium.[50]

Cryptopatces have been reported to appear at around 14-17 days after birth in mice[51] while ILFs are detected during the weaning period (21-28 days).[55] Dietary products such as polyphenols and glycosinolates that interfere with Aryl hydrocarbon receptor (Ahr) and vitamin A are required for the development of CPs/ILFs. Commensal microbiota also, modulate the maturation of CPs into ILFs via innate immune receptors such as TLR2/4, MyD88 and NOD2.[56,57] Although germ free mice develop ILFs with reduced size, their total number is not affected.[58]

2.3 Intestinal mesenchymal cells

Mesenchymal cells (MCs) are a heterogeneous population of mesoderm-derived multipotent cells. Intestinal mesenchymal cells (IMCs) comprise various cells types such as subepithelial myofibroblasts and fibroblasts that share common origin and common markers.[62] Their physiological role is to produce ECM components, growth factors and enzymes, while they interact with various cell types such as epithelial and immune cells.[63] MCs reside in the base of the crypts can provide the intestinal epithelial stem cells with essential factors for their expansion and proliferation under both homeostastic or inflammatory conditions.[64] Recently, $CD34^+$ MCs were identified to be located close to intestinal crypts and provide essential factors supporting intestinal epithelial stem cell niche both under homeostatic conditions and injury.[64] This $CD34^+$ MC population is distinct from sub epithelial myofibroblasts which are $CD34^-$.

IMCs have also an important role in orchestrating the immune responses inside the lymphoid tissues. During the development of PPs and mLNs mesenchymal cells defined as lymphoid tissue organizer cells (LTos) that express LTβR interact with LTi cells expressing $LT\alpha_1\beta_2$ and thus promote the further **development of intestinal SLOs**.[65] IMCs also support the structure and function of SLOs. **Follicular dendritic cells (FDCs)** which localize in the B cell area promote the B cell homing by secreting CXCL13 while **fibroblastic reticular cells (FRCs)** in the T-cell area secrete CCL19 and CCL21 to attract and maintain T cells.[66,67] **Marginal reticular cells (MRCs)** reside underneath the marginal sinus, but their exact role is still unclear. Finally, a mesenchymal cell population known as pericytes surround and support the function of blood and lymphatic vessels.[68] Inside the TLOs they have been identified FDC-like populations and MRC-like populations but they have been poorly studied.[69] Recently, $CD34^-$ MCs that reside inside the ILFs were found to produce oxysterol, a cholesterol metabolite that LTi-like cells sense, promoting with this interaction their migration in CPs and the formation of ILFs.[70] Interestingly, in the same study, $CD34^+$ MCs that surround ILFs produced the oxysterol degrading enzyme creating in this way an oxysterol gradient between ILFs and LP.

Furthermore, IMCs regulate the immune responses by **secreting** various cytokines, chemokines, growth factors and other immune mediators (Fig. 4). Depending on the microenvironment, IMCs can play an immunosupressive or immunostimulatory role. Intestinal fibroblasts and myofibroblasts express TLRs,[71] while in vitro stimulation of human duodenal fibroblasts with LPS, IL-1 and TNF induced the expression of pro-inflammatory cytokines and adhesion molecules such as ICAM-1 and VCAM-1, indicating that they are involved in the initiation and/or regulation of intestinal inflammation.[72] Colonic MHC-II^{+} myofibroblasts can function as non professional APCs presenting antigens to CD4^{+} cells.[73] IMCs can also participate in immune tolerance as they express the immunosuppressive PDL-1 and PDL2 molecules[74] and can also induce the expansion of Tregs.[75]

Although there is some evidence about the role of this heterogeneous cell population, IMCs are still poorly defined. Cre-mediated recombination has contributed to the further characterization of IMC subtypes and their role in homeostasis and disease. Several genetic tools has been developed and target different MC subpopulation. **Twist2-cre** is a broad MC-targeting cre line in the intestine targeting almost 95% of MCs.[76] **ColVI-cre mouse line** is also an important genetic tool developed in our lab that targets a subepithelial mesenchymal cell subset (30%)[77] as well as mesenchymal cells inside the SLOs and ILFs.[69] These ColVI-cre+ cells, through TNFR1, have been found as sufficient target for pathogenic TNF in TNF$^{\Delta ARE}$ mouse model, both for rheumatoid arthritis [77] and CD (unpublished data).

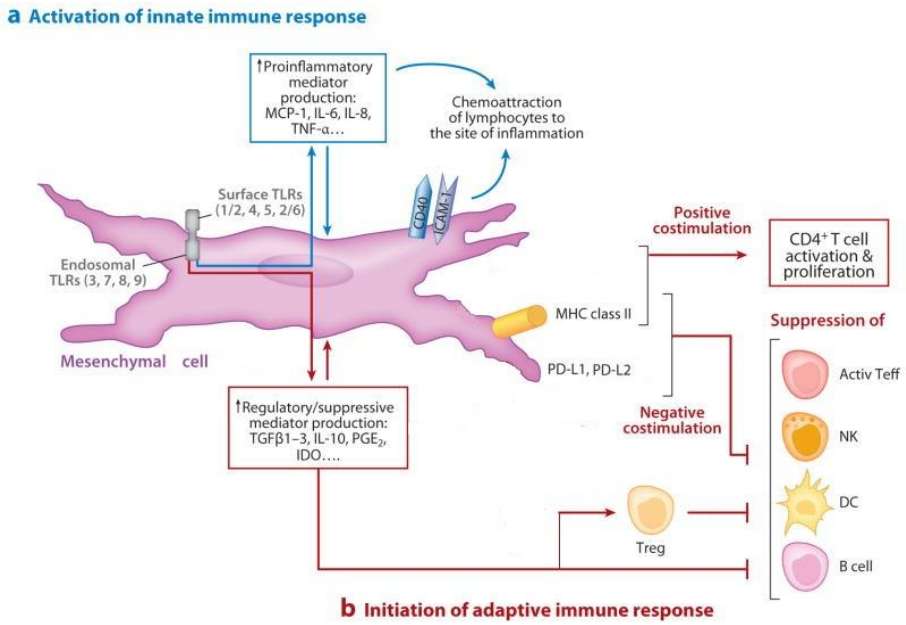

Figure 4. MCs as activators of both innate and adaptive immunity. a/MCs express TLRs that induce the expression of proinflammatory cytokines and adhesion molecules and the attraction of immune cells. b/ MCs express MHC class II molecule and act as non professional APCs activating $CD4^+$ T cells. Depending on the microenvironment MCs function in either a tolerogenic or an immunostimulatory role modulating the activity of T NK, DC, and B cells.[63]

2.4 Tumor necrosis factor (TNF)

Tumor necrosis factor is a major pro-inflammatory cytokine elevated during intestinal inflammation. TNF is expressed as a homotrimeric transmembrane form (26kDa) and it's cleaved into a soluble form (17kDa) by the metalloproteinase TNF-converting enzyme, TACE which is also known as ADAM17.[78] Both transmembrane and soluble TNF are augmented in IBD patients.[79] TNF signals via two different receptors, TNFR1 (55kDa) which is expressed by all cell types and TNFR2 (75kDa) which expression is restricted in specific cell types such as mast cells, Tregs, endothelial cells, mesenchymal cells and neuronal cells.[80] TNFR1 signaling cascade includes the activation of canonical NF-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathway which induce the expression of proinflammatory genes.[81] However, when NF-κB is inhibited, TNFR1 can induce cell death and necroptosis.[82] TNFR2 is responsible for the activation of both canonical and

alternative NF-κB signaling pathway while it induces the MAPK signaling via activation of JNK kinase.[81] TNF can be secreted by various cells and [29]depend on the target cell it can induce the expression of pro-inflammatory cytokines, promote angiogenesis and tissue destruction (Fig.5). [83]

Anti-TNFs were first approved as therapeutic agents for IBD in 1998 and until now 4 monoclonal antibodies are used in clinical practice: infliximab, adalimumab, golimumab and certolizumab pegol.[84] These drugs usually are used as a second line of therapy, after the failure of steroids and/or immunomodulators. However, need for new and more efficient TNF-agents has arise due to decreased response in many patients after a long period of administration. Putative mechanisms of anti-TNF function include monocyte and T-cell apoptosis,[85,86] antibody-dependent cell-mediated cytotoxicity, and complement-dependent cytotoxicity.[87,88] They also inhibit main TNF function such as of DC maturation, immune cell activation and proliferation, cytokine and chemokine release and Tregs supression.[84]

