ROLE OF LYMPHOTOXIN B RECEPTOR SIGNALING IN LYMPH NODE LYMPHANGIOGENESIS INDUCED BY IMMUNIZATION

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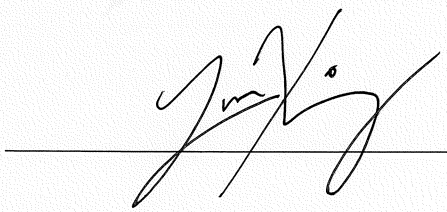
Declaration

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



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Summary

Lymphangiogenesis is the formation of new lymphatic vessels from pre-existing vasculature, where lymphatic vessels are important in the maintenance of tissue fluid homeostasis and immune surveillance. During inflammation, lymphangiogenesis can be observed in the draining lymph nodes (LNs) of inflamed peripheral tissues. Several immune cells such as B cells, macrophages and DCs have been linked to the induction of lymphangiogenesis in the LNs. However, the molecular mechanism underlying the remodeling of lymphatic vessels in the LNs during inflammation is still in its infancy. Of interest to us is the signaling pathway behind B cells-mediated LN lymphangiogenesis, as well as the role that the lymphotoxin β receptor (LTβR) signaling pathway may play in regulating LN lymphangiogenesis. Although the role of LTβR signaling in the control of splenic architecture is well recognized, aspects of the LN microenvironment that are dependent on LTβR remain uncertain.

In this study, we showed the requirement of B cells in the expansion of the LNs and lymphangiogenesis in response to immunization. The expression of LTα by B cells is critical for LN lymphangiogenesis. We demonstrated that LN expansion and lymphangiogenesis were reduced when LTβR signaling was inhibited by a decoy receptor prior to immunization. Expression of LTα, which forms the heterotrimer $LT\alpha_1\beta_2$ together with LTβ, increased in B cells after immunization. In addition, we found that LTβR signaling only exert its regulatory role in the early stages of lymphangiogenesis. These observations led us to investigate one of

the initial steps of the lymphangiogenesis process involving the degradation of the extracellular matrix (ECM) and basement membrane (BM) by matrix metalloproteinases (MMPs). The role of MMPs in lymphangiogenesis to date is not as well characterized compared to angiogenesis.

Our findings revealed that MMP-13 expression increased in the LNs after immunization, and this expression of MMP-13 is regulated by LTβR signaling. Study of the localization of MMP-13 in the LNs suggested that the proteinase might be involved in driving lymphangiogenesis through the remodeling of ECM and BM. Using a stable lymphatic endothelial cell (LEC) line, we showed that LECs express MMP-13. Through *in vitro* experiments, we demonstrated that MMP-13 mediates lymphangiogenesis through its proteolytic activites. Overall, this study identify LTβR signaling pathway as a key molecular mediator in the B cell-mediated lymphangiogenesis, as well as showing that LTβR signaling regulates the expression of MMP-13 that is required for the degradation of matrix components for sprouting of new lymphatic vessels.

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Abbreviations

ALK	activin receptor-like kinase
AM	adrenomedulin
Ang	angiopoietin
APC	antigen-presenting cell
APC	allophycocyanin
Aspp	apoptosis stimulating protein of p53
BAFF	B cell activating factor
BEC	blood endothelial cell
BM	basement membrane
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CALCRL	calcitonin receptor-like receptor
CD	cluster of differentiation
CFA	complete Freund’s adjuvant
CCL	chemokine (C-C motif) ligand
CCR	chemokine (C-C motif) receptor
CXCL	chemokine (C-X-C motif) ligand
Cy	cyanine
DC	dendritic cell
DcR	decoy receptor
DMEM	Dulbecco’s modified Eagle’s medium
DTH	delayed-type hypersensitivity
EDTA	ethylenediaminetetraacetic acid
ECM	extracellular matrix

FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FDC	follicular dendritic cell
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FITC	fluorescein-5-isothiocyanate
FLT4	fms-related tyrosine kinase 4
FRC	fibroblastic reticular cell
HBSS	Hank's Buffered Saline Solution
HEV	high endothelial venule
HGF	hepatocyte growth factor
HMVEC	human microvascular endothelial cells
HRP	horseradish peroxidase
HVEM	herpesvirus entry mediator
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IGF	insulin-like growth factor
IL	interleukin
JAK	Janus kinase
KLH	keyhole limpet hemocyanin
LEC	lymphatic endothelial cell
LIGHT	lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for herpesvirus entry mediator
LN	lymph node

LT	lymphotoxin
LTβR	lymphotoxin β receptor
LYVE-1	lymphatic endothelial hyaluronan receptor
MACS	magnetic activated cell sorting
MAdCAM	mucosal vascular addressin cell-adhesion molecule
MAPK	mitogen activated protein kinase
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MT-MMP	membrane-type matrix metalloproteinase
NF-κB	nuclear factor-kappaB
Nrp	neurophilin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PE	phycoerythrin
PerCP	peridinin-chlorophyll protein
PNAd	peripheral lymph node addressins
Prox1	prospero-related homeobox 1
qPCR	quantitative real-time polymerase chain reaction
RAMP	receptor activity-modifying protein
RPMI	Roswell Park Memorial Institute
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	short interfering RNA
SLP	SH2 domain containing leukocyte protein
Src	proto-oncogene tyrosine-protein kinase
STAT	signal transducer and activator of transcription

Syk	Spleen tyrosine kinase
TCR	T cell receptor
TGF	transforming growth factor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
VEGF	vascular endothelial grow factor
VEGFR	vascular endothelial grow factor receptor
WT	wild type

Chapter 1: Introduction

1.1 Lymphatic Vessels

The blood vasculature transports oxygen and nutrients, carrying out exchange of molecules and removal of waste products to and from the tissues. In this process, hydrostatic and osmotic pressure gradients cause plasma from the blood capillaries to enter the surrounding interstitial space. This extravasation of fluids and proteins from the blood vessels is balanced by a second vascular system, the lymphatic vascular system. The lymphatic vasculature drains and returns this fluid (lymph) back to the bloodstream. Unlike the pressurized circulatory system of the blood vasculature, there is no central pump in the lymphatic vascular system and the lymphatic vasculature is a hierarchical network of vessels with a unidirectional lymph flow from the periphery back to the blood circulation. The major roles of the lymphatic vasculature comprise the maintenance of tissue fluid homeostasis, immune surveillance and fat absorption in the small intestine (Oliver and Alitalo, 2005).

1.1.1 Lymphatic vasculature

The lymphatic vessels are part of the lymphatic system, which also includes the lymphoid organs, such as the lymph nodes (LNs), spleen, thymus, Peyer's patches and the tonsils. The lymphatic vasculature consists of five main categories of conduits: the lymphatic capillaries (or initials), collecting vessels, LNs, larger trunks and the thoracic duct (Swartz, 2001). The initial lymphatic capillaries that begin blind-ended in the periphery are made up of a single layer of lymphatic endothelial cells (LECs) and have a wider lumen compared to blood capillaries

(Swartz and Skobe, 2001). There is incomplete coverage of the lymphatic capillaries by basement membrane (BM) and, in its place, the LECs are attached to the surrounding extracellular matrix (ECM) through anchoring filaments that prevent the capillaries from collapsing (Gerli et al., 1990; Schmid-Schönbein and Schmid-Schönbein, 1990; Pflicke and Sixt, 2009). Interstitial fluid first enters the lymphatic capillary plexus through discontinuous button-like junctions between the LECs (Baluk et al., 2007). From there, lymph is drained into the collecting vessels. The collecting lymphatic vessels, unlike the lymphatic capillaries, are not anchored to the ECM. Instead, they contain perivascular smooth muscle cells that facilitate the propulsion of lymph, as well as valves that prevent retrograde flow (Figure 1.1) (Schmid-Schönbein and Schmid-Schönbein, 1990; Bridenbaugh et al., 2003; Randolph et al., 2005). The collecting vessels then pass through one or several clusters of LNs leading into the larger trunks, before draining into the thoracic duct where the lymph is discharged into the blood circulation.