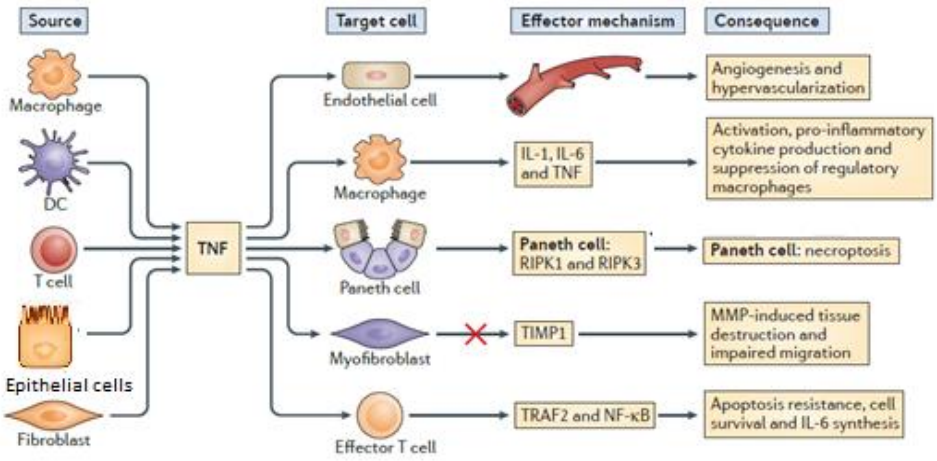

Figure 5. The central role of TNF in the pathogenesis of IBD. TNF is produced by various immune and mesenchymal cells. It exerts a proinflammatory role in the LP of IBD patients. It may promote angiogenesis and hypervascularization, induce the production of pro-inflammatory cytokines by macropahges and T-cells, alter the intestinal barrier function, induce cell death of intestinal epithelial cells (IECs) and Paneth cells. TNF can act also on mesenchymal cells where it promotes the increasing production of matrix metalloproteinases (MMPs) and thus the tissue destruction. Finally, it can induce resistance to apoptosis in T cells. [29]

2.5 TNF$^{\Delta ARE}$ mouse model

A major contribution for the dissection of the mechanisms during IBD pathogenesis and the development of novel therapeutic drugs was provided by the generation of more than 66 IBD animal models that are classified into chemically induced, cell-transfer, congenial mutant, and genetically engineered models.[89]

TNF$^{\Delta ARE}$ mice belong to the genetically engineered mouse models that develop spontaneously a granulomatous Crohn's disease-like disorder, as well as polyarthritis[90] and heart valve disease. TNF$^{\Delta ARE}$ mice were generated by a targeted deletion of a 69-bp gene segment including AU rich elements (ARE) in its 3'-UTR region. ARE regulatory elements are responsible for the destabilization of the transcript and translational signaling and thus lack of ARE in TNF$^{\Delta ARE}$ mice results in TNF overexpression (Fig.6). The intestinal inflammation in these mice is restricted to the terminal ileum and occasionally to the proximal colon and starts appearing in around 6-8 weeks old mice.[89] Histopathological analysis revealed that there is extended mucosal, submucosal and transmural infiltration of chronic and acute inflammatory cells, while villi are blunted and broadened.[91]

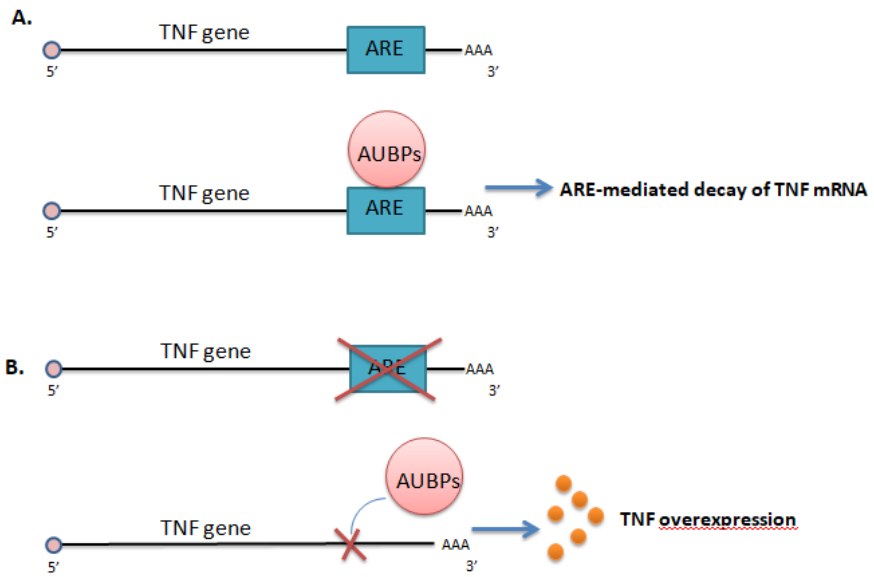

Figure 6. TNF overexpression in TNF$^{\Delta ARE}$ mice. A/ In the cytoplasm, AUBPs bind to ARE elements (AUUUA) in the 3'-UTR of TNF mRNA promoting its degradation. B/TNF$^{\Delta ARE}$ mice lack the ARE elements in the TNF gene disrupting the binding of AUBPs and thus promoting the TNF overexpression. [91]

TNF overexpression specifically by intestinal epithelial cells (IECs) or macrophages is sufficient to induce full Crohn's disease-like pathology in this model (Fig 7).[92,93] $TNF^{\Delta ARE}$ mice that lack TNFR1 didn't develop intestinal inflammation while deletion of TNFR2 attenuated the pathology indicating that both TNF receptor signaling promote intestinal inflammation.[91] However ColVI-cre specific reactivation of **TNFR1 on IMCs** was sufficient for the development of the disease, indicating that IMCs are the targets of pathogenic TNF in this model.[77] Crohn's disease-like pathology developed in $TNF^{\Delta ARE}$ mice is microbiota-dependent, as $TNF^{\Delta ARE}$ mice bred in germ-free conditions or treated with antibiotics don't develop intestinal pathology.[94]

The inflammatory ileitis that $TNF^{\Delta ARE}$ mice develop is **$CD8^+$ T cell-dependent** and is associated with increased Th1-like cytokine response including elevated TNF, IFN-γ and IL-12p40 production.[93] Additionally, β7 integrin has been shown to mediate intestinal pathogenesis as mice deficient for β7 integrin, showed a complete amelioration of the disease.[95] During the disease progression it has been shown that epithelial barier is defected as loss of Paneth cells[94] as well as decreased secretion of HCO^-_3 by IECs has been detected.[96] Finally, dysbiosis and antimicrobial peptide production is resulted from pathogenic alterations including loss of Paneth cells. Regarding the signaling pathways involved in the pathogenesis of this model, it has been found that Tpl2 and JNK2 promotes IBD pathogenesis while p38/MK2 exert a protective role.[93]

$TNF^{\Delta ARE}$ mice develop an increased number of TLOs with leukocyte infiltration in the deeper layers of the intestine.[60] RORγ-t deficient mice $TNF^{\Delta ARE}$ showed formation of intestinal TLOs in the terminal ileum showing TNF over expression overcome the requirement of RORγ-t^+ LTi cells.[61] Moreover, deletion of LTβR resulted in the absence of TLOs in these mice.[61] It hasn't been described yet if these mice lack CPs and which LTβR expressing population is responsible for the development of TLOs in this model.

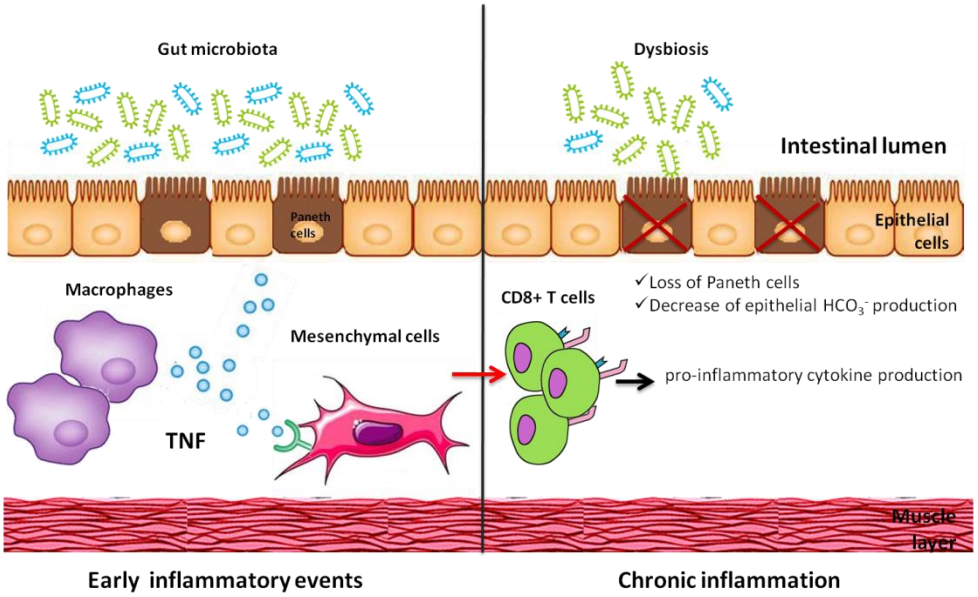

Figure 7. The proposed mechanism of TNF$^{\Delta ARE}$ model intestinal pathology. IECs and macrophages over-express pathogenic TNF in a microbiota-dependent manner. IMCs via expressing TNFR1 are the main targets of pathogenic TNF where it induces the secretion of pro-inflammatory cytokines such as TNF, IFN-γ and IL-12p40 and therefore the infiltration of CD8$^+$ T-cells. It also promotes decrease of the epithelial HCO^-_3 secretion, dysbiosis and loss of Paneth cells