1.1.2 Lymphatic vessels markers

Although the lymphatic system has been observed centuries ago, and its anatomy nearly entirely characterized by the 19th century, our understanding of the lymphatic system advanced at a much slower rate compared to the blood circulatory system in the last century (Swartz, 2001). This is primarily due to the fact that lymphatic vessels are difficult to distinguish from blood vessels histologically. Furthermore, the majority of blood vascular markers can also be found in the lymphatic vessels (Sleeman et al., 2001). It is only with the discovery

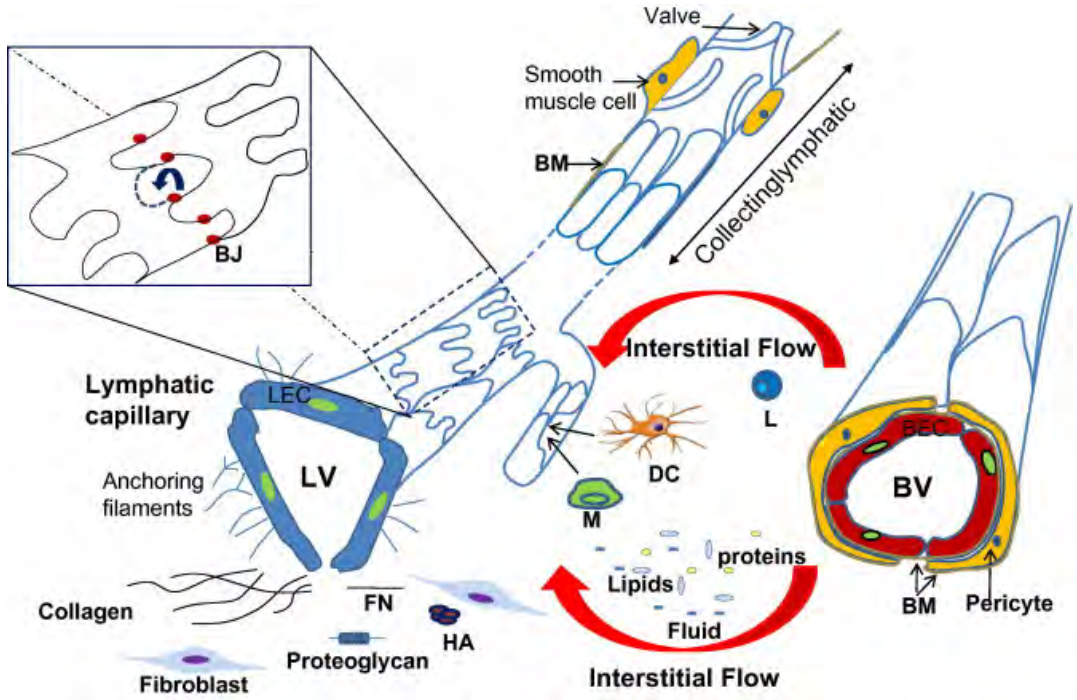


Figure 1.1: Schematic overview of the structure and function of the lymphatic vasculature. (Adapted from Mol Aspects Med, 32(2), Paupert et al., Lymphangiogenesis in post-natal tissue remodeling: lymphatic endothelial cell connection with its environment, 146-58, copyright 2011 with permission from Elsevier) Endothelial cells of lymphatic capillaries have an oak shaped with overlapping scalloped edges (flaps). These flaps are only sealed on the sides by discontinuous button- like junction (BJ) allowing fluid entry through these flaps without disturbing cell–cell cohesion. Immune cells (lymphocytes (L), macrophages (M), dendritic cells (DC)) likely enter lymphatic capillaries through the intermingled flaps. In contrast to BV, interstitial matrix constitutes the principal microenvironment of initial lymphatic since they are devoid of a continuous basement membrane (BM). Anchoring filaments connect lymphatic capillaries to extracellular matrix and modulate vessel diameter by pulling adjacent endothelial cells apart. Lymphatic vessels (LV), lymphatic endothelial cells (LEC), blood vessels (BV), blood endothelial cells (BEC), fibronectin (FN), hyaluronan (HA).

of specific LEC surface markers, as well as specific molecules that govern the development and growth of lymphatic vessels, that allowed advances and understanding of the lymphatic system (Oliver and Detmar, 2002).

Among the first lymphatic markers to be characterized is the vascular endothelial growth factor receptor-3 (VEGFR-3), also known as the fms-related tyrosine kinase 4 (FLT4), together with its ligand VEGF-C (Kaipainen et al., 1995; Kukk et al., 1996). During early embryonic development, VEGFR-3 was observed to be expressed in both the developing venous and the presumptive lymphatic endothelia (Kaipainen et al., 1995). However in adult tissues, VEGFR-3 is expressed mainly in the lymphatic endothelium, unlike VEGFR-1 and VEGFR-2 which are found on both the blood and lymphatic endothelia (Kaipainen et al., 1995; Veikkola et al., 2000; Kriehuber et al., 2001). Following that, a specific marker on the surface of LECs and macrophages was identified as the lymphatic endothelial hyaluronan receptor (LYVE-1) (Banerji et al., 1999). As the name suggests, LYVE-1, a CD44 homolog, is a receptor for hyaluronan. Hyaluronan is an ECM glycosaminoglycan that is abundantly found in the skin and mesenchymal tissues where it has roles in cell adhesion and cell migration (Knudson and Knudson, 1993; Jiang et al., 2011). With the discovery of the LYVE-1 marker on LECs, the lymphatic vessels have been visualized in tissue sections from several tissues including the skin (Skobe and Detmar, 2000). Another surface marker that can also be used to identify the lymphatic vasculature is podoplanin (Wetterwald et al., 1996; Breiteneder-Geleff et al., 1999).

Podoplanin is a transmembrane mucin-type glycoprotein that is expressed in osteoblastic cells, podocytes, lung alveolar type I cells, cells of choroid plexus and LECs (Wetterwald et al., 1996).

1.1.3 Development of the lymphatic vasculature

The development of the lymphatic vasculature, which begins only after the embryonic blood vascular system has been set up, is similar to that of blood vessel development in terms of morphology such as undergoing sprouting and outgrowing of a primary capillary plexus as well as the expansion of the capillary plexus (Oliver, 2004; Adams and Alitalo, 2007). However, the unique structure and function of the lymphatic vessels means the LECs need to acquire exclusive gene products from that of the blood endothelial cells (BECs) during their developmental process. Experimental data from mice revealed that one of the earliest recognized events in the lymphatic vasculature development is the expression of the transcription factor prospero-related homeobox 1 (Prox1) at embryonic day (E) 10.5 within a subset of endothelial cells in the cardinal veins (Wigle and Oliver, 1999). The $Prox1^{+}$ endothelial cells then bud from the veins in a polarized manner while proliferating and migrating to eventually form the embryonic lymph sacs and subsequently the lymphatic plexus (Wigle and Oliver, 1999). The importance of Prox1 in the development of the lymphatic vasculature is revealed in *Prox1* knockout mice, where the embryos do not have lymphatic vessels and develop severe edema before dying around midgestation (Wigle and Oliver, 1999; Wigle et al., 2002). Endothelial cells in these $Prox1^{-/-}$ embryos had

reduced and abnormal budding from the cardinal veins, and they fail to differentiate into the lymphatic lineage having no expression of specific LEC markers (Wigle et al., 2002). These findings supported the widely accepted theory regarding the venous origin of the lymphatic vasculature put forward by Florence Sabin over a century ago in 1902 (Oliver, 2004). While Prox1 is crucial for the initial commitment of the endothelial cells to the lymphatic lineage, another factor, VEGF-C is essential for the sprouting of the lymphatic vessels from the budding and sprouting of these endothelial cells from the embryonic veins to form the lymph sacs (Karkkainen et al., 2004). Similar to *Prox1*$^{-/-}$ embryos, embryos deficient in VEGF-C display a complete lack of lymphatic vessels and die around midgestation (Karkkainen et al., 2004). The subpopulation of Prox1^{+} venous endothelial cells is present in the *Vegfc1*$^{-/-}$ embryos, however, they do not sprout and remain confined to the cardinal vein before disappearing, likely due to apoptosis (Karkkainen et al., 2004). With the formation of the lymph sacs, the lymphatic vascular system goes on to develop separately from the blood vascular system, and only associates with the blood vasculature at specific spots for the return of the lymph to the bloodstream (Cueni and Detmar, 2008). Two of the key molecules involved in regulating the separation of the two vasculatures are the adaptor protein SLP-76 and the tyrosine kinase Syk (Abtahian et al., 2003). As these two proteins are expressed largely by hematopoietic cells, it seems to suggest that the circulating blood cells may also play a role in the development of the lymphatic vasculature (Abtahian et al., 2003).

1.2 Lymph nodes

The lymphatic vessels and the LNs are functionally inseparable in the immune system. LNs are encapsulated lymphoid organs where collecting vessels converge, and they are located at strategic positions of the body to allow a quick and efficient initiation of an immune response. Lymphatic vessels form a sinusoidal network within the LNs, and the afferent lymphatic vessels are the routes that dendritic cells (DCs) use to migrate to the LNs after antigen uptake. The major chemokine expressed by LVs for directing lymphatic entry of DCs is chemokine (C-C motif) ligand 21 (CCL21), which attracts DCs that express its cognate chemokine (C-C motif) receptor 7 (CCR7), a receptor required for DC migration to LNs (Förster et al., 1999; Ohl et al., 2004; Randolph et al., 2005). LNs have numerous essential roles in the immune system, such as to gather antigens and DCs from the peripheries, to recruit naive lymphocytes from the blood and to present the proper environment for antigen-specific tolerance or effective primary or secondary effector responses (Andrian and Mempel, 2003).

1.2.1 Structure of the lymph node

Recognizing the morphological features of the LN is fundamental to comprehending its functions. LNs contain either a singular or multiple lymphoid lobules bound by lymph-filled sinuses and enclosed by a capsule, where two core regions, the cortex and the medulla, can be distinguished (Willard-Mack, 2006). The cortex, which is the lymphoid compartment of the LN, is made up of a reticular meshwork of fibroblasts with separate areas for T and B cells (Gretz et

al., 1996; 1997). The route of entry for lymphocytes into the LNs from the blood circulation is primarily through specialized blood vessels called the high endothelial venules (HEVs). B cells are found in the more superficial cortex area made up of follicles and germinal centers with follicular dendritic cells (FDCs) cluster in the middle of these follicles, whereas the T cell zones are located in the paracortex of the LN entrenched in a scaffold of stromal cells known as the fibroblastic reticular cells (FRCs) (Junt et al., 2008; Mueller and Germain, 2009) The lymphoid region is an enclosed compartment where fluid can only enter through tubules originating from the sinus (Roozendaal et al., 2008). On the other hand, the medulla is highly vascularized, consisting mainly of lymph-draining sinuses that transport lymph out of the LN. Through the afferent lymphatic vessels, lymph enters the LNs into the subcapsular sinus. The lymph then flow through several radial cortical sinuses surrounding the lobules that lead into the medullary region, merging into larger medullary sinuses before finally exiting the LN via the efferent lymphatic vessel at the hilus (Figure 1.2) (Roozendaal et al., 2008).

1.3 Lymphangiogenesis

Lymphangiogenesis is the process by which new lymphatic vessels form from pre-existing lymphatic vessels. The formation of new lymphatic vessels is a complex dynamic process of several different steps, including the sprouting from a pre-existing vessel, cell proliferation, migration and differentiation into capillaries (Adams and Alitalo, 2007). While lymphangiogenesis and the

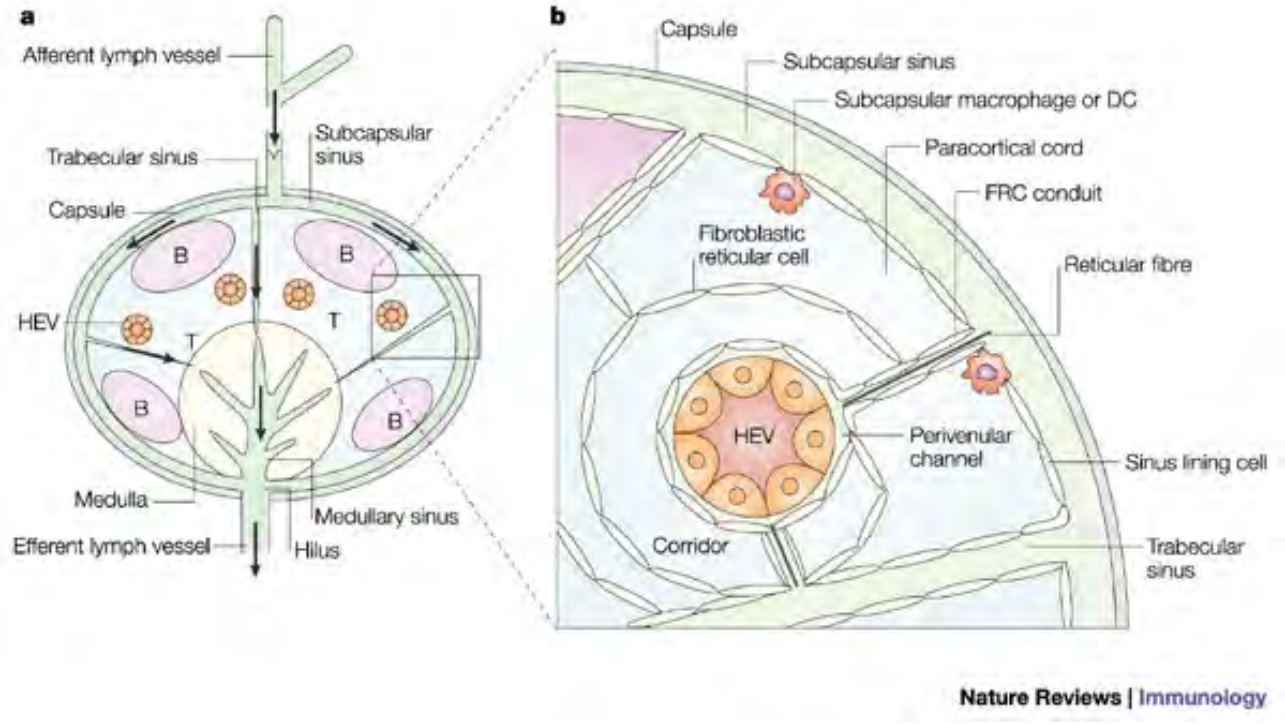


Figure 1.2: LN architecture. (Adapted by permission from Macmillan Publishers Ltd: Nat Rev Immunol (von Andrian and Mempel, 2003), copyright 2003) (A) Schematic diagram showing the major structural components of a LN. The main routes of lymph flow into and within LN are indicated by arrows. Blind-ending afferent lymph vessels collect and channel interstitial fluid into the subcapsular sinus. From here, the lymph is drained towards the hilus through the FRC conduit and trabecular sinuses that connect to medullary sinuses. (B) Schematic depiction of a paracortical cord. The T-cell-rich cord (light blue) is shown adjacent to a B-cell follicle (pink) and demarcated by lymph-filled sinuses (green). The cord is penetrated by reticular fibres consisting of type 1 and type 3 collagen that are contained within the sleeves of the FRCs forming a conduit. At the centre of each cord is a HEV that is surrounded by concentric layers of FRCs. The FRC conduit drains lymph into the perivenular channel.

remodeling of the lymphatic vessel occur spontaneously during embryogenesis, these processes are not restricted to this particular developmental stage (Oliver, 2004). However, lymphangiogenesis in adulthood is predominantly associated with pathological conditions such as inflammation, tissue injury and tumor dissemination (Cueni and Detmar, 2008; Alitalo, 2011).