3. Lymphotoxin-β receptor signaling

The *ltβr* gene resides on 12p13 chromosome in humans and on chromosome 6 in mice.[97] It encodes for LTβR, a transmembrane protein with 61kDa size. LtβR is mainly expressed on mesenchymal cell[98], monocytes,[97] alveolar macrophages,[99] mast cells[100] and DCs.[101] $LT\alpha_1\beta_2$, $LT\alpha_2\beta_1$ and LIGHT are the three distinct ligands for LTβR.[102,103] $LT\alpha_1\beta_2$ binds exclusively to LTβR while $LT\alpha_2\beta_1$ interacts also with TNFR1 and TNFR2. Additionally, LIGHT signals also via the herpes virus entry mediator (HVEM). Decoy receptor 3 (DcR3) is a soluble protein that competes with LIGHT for LTβR or HVEM.[104]

Binding of LTβR with its ligands induce the association of the cytosolic domain with TNF associated factors (TRAFs) 3,5 6. This can lead to:

1/ Activation of the **canonical** nuclear factor kappa-light-chain-enhancer of activated B cells **(NF-κB) pathway**. This includes the phosphorylation and activation of the IKK complex comprising catalytic subunits IKKα, IKKβ and two molecules of IKKγ (NEMO). IKK complex activation induce the phosphorylation and proteasomal degradation of IKβ which is an inhibitor of p50 and p65 heterodimmer (p50/p65). Therefore, p50/p65 translocate to the nucleus where it can activate the expression of proinflammatory genes such as VCAM1, IL-8 expression MIP-1β, MIP2.[105,106]

2/Activation of the **alternative NF-κB pathway**. This pathway involves activation of NF-kappa-B-inducing kinase (NIK) that phosphorylates IKKa dimmers which subsequently phosphorylate p100. This event along with the K48 polyubiquitination of p100 lead to the proteasomal degradation of p100 and its conversion into p52.[107] Then, RelB/p52 dimer translocate into the nucleus and activate the expression of homeostatic cytokines such as CCL21, CXCL13, and CXCL12.[108]

3/Activation of the c-Jun N-terminal kinase **(JNK) pathway.** LIGHT/LTβR-dependent activation of JNK/c-Jun has been reported in 293HEK, Hela cells and mouse embryo fibroblasts.[109,106]

4/Induction of **apoptosis**. LIGHT/LTβR can induce apoptosis in tumors cells by recruiting ciAPs and releasing with this way caspase-9,-7 and -3.[110]

3.1 The role of LTbR in lymphoid organogenesis and during intestinal inflammation.

LTBR signaling is important for the development of SLOs. The interaction between LTαβ expressing LTi cells and LTos expressing the LTβR is vital for the embryonic development of SLOs. More specifically, genetic deletion of LTa, LTβ, and LTβR results in the absence of PPs and LNs, defects in the architecture of the spleen[111,65] and impaired migration and activation of NK cells[112] and DCs.[113] However, $LT\beta^{-/-}$ mice retain some mesenteric lymph nodes.[114] Additionally, LTa and LTβ are required for the secretion of lymphoid tissue chemokines by stromal cells.[115,116]

Besides the important role of LTβR for the SLOs, it is required for the development of ILFs as mice deficient for LTα or LTβR lack ILFs.[117,118] Although, continuous treatment with LTβR-Ig led to ILF deficiency, mice treated *in utero* with LTβR-Ig or $LTa^{-/-}$ recipient mice of C57BL/6 bone marrow lack PPs and LNs but develop normal ILFs indicating that their development is inducible.[119] The first step for the development of CPs is the clustering of $ckit^{+}$ LTi-like cells, which belongs to the type 3 innate lymphoid cells (ILC3) and its presence depends on RORγ-t and the transcription factor Id2.[120,121] IL-7 signaling on hematopoietic cells induce the expression of $LT\alpha_1\beta_2$ that interacts with the LTβR on stromal cells. This interaction promotes via the activation of NF-κB, the further clustering of LTi-like cells and the maturation of CPs into ILFs through the secretion of CCL21, CCL19 and CXCL13. These chemokines are responsible for the recruitment of leukocytes in the ILFs. Moreover, the maturation of ILFs depends on the TNFR1 expressed on stromal cells.[119] $TNFR1^{-/-}$ mice lack mature ILFs but they develop normal PPs. Interestingly, RORγ-t mice that lack ILFs, under a TNF overexpression background, develop normal ILFs, indicating that TNFR1 pathway can bypass the requirement of RORγ-t^{+} LTi-like cells.[61]

Apart from LTβR signaling requirement for lymphoid organogenesis, LTβR expressed by stromal cells in the intestine has been proven important for the migration and maintenance of B cells in the LP. $LT\alpha^{-/-}$, $LT\beta^{-/-}$ and $LT\beta R^{-/-}$ mice show reduced levels of IgA in the LP,[122] while bone marrow transfer from wild type mice into irradiated $LT\alpha^{/-}$ reversed this effect in a PP/LN independent manner.[123]

LTβR signaling has been reported to promote intestinal inflammation in colitis mouse models by altering the lymphoid architecture in spleen and intestine.[124] LTβR blockade by LTbR-IgG treatment attenuated disease progression in two Th1-mediated mouse models[125] and in trinitrobenzene sulphonic acid (TNBS) induced colitis, a Th2-mediated colits model.[126] In dextran sulfate sodium (DSS)-induced colitis mice treated with LTbR-IgG show less severe disease with impaired leukocyte rolling.[127] However, in a more recent study it was found that LTβR-dependent IL-23 production by epithelial cells limits mucosal damage via induction of IL-22 by $CD4^{-}$ LTi cells in DSS colitis model.[128]

AIM

The contribution of LTβR in the lymphoid organogenesis and the regulation of the intestinal immune system have been acknowledged. However, its exact role during intestinal inflammation lacks evidence. Additionally, it is still unknown which is the actual LTβR expressing cells type that promotes ILF development. In this study we tried to:

- Elucidate the role of **LTβR** during **intestinal inflammation**
- Study the **MC-specific LTβR** role in **development of ILFs** both under homeostatic and inflammatory conditions.

To address these queries, we took advantage of the $TNF^{\Delta ARE}$ mouse model. To study the LTβR role during intestinal inflammation $TNF^{\Delta ARE}$ mice lacking LTβR were generated. Additionally, by deleting MC-LTβR in normal and $TNF^{\Delta ARE}$ mice we aimed to identify the MC-specific specific role during ILF development and intestinal inflammation.

The results will clarify the contribution of LTβR in IBD-like pathology of TNF-mediated IBD and provide new insights for the specific cell type responsible for the development and maintenance of intestinal TLOs under normal or inflammatory conditions.

MATERIAL AND METHODS

1. Mice

To delete LTβR we crossed LTβR$^{F/F}$, mice that have been previously described,[129] with deleter-cre mice, that constantly express a ubiquitously active general Cre deleter early in the embryogenesis, before implantation.[130] Through this, we managed to generate mice that lack LTβR (Deleter-cre LTβR$^{D/D}$). To delete LTβR specifically in MCs we crossed LTβR$^{F/F}$ mice with ColVI-cre mice,[77] ColVI-cre R26$^{mT/mG}$ reporter mice[69] and Twist2-cre mice.[76] ColVI-cre R26$^{mT/mG}$ mice express membrane-targeted tandem dimer Tomato (mT) in the Rosa26 locus and upon ColVI-Cre recombination express GFP. To study the LTβR role during intestinal inflammation, total or MC-specific LTβR deficient mice were crossed with TNF$^{\Delta ARE}$ mice.[90]

All mice were bred and maintained on a C57BL/6J genetic background in the animal facilities of the Biomedical Sciences Research Center "Alexander Fleming" under specific pathogen-free conditions. Experiments were approved by the Institutional Committee of Protocol Evaluation in conjunction with the Veterinary Service Management of the Hellenic Republic Prefecture of Attika according to all current European and national legislation and were performed in accordance with relevant guidelines and regulations.

2. Genotyping

For the genotyping of mice, DNA was isolated from the tail of newborn mice according to salt DNA extraction protocol. Briefly, the cells were lysed with a digestion buffer and proteinase K. The proteins were precipitated with NaCl 6M, the DNA was purified with isoprpopanol and precipitated with 70% ethanol. PCR was performed by using the primers:

for TNF$^{\Delta ARE}$: 5'-GAGCCAGCCCCCCTCGGAAGGCCGGGGTG-3' (forward), 5'-AATTAG GGTTAGGCTCCTGTTTCC-3' (reverse),

for LTβR: 5'-CAGTGGCTCCAAGTGGCTTG-3' (forward), 5'-GCAAACCGTGTCTTGGCTGC-3' (reverse),

for cre: 5'-ATTACCGGTCGATGCAACGAGT-3' (forward), 5'-CAGGTATCTCTGACCAG AGTCA-3' (reverse).

The following table shows the PCR programs followed.

	LTβR			**TNF$^{\Delta ARE}$**			**CRE**		
Step	**Temp. °C**	**Time (min)**	**Cycles**	**Temp. °C**	**Time (min)**	**Cycles**	**Temp. °C**	**Time (min)**	**Cycles**
Initial denaturation	95	5		94	3		94/94	4	x5
Denaturation	95	0:30	x35	93	1:30	x30	94/94	1/0:30	x25
Annealing	60	1		57	1		57/55	1/0:40	
Extension	72	1		72	1		72/72	1/1:10	
Final extension	72	5		72	10		72	10	

3. Histopathological analysis

Mice were euthanized and the terminal ileum (last 6cm) was harvested, flashed with modified Bouin's fixative (50% ethanol/5% acetic acid in dH2O) and prepared in swiss rolls.[131] Swiss rolls were fixed with formalin (O/N, 4°C), embedded into paraffin and cut in the microtome. Tissues were stained with Hematoxylin and Eosin (H&E) and observed in Nikon Eclipse E800 microcope, equiped with Q Imaging ExiAqua camera. Pictures were captured using Bioquant Q-capture Pro7 program. The inflammation in the terminal ileum was evaluated according to scoring scale ranging from 0 to 4. 0: normal intestinal morphology, 1: mucosal infiltration of immune cells, 2: mucosal with sporadic submucosal immune cell infiltration, 3: mucosa and extended submucosal infiltration of immune cells, 4: mucosal, sumucosal and transmural

infiltration of immune cells. Total disease score per mouse was calculated by the summation of inflammatory scores for each mouse (data represented as mean +/- SEM).

4. Immunohistochemistry

Paraffin embedded swiss roll sections from the terminal ileum (6cm) of mice were deparaffinized by incubating them at 60°C for 30min, embedded in xylene and rehydrated in descending grades of ethanol to water. Then the antigen retrieval was performed by microwaving the sections at full power for 20min in citrate buffer. The endogenous peroxidase was blocked with 3% H_2O_2 and the unspecific binding sites were blocked by 1%BSA/PBS. The sections were incubated (O/N, 4°C) with anti-B220 antibody diluted (BD Pharmigen) in 1%BSA/PBS. The following day, sections were rinsed in PBS and incubated for 1h at RT in biotinylated goat anti-rat antibody (Vector, 1/500) in 1%BSA/PBS followed by 45 min in ABC solution (prepared according to the manufacturer's instructions). Visualization of bound peroxidase was achieved DAB staining (Vector) according to manufacturer instructions. The reaction was stopped by several washes with dH_20. The sections were counterstained with hematoxylin and mounted with DPX mounting medium. Control sections were prepared in the absence of primary antibody (data not shown). The number of ILFs/cm were quantified as B cell clusters/cm of the terminal ileum.

5. Immunofluoresence

Mice were euthanized by CO_2 asphyxiation, according to institutional animal care and use committee (IACUC) guidelines and perfused with PBS and PFA 4%. The last 6cm of the terminal ileum were harvested, washed with PBS and fixed with PFA 4% (O/N, 4°C). Fixed terminal ileum was immersed in 15% and 30% sucrose/PBS for cryoprotection and snap-frozed in an OCT-filled mold on a liquid nitrogen-cooled metal surface. Tissue cryosections of 10 µm thickness were acquired, rehydrated in wash buffer (0.1% saponin in PBS), blocked in 0,1 saponin/PBS with 1% BSA and stained with the following antibodies: anti-CD35 (BD Pharmigen), anti-ColIV (Abcam), Alexa Fluor 594 conjugated anti-CD3 (Biolegend), biotinylated anti-B220 (Biolgend),.

Unconjugated antibodies were detected with the following secondary antibodies: Alexa Fluor 594-conjugated anti-rat-IgG (Invitrogen), Alexa Fluor 647-conjugated anti-rabbit-IgG (Invitrogen), Alexa Fluor 647-conjugated Streptavidin (Invitrogen).

6. Stromal cell isolation

SI and colon were harvested and washed with cold PBS. Epithelial cells were removed after incubation in Hank's balanced salt solution (HBSS) supplemented with 5 mM EDTA, 1mM DTT and 20 µM HEPES for 30 minutes at 37 °C. Then, both SI and colon were dissected into small pieces and digested using an enzyme mix comprised of 10%FBS/PBS containing 0.8 mg/ml Dispase (Roche), 0.1 mg/ml Collagenese P (Roche) and 0.1 mg/ml DNase I (Sigma) for 50 min at 37 °C, as it was described previously. Cells were counted and resuspended in FACs buffer (PBS supplemented with 0.5% bovine serum albumin (BSA) and 5 mM EDTA in PBS) at 10^7cells/m.

7. Isolation of LP lymphocytes

The last 6cm of the terminal ileum were harvested, flushed, washed with cold PBS and incubated with with Hank's balanced salt solution (HBSS) supplemented with. mM EDTA, 1mM DTT and 20 µM HEPES for 30 minutes at 37 °C. PPs were removed, and intestinal tissue was cut longitudinally in small pieces. Intestinal pieces were digested with 10%FBS/PBS containing 50u/ml DNase type I (Sigma) and 300u/ml Collagenase type XI (Sigma) for 45min at 37 °C as it was previously described. Cells were filtered through 70µm strainers, counted and resuspended in 2mM EDTA/PBS at $5x10^6$ cells.

8. Flow cytometry analysis

Stromal cells were stained for 20 min at 4 °C using the following antibodies: PerCP-Cy5.5 conjugated-anti-CD31 (Biolegend) PE-Cy7 conjugated anti-podoplanin (Biolegend) Alexa Fluor 700 conjugated anti-CD45 (Biolegend), APC-A780 conjugated anti-Ter119 (eBioscience) APC-Cy7 conjugated anti-EpCAM1 (Biolegend) and anti-LTβR (eBioscience). Alexa Fluor 647-conjugated anti-rat-IgG (Invitrogen)

was used as a secondary antibody to detect LTβR expression. LP lymphocytes were stained with Zombie NIR (Biolegend) for 15 min at 4 °C, blocked with a solution containing 5% normal mouse serum, 5% normal rat serum (Sigma) and anti-CD16/CD32 (Biolegend) and stained with Alexa Fluor 700 conjugated anti-CD45. To detect myeloid cells, cell suspension was further stained with: FITC conjugated anti-CD11b (eBioscience), biotinylated anti-GR1 (BD Pharmigen,), Pe-Cy7 conjugated anti-Cd11c (Biolegend) and APC-conjugated anti-MHCII (eBioscience). Alexa Fluor 647-conjugated Streptavidin (Invitrogen) was used as a secondary antibody to detect GR1 expression. To detect T cells, the suspension was stained with: PE-Cy7 conjugated anti-CD3e (eBioscience), PE conjugated anti-CD8a (BD Pharmigen), PE-Cy5 conjugated anti-CD4 (BD Pharmigen) and FITC conjugated anti-TCRβ (eBioscience). For the detection of B cells PercP-Cy5.5 conjugated anti- B220 (eBioscience) was used and then the cells were fixed with 100µl fixation buffer for 20min at 4 °C, washed with permeabilization buffer and stained with FITC-conjugated anti-IgA (). Finally, cells were acquired on a FACSCANTO II (BD Bioscience) and data were analyzed using Flowjo software (Tree star Inc).The absolute number of recovered cells following the LP lymphocyte isolation procedure was estimated using 123eBeads counting beads (Invitrogen).

9. Statistical analysis

Data are represented as mean± SEM (standard error of the mean). Statistical significance was determined by performing student t-test or multiple t-tests. p-value≤ 0.05 were accepted as statistical significant. Data were analyzed using GraphPad Prism 6.

RESULTS

1.ColVI cre-specific LTβR deficiency results in normal ILF number development.

To study the mesenchymal role of LTβR in the development of ILFs, mice with MC-specific LTβR deficiency were generated (ColVI-cre LTβR$^{F/F}$). The deletion of LTβR in ColVI-cre^{+} cells was assessed by crossing LTβR$^{F/F}$ mice with ColVI-cre R26$^{mT/mG}$ reporter mice. Flow cytometric analysis for the detection of LTβR in IMCs confirmed the deletion of the LTβR allele in these mice. Indeed GFP^{+} cells derived from the SI and colon did not show LTβR expression compared with control mice, in contrast with LTβR expression in Tomato^{+} cells that wasn't affected (Fig.8A). Next, we aimed to investigate whether the MC-specific LTβR deletion impaired the development of ILFs in the SI. ILFs were identified and quantified as B220+ clusters distributed in the intestinal wall of SI. Unexpectedly, normal numbers of ILFs were found in the SI of these mice, suggesting that ColVI-cre^{+} specific LTβR is not required for ILF formation (Fig.8B).

Unpublished data from our lab have shown that LTβR is required for the localization of MCs inside of SLOs. To examine whether it is also required for ILF MC localization, we analyzed the location of ColVI-Cre+ cells in MC networks of normal and LTβR$^{F/F}$ mice by confocal microscopy. In contrast to normal mice, where an extensive ColVI-cre^{+} MC network was formed inside the ILFs, LTβR-deficient ColVI-cre^{+} cells were located just only under the epithelium or surrounding blood vessels (Fig.9A). Furthermore, no differences were detected in the localization as B and T cell inside the ILFs (Fig.9B). These results indicate that non-Cre-expressing MCs are able to organize ILFs in ColVI-cre LTβR$^{F/F}$ mice, and reveal that mesenchymal LTβR plays an important role during their development.