1.3.1 Lymphangiogenic growth factors & receptors

The first known, and best-characterized, signaling pathway involved in lymphangiogenesis is induced by the interactions between VEGF-C and the structurally similar VEGF-D and their receptor VEGFR-3 (Jeltsch et al., 1997; Oh et al., 1997; Veikkola et al., 2001). Overexpression of VEGF-C and VEGF-D in the skin of transgenic mice induced hyperplasia of cutaneous lymphatic vessels (Jeltsch et al., 1997; Veikkola et al., 2001). VEGF-C/VEGFR-3 signaling has also been shown to stimulate the growth, migration and survival of cultured human LECs (Mäkinen et al., 2001). Other than VEGFR-3, VEGF-C and VEGF-D can also bind to neuropilin 2 (Nrp2), a semaphorin receptor in the nervous system that is expressed in the lymphatic capillaries (Kärpänen et al., 2006). Homozygous deletion of Nrp2 in mice leads to either absence or drastic reduction of small lymphatic vessels and capillaries (Yuan et al., 2002). After proteolytic cleavage, VEGF-C and VEGF-D can also bind to a third receptor, VEGFR-2 (Joukov et al., 1996; Achen et al., 1998). Although VEGFR-2 is well known for its role in angiogenesis, studies have shown that VEGFR-2 can also promote lymphangiogenesis both *in vitro* and *in vivo* upon activation by its ligand VEGF-

A (Nagy et al., 2002; Hong et al., 2004; Kunstfeld et al., 2004). However, VEGF-A cannot replace VEGF-C's role in lymphatic development (Karkkainen et al., 2004). Besides inducing inflammatory lymphangiogenesis directly through VEGFR-2 signaling on LECs, VEGF-A can also promote lymphangiogenesis indirectly by the recruitment of inflammatory cells such as macrophages that produce VEGF-C and VEGF-D (Cursiefen et al., 2004b; Baluk et al., 2005).

While the VEGF family of growth factors represents the key lymphangiogenic factors, various non-VEGF-related lymphangiogenic factors have also been identified. Angiopoietins (Ang) are a family of growth factors known to regulate angiogenesis. The endothelial tyrosine kinase Tie2, specific receptor of angiopoietin 1 (Ang1), is expressed in cultured LECs as well as in lymphatic vessels *in vivo* (Kriehuber et al., 2001; Morisada et al., 2005). Mice deficient for Ang2 exhibit defects in the patterning and function of the lymphatic vessels, and Ang1 is sufficient to rescue the lymphatic phenotype in the Ang2 mutant mice (Gale et al., 2002). All four angiopoietins have been demonstrated to induce lymphangiogenic sprouting, with Ang1 being the most potent (Morisada et al., 2005; Tammela et al., 2005; Kim et al., 2007). In addition to activating Tie2 directly, Ang1 may also induce lymphangiogenesis indirectly through the VEGF-C/VEGFR-3 pathway (Morisada et al., 2005; Tammela et al., 2005). Fibroblast growth factor-2 (FGF-2), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1) and IGF-2, and platelet derived growth factor-BB (PDGF-BB) have all been shown to induce lymphangiogenesis in various experimental models

(Figure 1.3) (Cueni and Detmar, 2006). However, many of these effects may be secondary to the induction of VEGF-C and VEGF-D in several different cell types (Cueni and Detmar, 2006; Tammela and Alitalo, 2010). The continued discovery of new lymphangiogenic factors in recent years, such as adrenomedulin (AM) together with its calcitonin receptor-like receptor (CALCRL) and the receptor activity-modifying protein 2 (RAMP2) (Fritz-Six et al., 2008); apoptosis stimulating protein of p53 (Aspp1) (Hirashima et al., 2008); activin receptor-like kinase 1 (ALK1) (Niessen et al., 2010); and liprin β1 (Norrmén et al., 2010), have widely expanded our knowledge of lymphangiogenesis (Norrmén et al., 2011).

1.3.2 Inflammatory Lymphangiogenesis

1.3.2.1 Inflammation

Inflammation is a tightly controlled physiological response for repairing damage against injurious insults such as microbial infections, tissue injury or tumor growth. Immediate response to the injury often results in an acute inflammation response, while a slower and prolonged response would lead to a chronic inflammation process. The major steps in an inflammatory cascade typically involve the recruitment and activation of leukocytes to the injury site, followed by construction of a physical barrier to limit the damage, and lastly to initiate a resolution phase for the repairing and healing of the injured tissue (Krishnamoorthy and Honn, 2006). While inflammation is fundamentally a protective mechanism leading to recovery, it can cause persistent tissue damage if

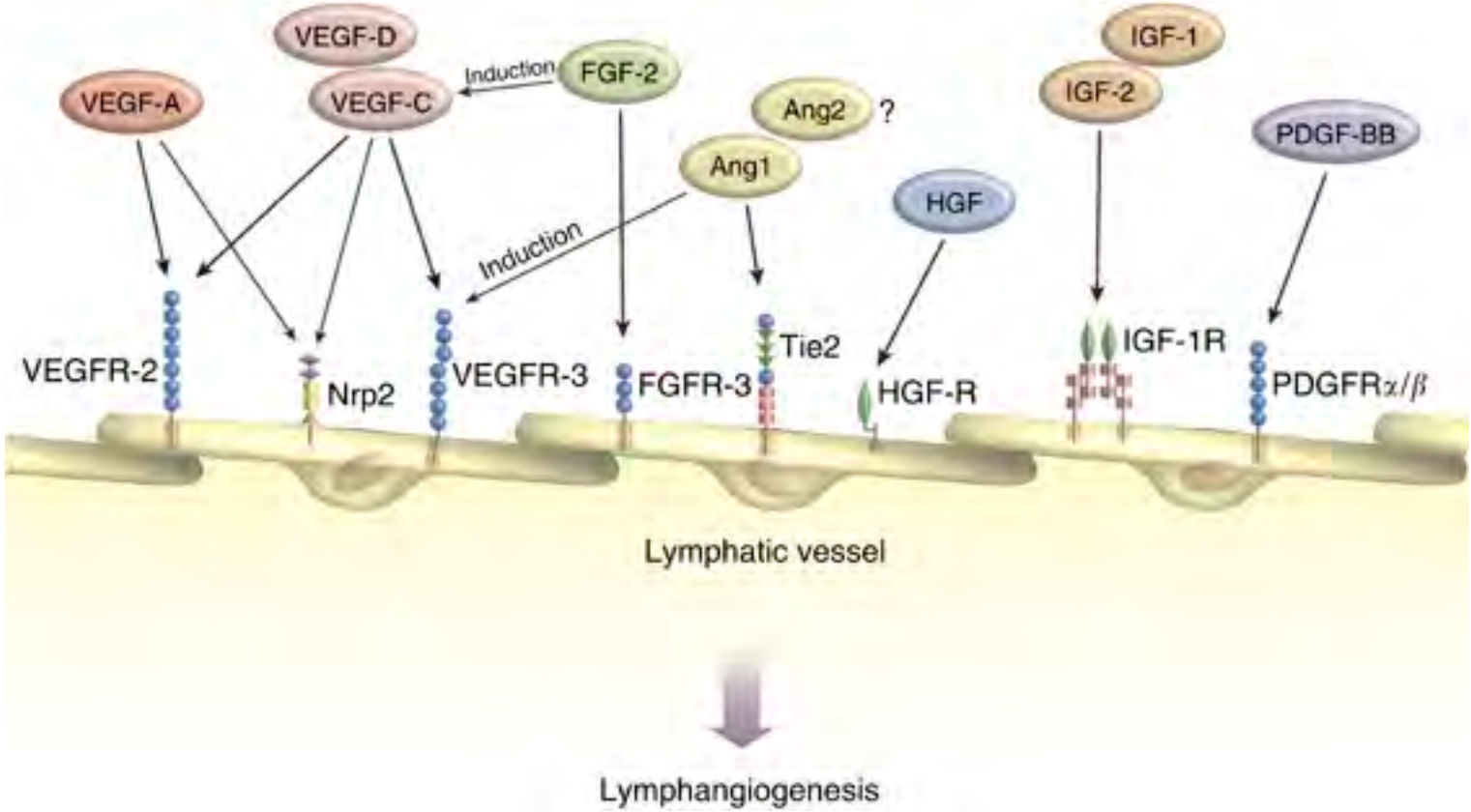


Figure 1.3: Schematic representation of lymphangiogenic growth factors and their receptors expressed by lymphatic endothelium. (Adapted by permission from Macmillan Publishers Ltd: J Invest Dermatol (Cueni and Detmar, 2006), copyright 2006) Several vascular endothelial growth factors (VEGF-A, VEGF-C, VEGF-D) promote lymphangiogenesis by activation of distinct VEGFRs and Nrp2. FGF-2 acts directly through FGFR-3 and also via induction of VEGF-C. Ang1 activates Tie2 and upregulates VEGFR-3. HGF, IGF, PDGF-BB act directly through their respective receptors HGF-R, IGF-1R, and PDGFR.

the steps of the inflammatory process are not properly phased or controlled (Nathan, 2002).