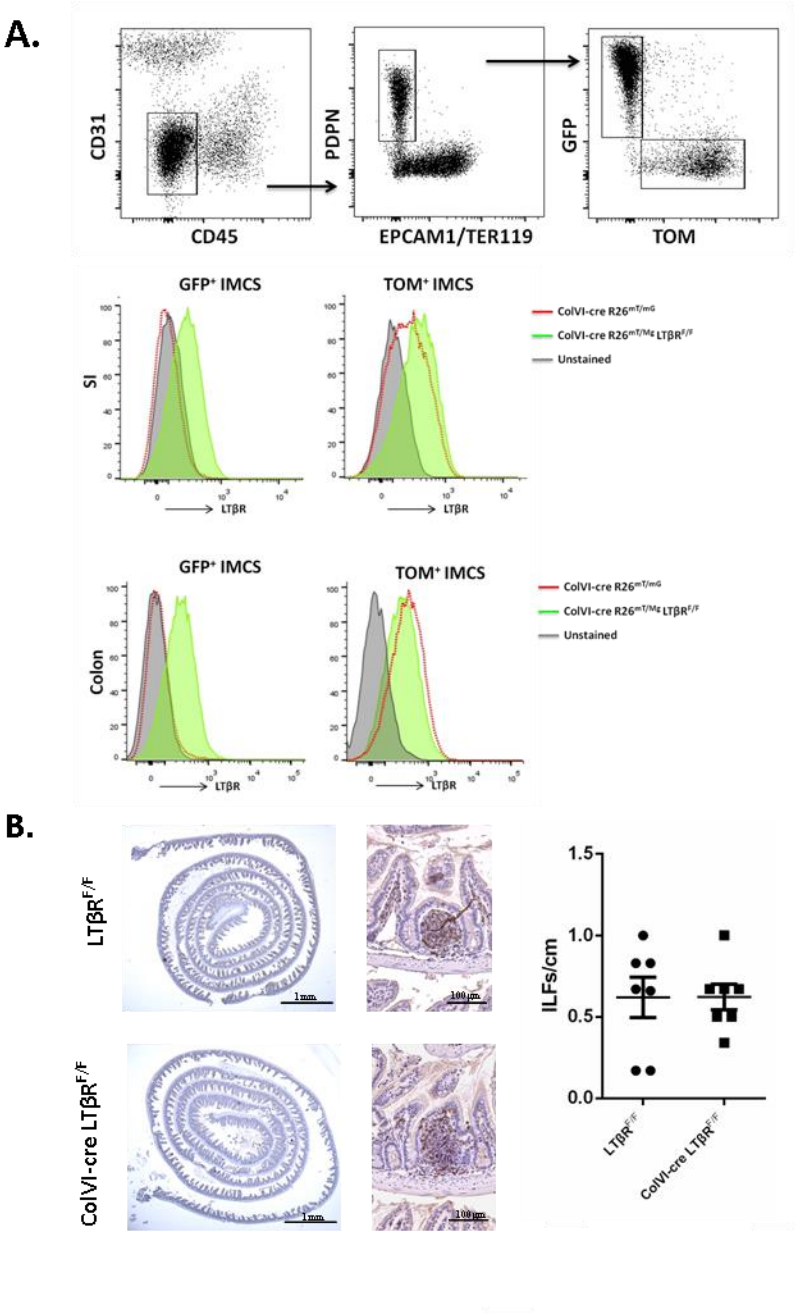

Figure 8. MC-specific LTβR deficient mice develop normal ILF number. A/ Flow cytometric analysis for the detection of LTβR expression in IMCs derived from ColVI-cre R26$^{mT/mG}$ LTβR$^{F/F}$ mice. IMCs were negatively selected as non endothelial, non hematopoietic, non epithelial and non erythrocyte cells (CD45$^-$CD31$^-$EPCAM1$^-$TER119$^-$) and positively selected as podoplanin+ cells. LTβR expression in TOM+ and GFP+ cells from SI and colon is represented by histograms. Three different mice of the indicated genotypes were analyzed. B/Representative immuno-histochemical staining for the B-cell marker, B220, in swiss roll sections and representative ILF images. Quantification of the ILF number from 12 week old mice. Data are presented as mean ± SEM.

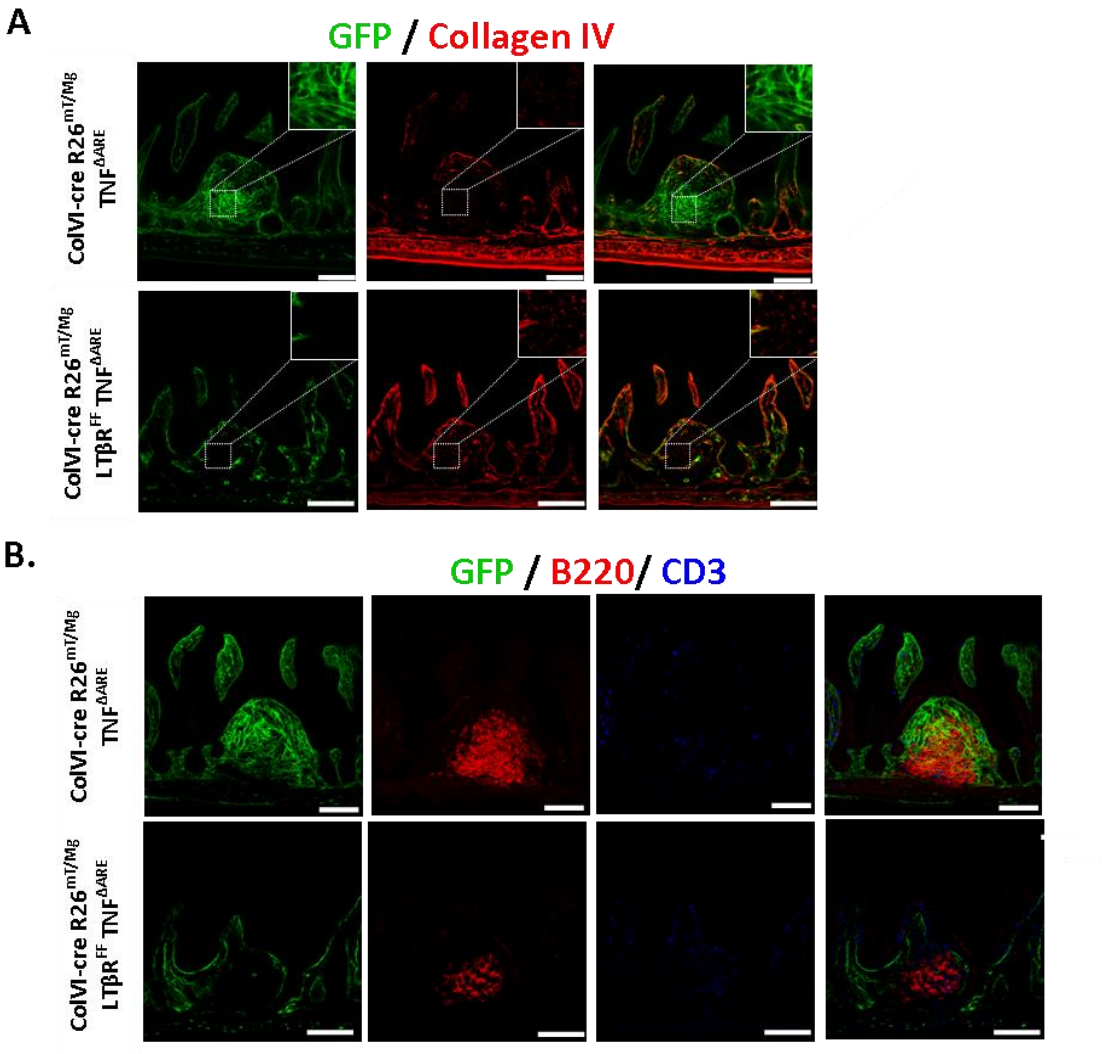

Figure 9. ColVI-cre specific LTβR deletion impairs the GFP$^+$ MC localization inside the ILFs.

A/ Representative confocal images of ILFs showing Cre-mediated GFP expression from ColVI-cre R26$^{mT/mG}$ LTβR$^{F/F}$ mice colocalizing with ColIV (A) and immune cell distribution (B). B cells are marked as B220$^+$ cells and T cells as CD3$^+$ cells. Scale bar, 100 μm, Several ILFs from 4 different mice (12 week old) were analyzed.

2. MC specific LTβR is dispensable for the disease progression in TNF$^{\Delta ARE}$ mice

To examine whether MC-LTβR plays a role in the development of ILFs under inflammatory conditions, we crossed ColVI-cre specific LTβR deficient mice with TNF$^{\Delta ARE}$ mice. The number of ILFs in the inflamed SI was quantified as previously described and did not reveal differences in 12 week (Fig.10A) and 20 week old mice (Fig.10B) compared with control littermates. Taking into consideration that GFP$^+$ MC network was impaired in mice lacking LTβR in MCs, we examined the cell

compartmentalization of ILFs in TNF$^{\Delta ARE}$ MC-LTβR deficient mice backcrossed with ColVI-cre R26$^{mT/mG}$ reporter mice. Indeed, when we analyzed the location of ColVI-Cre^{+} cells, they were restricted to the subepithelial layer and in the perivascular area. Interestingly, the FDCs were normally developed inside the ILFs (Fig.11B), indicating that also under intestinal inflammatory conditions another source of MCs which is not targeted by ColVI-cre is responsible for the proper organization of ILFs.