Lymphangiogenesis has been observed in various forms of inflammation, and the biological changes in the LECs are often a result of the accumulation of inflammatory cells and the inflammatory mediators such as tumor necrosis factor α (TNFα), VEGF-A, VEGF-C and interleukin (IL)-6 that these cells secrete (Ji and Ji, 2007; Huggenberger et al., 2011a). The remodeling of lymphatic vessels in relation to its role in the transport of immune cells plays an important role in the regulation of the inflammatory response.

1.3.2.2 Lymphangiogenesis in peripheral tissues

Recent work have shown that during inflammation, lymphangiogenesis can be observed both in the inflamed peripheral tissues as well as the draining LNs of these tissues (Ji, 2009; Kim et al., 2012). Lymphangiogenesis occur in the peripheral tissues where the initial lymphatics drain antigen and antigen-presenting cells (APCs) into the LNs. Remodeling of the lymphatic vessels in the LNs then function as bottleneck filters that congregate the afferent lymph to initiate the adaptive immune response, as well as inflammation resolution (Kim et al., 2012). Majority of the research on inflammatory lymphangiogenesis has been focused on the peripheral tissues, where the VEGF-C/VEGFR-3 signaling pathway is the key molecular regulator for lymphangiogenesis, followed by the

direct and indirect effect of the VEGF-A/VEGFR-2 signaling (Ji and Ji, 2007; Tammela and Alitalo, 2010; Kim et al., 2012). Recent work on a mouse model of inflammatory peritonitis has shown that the primary mediator of the inflammatory response, the nuclear factor-kappaB (NF-κB) family of transcription factors, is able to promote lymphangiogenesis upon activation by inflammatory stimuli through the activation of Prox1 and subsequent upregulation of VEGFR-3 (Flister et al., 2010).

1.3.2.3 Lymphangiogenesis in lymph nodes

LNs, as discussed above, have an important role in the regulation of inflammation. During immune responses, the recruitment of lymphocytes causes the LN to grow in size, and this growth is accompanied by growth in the vasculatures. Contrary to inflammatory lymphangiogenesis in the inflamed peripheral tissues, research on LN lymphangiogenesis have mainly focused on the VEGF-A/VEGFR-2 signaling pathway. Our previous study first showed that the expansion of the lymphatic vessel network in activated LNs of immunized mice requires the recruitment of B cells within the LN, and the LN lymphangiogenesis then results in enhanced DC migration from the periphery (Angeli et al., 2006). This finding demonstrated that signals initiated within activated draining LNs could increase the migration of DCs, despite the latter being in an “upstream” location against the unidirectional flow of lymph. In addition, the newly formed lymphatic vessels in the LNs were in vicinity with B cells, and B cell follicles co-localized with the expression of VEGF-A (Angeli et al., 2006). Therefore, B cells entering the activated LN were

argued to respond to inflammation by secreting VEGF-A to stimulate lymphangiogenesis via VEGFR-2 and it was demonstrated that blocking VEGFR-2 signaling significantly reduced lymphatic growth and DC trafficking in response to immunization (Angeli et al., 2006).

Using a different immunization model, it was revealed that the remodeling of lymphatic vessels in the LNs first suffered a transient insufficiency in the function of the afferent lymphatic vessels after immunization, followed by recovery at a later time point (Liao and Ruddle, 2006). It is most likely due to the difference in immunization regimens that we did not observe the transient insufficiency of the lymphatic vessels in our previous work. B cells, while important in the early phase of lymphangiogenesis, were demonstrated to be dispensable in the latter stages of the process (Liao and Ruddle, 2006). Furthermore, the lymphotoxin β receptor (LTβR) was shown to be critical in the regulation and maintenance of HEVs in the LNs, and together with B cells, important in mediating HEVs and lymphatic vessels synchrony and cross talk after immunization (Liao and Ruddle, 2006). Besides B cells, DCs have also been reported to drive LN vascular growth (Webster et al., 2006). Endothelial cells in the LNs proliferate and increase in cell number upon immunization, and this was driven by an increase in VEGF-A levels in the LNs (Webster et al., 2006). Although DCs are unlikely to be the source of the increased VEGF-A, DCs promote LN vascular growth by upregulating VEGF-A in a cell recruitment-dependent manner (Webster et al., 2006). Another study using a delayed-type hypersensitivity (DTH) response has also shown that

inflammatory lymphangiogenesis in LNs was independent of the presence of nodal B cells (Halin et al., 2007). In this chronic inflammation model, tissue inflammation induced both lymphangiogenesis and angiogenesis in the inflamed ears, but only specifically induced lymphangiogenesis and not angiogenesis in the draining LNs (Halin et al., 2007). Similar to the acute inflammation model in the previous studies, VEGF-A is required for LN lymphangiogenesis (Halin et al., 2007). In contrast to our previous work, it was demonstrated that VEGF-A was only produced at the sites of inflammation and then transported to the draining LNs through the afferent lymphatic vessels, implying that LN lymphangiogenesis can be regulated by distantly produced lymphangiogenic factors on top of signals produced locally (Halin et al., 2007). This phenomenon, however, may be unique to chronic inflammation models.

FRCs, stromal cells that are mainly found in the T cell zone and medullary cords of the LN, are important in defining the three-dimensional network of the LN. In addition to its structural role in the LN, FRC also plays a critical role in the migration and survival of lymphocytes in the LN (Buettner et al., 2010). Supporting the production of VEGF-A in LN itself, subsequent work has illustrated that FRCs are the principal VEGF-A-expressing cells both during homeostasis and upon LN stimulation (Chyou et al., 2008). While VEGF-A has been known to be important in driving LN endothelial cell proliferation upon stimulation, VEGF-A is also demonstrated to mediate homeostatic LN endothelial cells proliferation (Webster et al., 2006; Chyou et al., 2008). Additionally, LTβR

signaling was also suggested to play a role in LN lymphangiogenesis where it was shown that inhibiting LTβR signaling in the LN reduces VEGF-A levels as well as endothelial cell proliferation, while stimulation of the LTβR signaling on FRCs *in vitro* upregulates VEGF-A expression (Chyou et al., 2011).

$CD11b^+$ macrophages have also been shown to play a role in LN lymphangiogenesis by being the main mediators or sources of VEGF ligands including VEGF-A, VEGF-C and VEGF-D in the draining LNs as well as at the site of inflammation through a study using a bacterial pathogen-induced acute inflammation model in the skin (Kataru et al., 2009). The inflammatory lymphangiogenesis that occurs in the draining LNs, as well as in the inflamed skin, driven by the upregulation of VEGF ligands expression facilitates lymph flow, inflammatory cell migration and antigen clearance, and subsequently inflammation resolution (Kataru et al., 2009). In contrast to the previous studies that focused on the role of VEGF-A in LN lymphangiogenesis, results from the K14-VEGF-C transgenic mice, mice with overexpression of VEGF-C, indicated that increase VEGF-C levels was sufficient to promote LN lymphangiogenesis (Kataru et al., 2009). Similarly, a study using TNFα-transgenic mice as a model of chronic inflammatory arthritis also highlighted the critical role of VEGF-C/VEFGR-3 signaling in LN lymphangiogenesis where inhibition of VEGFR-3 specifically reduces inflammatory lymphangiogenesis in the draining LNs (Guo et al., 2009). Inhibition of VEGFR-2 in this model of chronic inflammatory arthritis also neutralizes LN lymphangiogenesis, however, it was suggested that VEGF-

A/VEGFR-2 promotes lymphangiogenesis indirectly through its stimulatory effects on angiogenesis as well as its recruitment of VEGF-C-producing inflammatory cells (Guo et al., 2009).

Reinforcing the role of B cell-derived VEGF-A in promoting LN lymphangiogenesis, creation of transgenic mice that express human VEGF-A specifically in B cells leads to an increase in lymphangiogenesis as well as HEVs expansion in the LN (Shrestha et al., 2010). Although increased lymphangiogenesis and angiogenesis were observed in the LNs of these mice, the B cell-derived VEGF-A suppresses certain aspects of the ensuing immune responses, consistent with the hypothesis that VEGF-A function to promote homeostasis (Shrestha et al., 2010). As macrophages were also observed to accumulate in these LNs, the role that VEGF-A plays in promoting lymphangiogenesis is either directly through the activation of VEGFR-2 or indirectly via the upregulation of VEGF-C by macrophages (Shrestha et al., 2010).