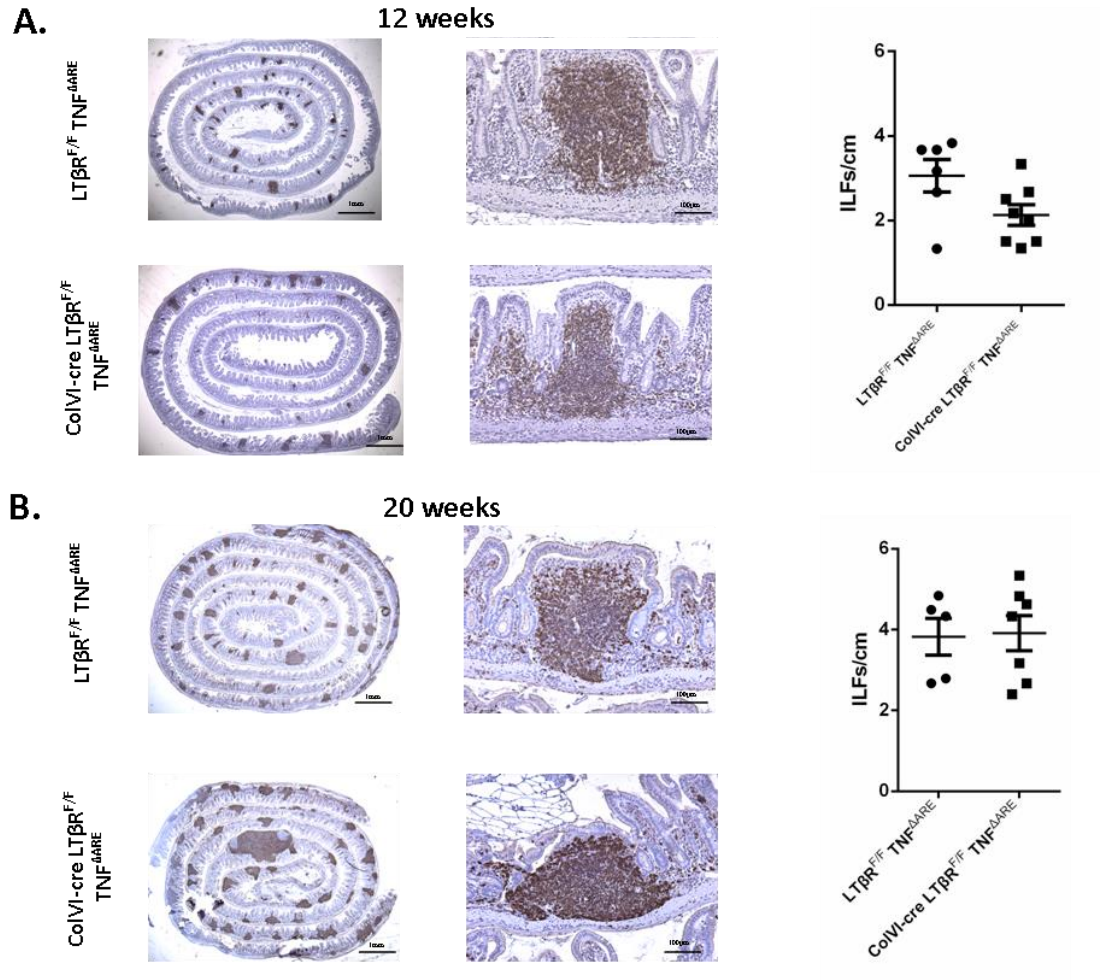

Figure 10. LTβR ablation in MCs does not alter the ILF number development in TNF$^{\Delta ARE}$model . Representative immunohistochemical staining of B220^{+} cells in swiss roll sections, representative ILF images and quantification of the ILF number from 12 week (A) and 20 week old mice (B). Data are presented as mean ± SEM.

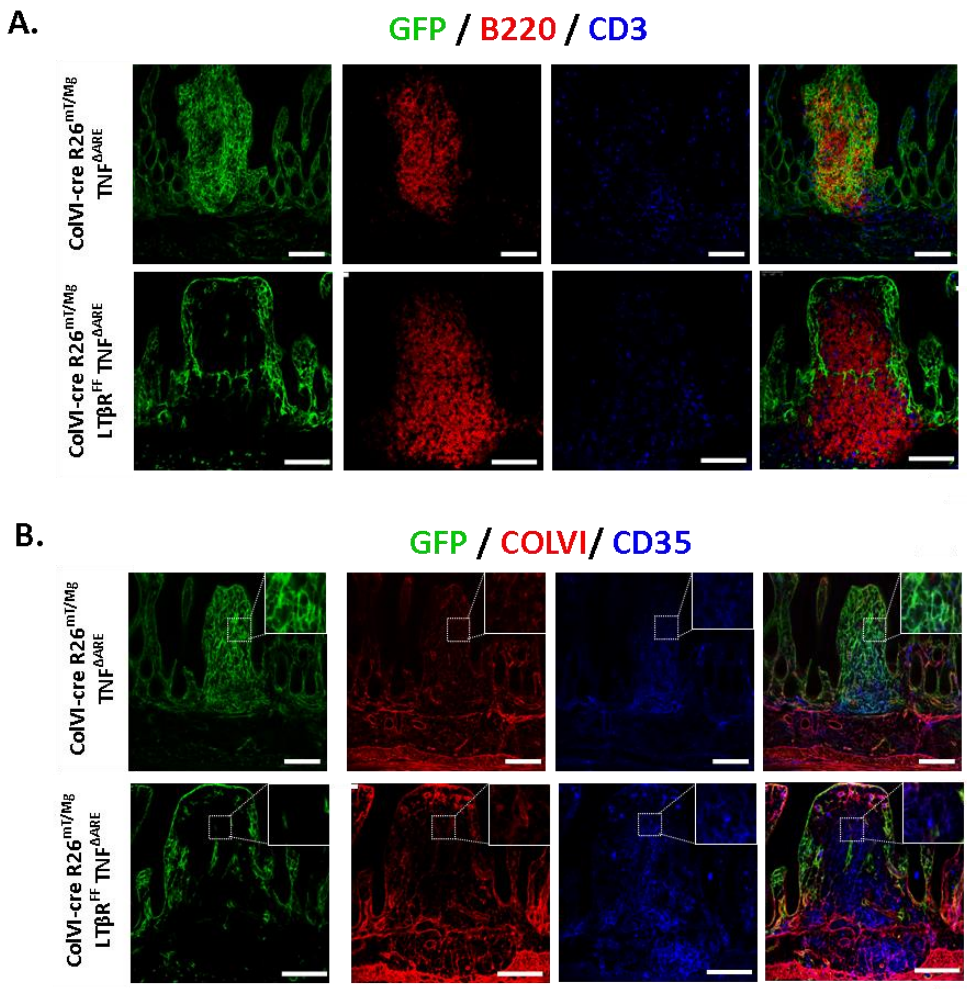

Figure 11. ILFs from TNF$^{\Delta ARE}$ mice deficient for MC-LTβR show defects in GFP^{+} MC localization

Representative images of ILFs showing Cre-mediated GFP expression and stained for A/ T cells (CD3 in red), B cells (B220 in blue), B/FDCs (CD35^{+} cells), podoplanin^{+} MCs. Scale bar, 100μm. Several ILFs from 4 different mice (12 week old) were analyzed.

Considering that these mice lack the ColVI-cre^{+} network inside the ILFs but we still target an intestinal subepithelial MC subpopulation, we examined the development of intestinal disease, independently of TLOs. Interestingly, mice exhibited inflammation with similar progression and severity compared to their littermate LTβR$^{F/F}$ TNF$^{\Delta ARE/+}$ mice, both in terminal ileum and proximal colon. Histopathological analysis of 12 (Fig.12A) and 20 week old mice (Fig.12B) didn't reveal any difference either in immune cell infiltration or villus blunting. We note that, unlike ILFs, GFP^{+}/Tom^{+} MC ratio in the intestine did not change (data not shown) suggesting that MC-specific LTβR expression does not affect the progression of intestinal inflammation the TNF$^{\Delta ARE}$ mouse model.