While most of the papers reviewed so far have focused on the pro-lymphangiogenic effects of the residing cells of the LNs, a recent study by Kataru et al. (2011) examined the anti-lymphangiogenic effects of T cells on LN lymphangiogenesis. T cells were shown to play a role in the regulation of the LN lymphatic vessel network density both in the steady and inflammatory states, by

which the level of LN lymphangiogenesis is inversely proportional to the number of T cells (Kataru et al., 2011). LN lymphangiogenesis is kept in check by T cell mainly through the secretion of interferon-γ (IFN-γ), which suppresses lymphatic growth by downregulating the expression of LEC-specific genes, particularly Prox1 through the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway (Kataru et al., 2011). Interestingly, a compensatory mechanism for antigen presentation to T cells was also implied through the increased recruitment of DCs to the LN via the increase in LN lymphangiogenesis in T-cell deficient LNs (Kataru et al., 2011). This suggested a feedback mechanism of T cells, alongside the pro-lymphangiogenic effects of the B cells, in regulating LN lymphangiogenesis to increase the chance of DCs presenting antigens to T cells.

Moreover, the plasticity of the newly formed inflammation-induced LN lymphatic vessels is highlighted by their ability to regress on the resolution of inflammation (Kataru et al., 2009; Mumprecht et al., 2012). While the mechanisms behind this observation are unclear, it does draw attention to the dynamic nature of LN lymphangiogenesis, as well as underlining the regulation of inflammation by LN lymphangiogenesis along with the homeostatic maintenance of the LN lymphatic vessel network. Understanding the pro-lymphangiogenic and anti-lymphangiogenic effects of the residing cells in the LN may provide us with a better account of how the LN maintain the balanced regulation of LN lymphangiogenesis, especially since the roles of the residing lymphocytes are of

utmost interest as they constitute the majority of the cells in the LN. Looking at the studies conducted so far in totality has proposed that the inflammatory LN lymphangiogenesis involves several intricate interactions between the LECs and the immune cells. Although the molecular mechanisms and signaling pathways involved in the growth of lymphatic vasculature during embryonic development have been relatively well recognized, those underlying inflammatory LN lymphangiogenesis are not as well characterized, mainly due to the discrepancies in the models examined, such as differences in the inflammatory stimuli used, disparities in the route of administration of the stimuli, and the varying time points adopted to determine lymphangiogenesis. Particularly of interest to us is the signaling pathway behind the B cells mediated LN lymphangiogenesis as well as the role(s) that the LTβR signaling pathway may play in regulating LN lymphangiogenesis.

1.3.2.4 Lymphangiogenesis in tertiary lymphoid structures

A study using a transgenic mice model mimicking Hashimoto's thyroiditis, where intra-thyroidal lymphoid follicles form spontaneously, has shown that LTβR signaling was required for *de novo* inflammatory lymphangiogenesis in tertiary lymphoid structures (Furtado et al., 2007). Tertiary lymphoid structures are similar to secondary lymphoid structures with organized lymphocyte compartments and specialized lymphatic vasculature within these lymphoid aggregates (Furtado et al., 2007). In this model of *de novo* lymphangiogenesis in the thyroid, VEGFs are not required and instead of B cells, T cells were found to

be indispensable for the formation of new lymphatic vessels (Furtado et al., 2007). These findings suggest that B cells may only be required for the expansion of already existing lymphatic vessels, and T cells while having shown to negatively regulate LN lymphangiogenesis, may promote *de novo* lymphangiogenesis, at least in tertiary lymphoid structures. A follow up study by the same group has gone on to show that the recruitment of DCs into the tertiary lymphoid aggregates are critical in the formation of lymphatic vessels in these structures, and the expression of the LT ligands on DCs could be essential in the activation of the LTβR signaling necessary for *de novo* lymphangiogenesis (Muniz et al., 2011). These studies are interesting; although tertiary lymphoid structures are fundamentally different from LNs and other secondary lymphoid organs in that they only formed during inflammation, they have similarities in the organization of the structures and may provide insight into the regulation of the LN lymphangiogenesis process.

1.4 Lymphotoxin β receptor signaling

The term LT was first introduced in 1968 to depict a cytotoxic molecule produced by lymphocytes *in vitro* (Ruddle and Waksman, 1967; Granger and Williams, 1968; Ruddle and Waksman, 1968). Subsequent purification and characterization of LTα, as well as TNFα, exposed the close relationship between these 2 molecules (Aggarwal et al., 1984; 1985a; 1985b). LTα, along with TNFα, are the first described members of the TNF superfamily (Ruddle et al., 1992). Members

of this superfamily of cytokines have varied functions and are generally considered to be primary mediators of immune and inflammatory response.

1.4.1 Lymphotoxins and their receptors

LTα and TNFα were initially believed to have similar functions, as the secreted $LT\alpha_3$ homotrimers bind to the same TNF receptors (TNFRs) as TNFα (Smith et al., 1994). It was only with the discovery of the LTβ molecule (Browning et al., 1993) and LTβR (Crowe et al., 1994) that changed this view. By itself LTβ does not appear to have any function, as it is solely tethered to the cell membrane as a type II transmembrane protein (Browning et al., 1993; 1995). When LTβ is co-expressed with LTα, they predominantly form a membrane-anchored $LT\alpha_1\beta_2$ heterotrimer (Browning et al., 1993; 1995). A minor form, $LT\alpha_2\beta_1$, can also be found on the cell membrane (Browning et al., 1995). $LT\alpha_1\beta_2$ heterotrimers bind to a unique LTβR instead of TNF receptors (Ware et al., 1995). In addition, LTβR can also be signaled by a second ligand, LIGHT (lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus [HSV] glycoprotein D for herpesvirus entry mediator [HVEM], a receptor expressed by T lymphocytes) (Mauri et al., 1998). Other than LTβR, LIGHT can also bind to HVEM or the TNF receptor superfamily Decoy Receptor 3 (DcR3) (Figure 1.4) (Mauri et al., 1998). The expression pattern of $LT\alpha_1\beta_2$, LIGHT and LTβR hints that they are critical for immune cell communication (Table 1.1) (Schneider et al., 2004; Ware, 2008). Particularly of interest, $LT\alpha_1\beta_2$ is expressed on B and T cells while LTβR is expressed on stromal cells and the endothelial cells.