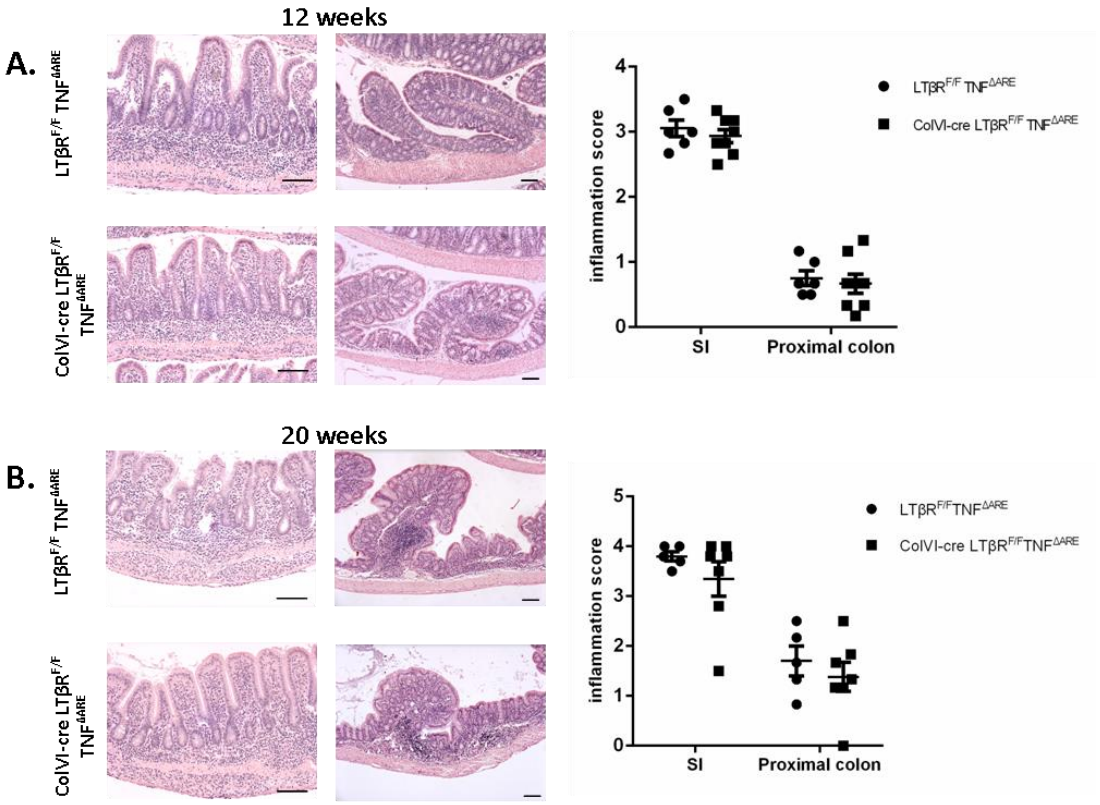

Figure 12. MC specific LTβR is dispensable for the disease progression in TNF$^{\Delta ARE}$ mice. Representative images of H&E-stained swiss roll sections and histopathological analysis performed in the terminal ileum and colon of 12 week (A) and 20 week old (B) mice. Scale bar (100µm). Data are presented as mean ± SEM.

To further confirm the histopathological assessment of the disease progression in TNF$^{\Delta ARE}$ mice lacking LTβR in MCs, we aimed to examine the immune cell infiltration in the LP of these mice. Flow cytometric analysis of the terminal ileum revealed a tendency towards a decreased number of infiltrating immune cells in the LP of SI including T cells, B cells and myeloid cell populations (Fig.13 A-C). Specifically, neutrophils showed a statistically significant reduction in their total numbers (Fig. 13C) indicating that MC LTβR plays a role in the neutrophil recruitment during inflammation.

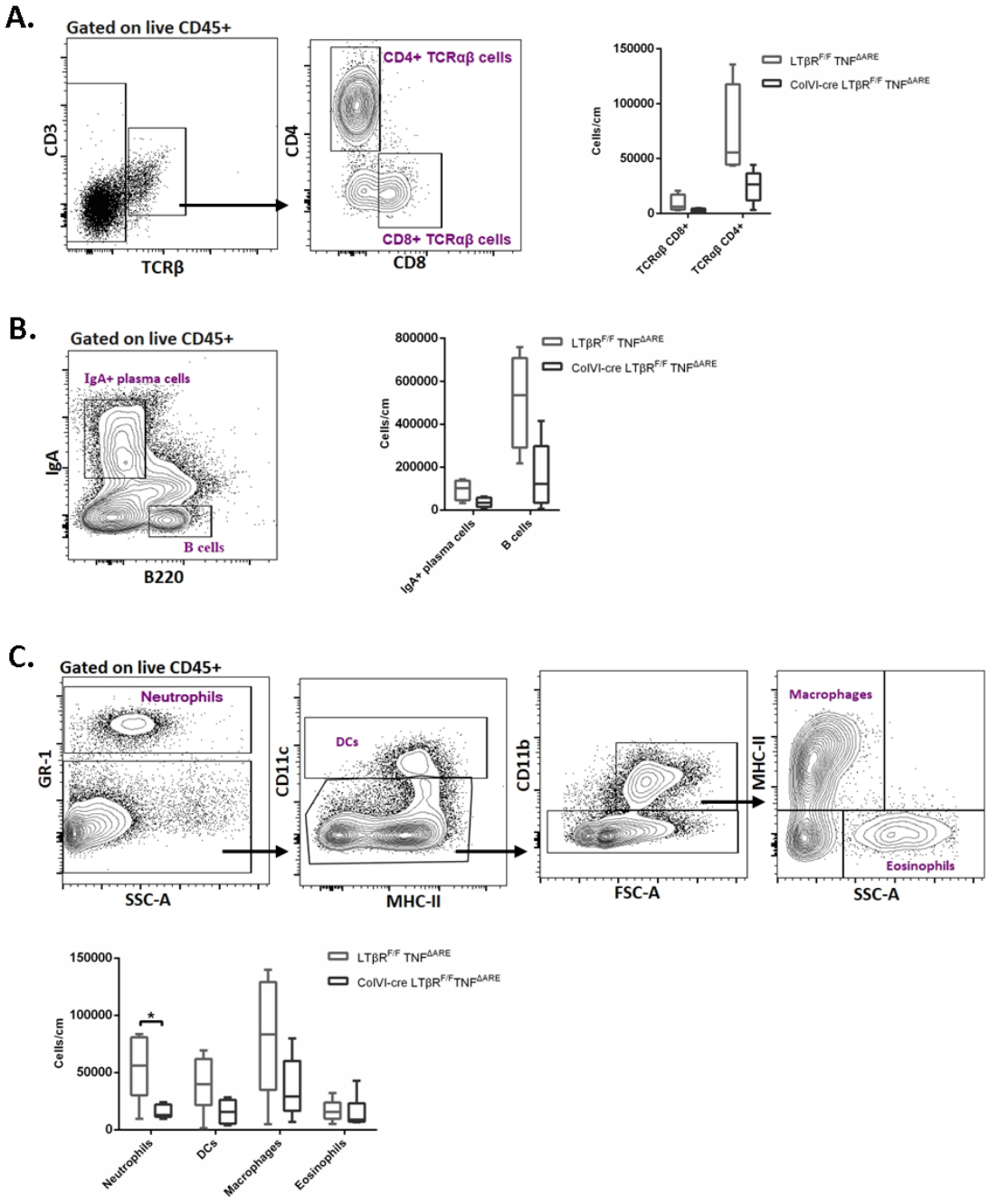

Figure 13. Immune cell infiltration in the SI LP from TNF$^{\Delta ARE}$ MC-specific LTβR deficient mice. Isolated LP lymphocytes LP from the ileum of 12-week-old mice ColVI-cre R26$^{mT/mG}$ LTβR$^{F/F}$ TNF$^{\Delta ARE}$ (n=4) were stained for A/ TCRβ, CD3, CD8, CD4, B/ IgA,B220, C/GR1, MHC-II, CD11c, and CD11b. Plots shown were gated on live CD45+ cells for (A) CD4+TCRαβ+, CD8+TCRαβ, (B) IgA+ plasma cells, B-cells, (C) neutrophils, dendritic cells, macrophages and eosinophils. Results are representative of 2 independent experiments

3. Normal ILF numbers are developed in the SI of Twist2-cre specific deficient mice.

To further explore whether the mesenchymal specific LTβR affect the development of ILFs, $LT\beta R^{F/F}$ mice were crossed with Twist2-cre mice, a broader mesenchymal specific cre line compared to ColVI-cre. Quantification of the ILF number as $B220^{+}$ clusters did not reveal any difference in the ILF formation compared with control littermates. (Fig.14) This indicates that LTβR in Twist2-cre^{+} cells does not affect the ILF number formation, without excluding that non-Cre-targeted MCs were responsible for ILF formation, similarly with the previous results .

4.LTβR limits colonic inflammation in $TNF^{\Delta ARE}$ mice

Given that LTβR KO $TNF^{\Delta ARE}$ mice do not develop ILFs we proceed to examine the role of TLOs under inflammatory conditions. To generate LTβR deficient mice, deleter-cre mice were crossed with $LT\beta R^{FF}$ mice. Then, deleter-cre $LT\beta R^{DD}$ were backcrossed to $TNF^{\Delta ARE/+}$ mice and the intestinal inflammation in the SI and colon was evaluated. Interestingly, we observed that inflammation in the SI didn't change between LTβR deficient mice and control mice (Fig.15A-B). However, $TNF^{\Delta ARE/+}$ LTβR deficient mice exhibited exacerbated inflammatory infiltration in the submucosa layer of the proximal colon, characterized by accelerated onset compared to $TNF^{\Delta ARE/+}$ LTβR sufficient mice (Fig.8A-B). These results show that LTβR exert a protective role, abrogating inflammation in the colon of the $TNF^{\Delta ARE}$ mice, independently of its redundant role for the development of ileitis in the same model.