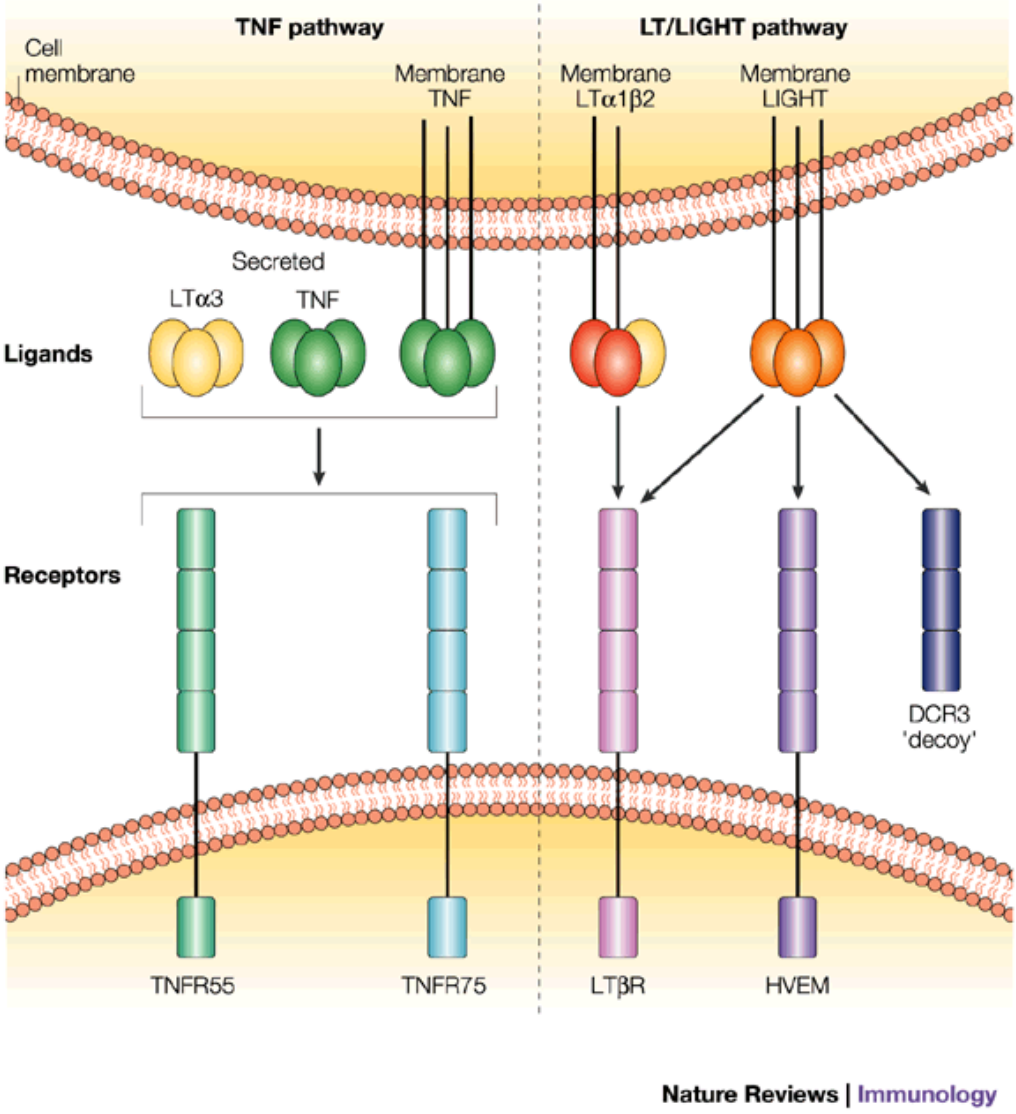


Figure 1.4: Ligands and receptors of the tumour-necrosis factor/lymphotoxin system. (Adapted by permission from Macmillan Publishers Ltd: Nat Rev Immunol (Gommerman and Browning, 2003), copyright 2003) Two fundamental pathways can be defined. The TNF pathway is activated by TNF or LTα-induced signalling through the two TNFRs. The LT/LIGHT system is composed of a typical TNF family receptor, LTβ receptor (LTβR), which binds to two ligands, the $LT\alpha_1\beta_2$ heteromer and homotrimeric LIGHT. LIGHT also binds to two additional receptors in the TNF family, decoy receptor 3 (DCR3) and herpes-virus entry mediator (HVEM).

Table 1.1: Expression and regulation of lymphotoxin, LIGHT and their receptors. (Adapted by permission from Macmillan Publishers Ltd: Nat Rev Immunol (Gommerman and Browning, 2003), copyright 2003)

Expression and regulation of lymphotoxin, LIGHT and their receptors		
Molecule	**Expression**	**Regulation**
Ligands		
LTα/LTβ	***Haematopoietic***	
	T-, T_H1-, B- and NK-cell lineages	Activation of T, B and NK cells
	Subset of follicular B cells	CXCL13
	T cells	IL-4, IL-7, CCL19/CCL21
	Lymphoid progenitor cells	IL-7, RANKL
	Inflamed sites	N.D.
LIGHT	***Haematopoietic***	
	T cells	T-cell activation
	Immature DCs	N.D.
	Granulocytes and monocytes	N.D.
	Non-haematopoietic	
	Breast epithelial-cell line	N.D.
Receptors		
LTβR	***Haematopoietic***	
	DCs and monocytes	N.D.
	Non-haematopoietic	
	Most lineages	Glucocorticoids?
	FDCs and HEVs	N.D.
HVEM	***Haematopoietic***	
	T cells	T-cell activation
	Immature DCs, macrophages and foam cells	N.D.
	Non-haematopoietic	
	Epithelial cells and hepatocytes	N.D.
DCR3*	Colorectal tumours	N.D.

1.4.2 Lymphotoxin β receptor and the NF-κB signaling pathway

The NF-κB family of transcription factors is involved in a variety of biological processes such as immune response, inflammation, cell survival or apoptosis and the development and maintenance of lymphoid organs (Weih and Caamaño, 2003; Bonizzi and Karin, 2004; Karin and Greten, 2005; Hayden and Ghosh, 2008; Vallabhapurapu and Karin, 2009). Similar to TNFR, LTβR signaling activates the canonical NF-κB signaling pathway, which is mainly involved in immune and inflammatory responses (Warzocha et al., 1995). In addition to the canonical pathway, LTβR signaling can also activate another NF-κB signaling cascade known as the noncanonical pathway (Figure 1.4) (Dejardin et al., 2002; Pomerantz and Baltimore, 2002; Müller and Siebenlist, 2003). The noncanonical NF-κB signaling pathway mainly regulates the development and maintenance of lymphoid organs, as well as B cell survival and maturation, DC activation and bone metabolism (Weih and Caamaño, 2003; Dejardin, 2006).

1.4.3 Role of lymphotoxin β receptor signaling in the development & maintenance of lymphoid structures

LTβR signaling pathway plays a vital role in the development of secondary lymphoid organs during embryogenesis as well as the maintenance of these organized structures (Fu and Chaplin, 1999; Mebius, 2003; Drayton et al., 2006; van de Pavert and Mebius, 2010). Deletion of LTα in the mice, as well as knocking out LTβ and LTβR in the mice, results in the deficiency in LN and Peyer's patch formation, disruptive lymphoid architecture and absence of mature

FDCs (De Togni et al., 1994; Koni et al., 1997; Fütterer et al., 1998). While the development of the spleen is independent of LTβR signaling, this pathway has been described to be vital for the spleen's lymphocyte organization, as well as for maintaining aspects of the marginal zone (Gommerman and Browning, 2003; Weih and Caamaño, 2003; McCarthy et al., 2006).).

1.4.4 Lymphotoxin β receptor signaling in lymph node homeostasis and remodeling

Studies have shown that LTβR signaling is required for maintaining homeostasis of lymphatic vessels and HEVs under steady state, as well as regulating their growth and functions in the LNs (Browning et al., 2005; Liao and Ruddle, 2006; Chyou et al., 2008). Supporting the contributions of LTβR signaling on vascular endothelial cells in LN remodeling, *in vitro* studies have shown that activation of LTβR on human dermal microvascular endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HDMECs) could induce proinflammatory gene expression via both the canonical and noncanonical NF-κB pathways (Cavender et al., 1989; Madge et al., 2008). In addition to controlling the growth and functions of lymphatic vessels and HEVs directly during LN remodeling, LTβR signaling could exert its influence indirectly through the LTβR-expressing FRCs in the LNs via the production of VEGF-A (Chyou et al., 2008). However as discussed previously, the extent to which this mechanism regulates VEGF-A production in regulating lymphangiogenesis and angiogenesis is unclear due to the various sources producing VEGF-A during inflammation such as macrophages or

B cells for example, and whether their actions are due to any control by LTβR signaling.

Another type of stromal cells present in the LNs known to be subjected to LTβR signaling regulation is the FDCs. Inhibition of the LTβR signaling leads to the loss of FDCs in lymphoid structures including LNs (Mackay and Browning, 1998). In addition, $LT\alpha_1\beta_2$ on B cells are necessary for FDCs development and chemokine (C-X-C motif) ligand 13 (CXCL13) production by FDCs, a B cell chemoattractant required for B cell homing to follicles in LNs (Ansel et al., 2000). Furthermore, CXCL13 induces B cells to upregulate the expression of $LT\alpha_1\beta_2$, establishing a positive feedback loop likely important in maintaining homeostasis (Ansel et al., 2000). Considering the vital role of FDC networks in the formation of germinal centers and their functions in antigen presentation and B cell memory, the maintenance of FDCs by LTβR signaling may be critical for LN remodeling (Klaus et al., 1980; Tew et al., 1997).

In contrast to our knowledge on the role of LTβR in the control of splenic architecture, the aspects of the LN microenvironment in mice that are dependent on LTβR are less defined, particularly in the context of LN remodeling during inflammation. Nevertheless, this is a relevant question as the organization of the LN is the central determinant of an efficient immune response and, conversely, alteration of this lymphoid environment may be at the origin of chronic inflammatory disorders {SainteMarie:2010hi}.