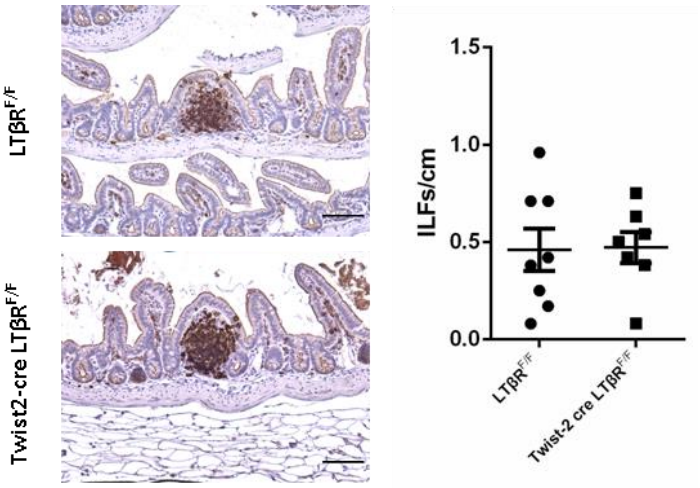

Figure 14. Twist2-cre specific LTβR deletion results in normal ILF number formation. Representative immunohistochemical staining of ILFs, stained as B220+ clusters and quantification of the ILF number from 12 week old mice. Scale bar (100µm). Data are presented as mean ± SEM.

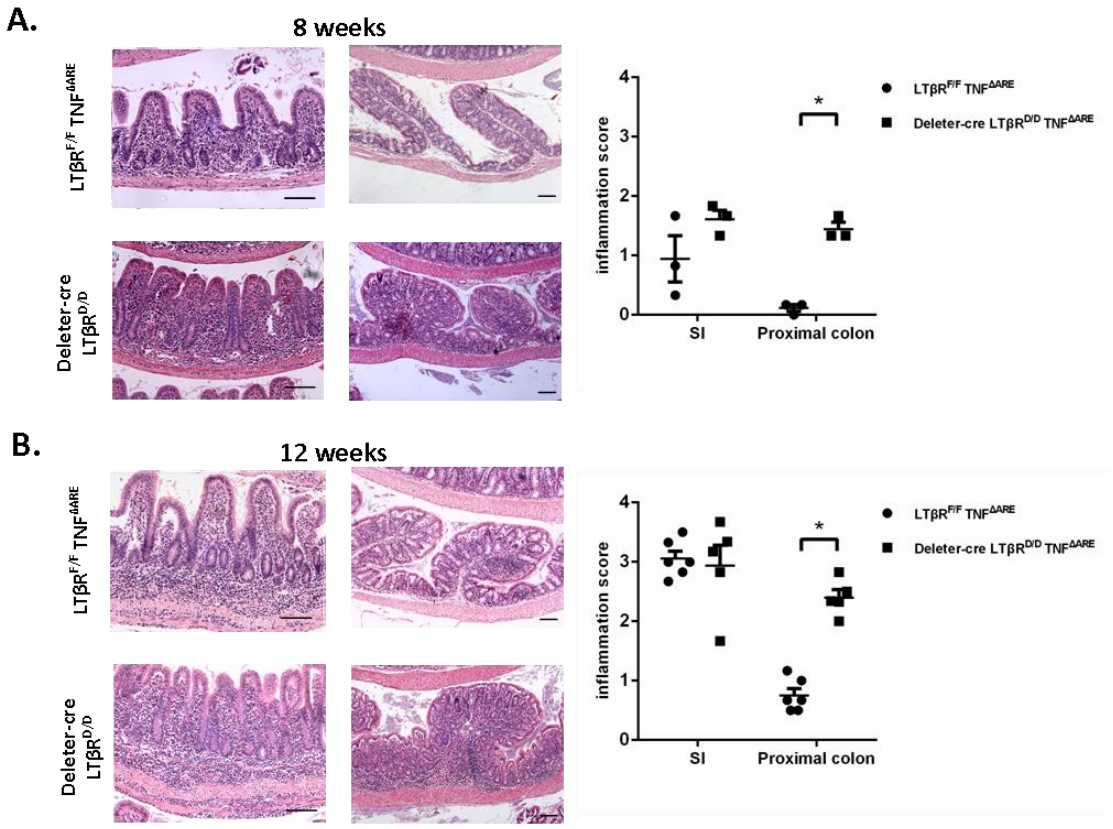

Figure 15. LTβR deficiency results in exacerbation of inflammation in the colon of TNF$^{\Delta ARE}$ mice. Representative images of H&E-stained swiss roll sections and histopathological analysis performed in the terminal ileum and colon of 12 week (A) and 20 week old (B) mice. Scale bar (100µm). Data are presented as mean ± SEM.

DISCUSSION

Intestinal LTβR signaling has gained more research interest as it is important for intestinal lymphoid organogenesis and participates in intestinal inflammation.[124] Stromal LTβR is required for ILF development in the intestine[119] but the exact LTβR expressing cell type responsible for the ILF formation is unknown. In addition, LTβR role during intestinal inflammation is still controversial. In this study we demonstate that LTβR on MCs is important for ILF development both under normal and intestinal inflammatory conditions as its MC-specific deletion impaired the MC localization inside the ILFs. Our work also provides evidence that TLOs in the intestine are not detrimental under inflammatory conditions, since ILF deficiency didn't attenuated intestinal pathology in $TNF^{\Delta ARE}$ model. We finally reveal that LTβR exerts a protective role in $TNF^{\Delta ARE}$ mouse model, suggesting an LTβR-dependent anti-inflammatory mechanism during intestinal inflammation.

Different studies have pinpointed contradictory roles for LTβR during intestinal inflammation. Although LTβR-Ig treatment attenuated inflammation in several colitis models,[125,126,127] mice with genetically LTβR ablation showed exacerbation during DSS colitis.[128] Indeed, our results from LTβR KO $TNF^{\Delta ARE}$ mice support that LTβR regulates colonic inflammation. We hypothesize that LTβR on epithelial cells may be responsible for this protective effect, as it has been previously proven that epithelial-specific LTβR limits mucosal damage by inducing IL-23 expression that promotes IL-22 production by RORγ-t^+ ILC3s.[128] The selective effect that LTβR has in the colon is further supported by previous studies that discuss the heterogeneity and the different molecular requirements between colon and SI. [132,133,70] However, it should be taken into consideration that LTβR KO mice show defects in the lympho–epithelial cross talk inside the thymus, and thus defects in the T-cell selection[134] that may affect T-cell responses in the inflamed intestine. Collectively, these findings reveal the importance of LTβR in regulating intestinal inflammation and raise more questions about the mechanism underlying this effect.

Our results from MC-LTβR deficient mice showed that LTβR on MCs is dispensable for the disease progression and exclude the possibility that MCs are responsible for the protective LTβR role in the $TNF^{\Delta ARE}$ mouse model. Unpublished data from our lab

have shown that MCs in TNF$^{\Delta ARE}$ mice express high levels of neutrophil chemo-attractants CXCL2 and CXCL5. It is possible that MC-LTβR signaling regulates the production of these chemokines in this model, as reduced neutrophil infiltration was detected in mice with MC-specific LTβR deletion.

TLO's role during disease hasn't been defined yet. Increased TLOs number has been observed during inflammation, autoimmunity and cancer.[43] However, a recent study proposed a beneficial role for TLOs during atherooscleroses. Indeed, deletion of artery TLOs in mice with atherosclerosis suggest that they attenuate rather than enforce chronic inflammation. Interetingly, ILFs represent intestinal TLOs and LTβR KO TNF$^{\Delta ARE}$ mice lack PPs and ILFs[61]. To this end, we monitored and identified the TLO's role during TNF-mediated intestinal inflammation. Interestingly, we observed that in the absence of ILFs the intestinal pathology was more severe in the colon and unaffected in the SI. These data exclude the possibility that ILFs are detrimental for intestinal inflammation. In agreement with this, a previous study showed that depletion of B cells in TNF$^{\Delta ARE}$ mice, the major immune component of ILFs, didn't affect the progression of inflammation.[93] Collectively these data demonstrate that ILFs may not be actively involved in the development of the disease in TNF$^{\Delta ARE}$ model.

Bone marrow transfer experiments have proven that stromal LTβR is required for ILF development.[119] We show that LTβR on MCs is important for ILF organization since subepithelial localization of ColVI-cre^{+} MCs inside the ILFs was detected in MC-specific LTβR deficient mice. This suggests that another MC subpopulation, not targeted by ColVI-cre, was able to promote the development of ILFs. This hypothesis is further supported by unpublished data from our lab showing at least two different mesenchymal subpopulations responsible for the PPs development. Deletion of ColVI-cre specific LTβR resulted in smaller PPs with ColVI-cre^{+} cells only under the epithelium and around vessels. On the other hand, deleting LTβR in the majority of MCs targeted by Twist2-cre, again didn't allow Twist2-cre^{+} MCs to migrate from the subepithelial area to the bottom part of ILFs. All these data suggest that MCs require LTβR to properly organize ILFs. We believe that the same phenomenon applies also in TNF$^{\Delta ARE}$ model, as a subepithelial line and perivascular ColVI-cre^{+} cells were found in ColVI-cre specific LTβR deficient TNF$^{\Delta ARE}$ mice.

Overall, we propose that MC-specific LTβR is significant for ILF organization both in health and disease. However, we demonstrate that ILFs are not actively involved during intestinal inflammation, emerging new queries about their actual role during disease. We further reveal that LTβR signaling limits colonic inflammation and exert a protective role during disease progression in $TNF^{\Delta ARE}$ mouse model. Future studies will better define the LTβR-expressing cell type and the mechanism responsible for this beneficial effect.

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