1.5 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of structurally related zinc-dependent endopeptidases involved in numerous diverse physiological and pathological processes such as angiogenesis, inflammation, cellular migration, cardiovascular, lung and rheumatic diseases and cancer (Nagase and Woessner, 1999; Lemaître and D'Armiento, 2006; Vargová et al., 2012). The first member of this enzyme family was identified in 1962 as a collagenase secreted by cultured tissue fragments of tadpole tail undergoing metamorphosis (Gross and Lapiere, 1962). Since their discovery, the main function of the MMPs has been thought to be in the regulation and remodeling of the ECM through the proteolytic degradation of the ECM components during pathophysiological events (Woessner, 1991). However, subsequent studies have shown that MMPs can also act on non-ECM substrates such as cytokines, chemokines, growth factors and growth factor-binding proteins (McCawley and Matrisian, 2001; Parks et al., 2004; Klein and Bischoff, 2011). In the last decade, more comprehensive system-wide approaches by proteomics and degradomics, along with murine models have substantially widened our knowledge of the exceptionally differing and complicated role of MMPs in development and disease (Butler and Overall, 2009; Morrison et al., 2009; Rodríguez et al., 2010).

With the exception of the membrane-anchored membrane-type (MT)-MMPs, the majority of MMPs are extracellular proteinases excreted in their inactive proenzyme forms. The general structure of MMPs is made up of the following

conserved domains: a signal peptide for secretion or membrane insertion; a prodomain that keeps the enzyme in its inactive form by occupying the active site; a zinc containing catalytic domain; a hemopexin domain for substrate specificity; and a hinge region that connects the catalytic domain to the hemopexin domain (Nagase et al., 2006). There are variations in the structures of different MMPs, giving rise to a wide range of substrate specificity and distribution. Based on the assessment of substrate specificity, organization and homology of the domains, and cellular localization, MMPs are widely classified into six groups: collagenases, gelatinases, stromelysins, matrilysins, MT-MMPs and other types of MMPs (Table 1.2) (Lemaître and D'Armiento, 2006).

1.5.1 Regulation of matrix metalloproteinase activity

The regulation of MMP catalytic activity takes place at four different stages: transcription, proenzyme activation, compartmentalization and enzyme inactivation (Parks et al., 2004). Expression of MMPs is strictly controlled at the transcriptional level, both spatially and temporally by signaling pathways that can be triggered by cytokines, growth factors, cell-to-cell and cell-to-ECM interactions (Chakraborti et al., 2003; Lemaître and D'Armiento, 2006; Yan and Boyd, 2007). The NF-κB signaling pathway, the mitogen activated protein kinase (MAPK) pathway and the signal transducer and activators of transcription (STAT) pathway are some of the main signaling pathways involved in the regulation of MMP gene expression (Vincenti and Brinckerhoff, 2007; Yan and Boyd, 2007; Clark et al., 2008).

Table 1.2: Members of the matrix metalloproteinase family. (Adapted from Onkologie, 35(1-2), Vargova et al., 2012, copyright @ 2012 Karger Publishers, Basel, Switzerland)

Subfamily	MMP	Main substrates
Gelatinases	MMP-2, MMP-9	gelatins; elastin; fibronectin; collagens I, IV, V, VII, X, XI; laminin; β-amyloid protein precursor
Collagenases	MMP-1, MMP-8, MMP-13	collagens I, II, III, VII, X; gelatins; entacin; aggrecan; link protein
Stromelysins	MMP-3, MMP-7, MMP-10, MMP-11, MMP-12	aggrecan; collagens III, IV, IX, X; fibronectin; laminin; elastin; gelatins; casein; IGFBP-1
Membrane-type MMPs	MMP-14, MMP-15, MMP-16, MMP-17, MMP-23A, MMP-23B, MMP-24, MMP-25	collagens I, II, III; fibronectin; laminin-1; dermatan sulphate; entactin
Other	MMP-19	gelatin
	MMP-20	amelogenin
	MMP-26	collagen IV; gelatin; fibronectin
	MMP-27	no data
	MMP-28	casein

Activation of inactive proMMPs requires a disruption in the interaction between the prodomain and the catalytic site in a process known as the cysteine-switch mechanism (Van Wart and Birkedal-Hansen, 1990). While the MT-MMPs are already active at the cell membrane due to intracellular activation, most of the secreted MMPs are activated outside the cell (Sternlicht and Werb, 2001; Zucker et al., 2003). Extracellular activation of proMMPs can be induced either through a proteolytic cleavage of the prodomain by active MMPs or serine proteinases such as plasmin, or through a allosteric activation involving the displacement of the prodomain from the catalytic site (Ra and Parks, 2007; Hadler-Olsen et al., 2011). Some activated MMPs have also been reported to degrade plasminogen in a negative feedback mechanism to keep the level of MMP activity in control (Kessenbrock et al., 2010).

Because the substrate specificity of the MMPS overlaps substantially, as well as the large number of substrates that each individual MMP can cleave, a regulatory mechanism needs to exist to localize and concentrate the proteases close to their target substrates. Secreted MMPs are known to localize on specific regions of the cell membrane and the ECM through binding to membrane-anchored proteins and receptors or cell-associated ECM molecules (Hadler-Olsen et al., 2011; Murphy and Nagase, 2011). This compartmentalization of MMPs is important in controlling the specificity of the proteolytic activity of the MMPs and, thus, determining their biological functions (Ra and Parks, 2007; Kessenbrock et al., 2010).

The proteolytic activities of activated MMPs can be inhibited by endogenous inhibitors such as the α2-macroglobulin and the tissue inhibitors of metalloproteinases (TIMPs) (Baker et al., 2002). While α2-macroglobulin is a general proteinase inhibitor with a broad spectrum of targets, TIMPs are specific inhibitors of MMPs (Sottrup-Jensen, 1989; Visse and Nagase, 2003). All active MMPs can be inhibited by TIMPs, although there is some difference in the inhibitory efficacy against different MMP members (Baker et al., 2002). In addition to MMP activity inhibition, TIMPs also have a role in the activation of proMMPs (Brew and Nagase, 2010).

1.5.2 Role of matrix metalloproteinases in physiological & pathological conditions

The ability of MMPs to cleave an extensive range of substrates including all the components of the ECM, cytokines, chemokines, growth factors and receptors, and other proteinases and proteinases inhibitors indicates that any alterations in the proteolytic activities of MMPs may result in numerous diseased states. The regulation of ECM homeostasis by MMPs activity is remarkably important because remodeling of the ECM is central to several physiological and pathological conditions (Mott and Werb, 2004). Degradation of the ECM by MMPs not only breaks down physical barrier, but also releases biologically active fragments and growth factors that influence cellular behavior (Schenk and Quaranta, 2003).

1.5.2.1 Matrix metalloproteinases in inflammation

Increased levels of MMPs are observed in all inflammatory processes, signifying the role of MMPs as modulators of inflammation (Parks et al., 2004). Although matrix proteolysis is a characteristic of the inflammatory process, degradation of the ECM is not the sole function of MMPs in inflammation. MMPs can also activate or inactivate chemokines by proteolytic processing, and establish chemokine gradients influencing leukocyte migration (Van Lint and Libert, 2007; Manicone and McGuire, 2008). Furthermore, TNFα and IL-1β are two of the most important proinflammatory cytokines that require proteolytic cleavage by MMPs for activation (Gearing et al., 1994; Schönbeck et al., 1998).

1.5.2.2 Matrix metalloproteinases in tumorigenesis

Extensive studies on the role of MMPs in cancer have been carried out for more than 30 years since the link between cancer cell invasion and MMP-mediated ECM degradation was first established (Liotta et al., 1980). The ability of MMPs to cleave all the ECM components together with findings showing upregulation of MMPs in almost all types of human cancers, made MMPs promising targets for cancer therapy (Coussens et al., 2002). Indeed, ECM degradation and remodeling is one of the most important roles of MMPs in cancer, leading to tumor cell invasion and metastasis (Kessenbrock et al., 2010). Several MMP members have been implicated in this process, with MMP-2, MMP-9 and MT1-MMP considered to be among the most critical (Pytliak et al., 2012). However, it is now clear that the role of MMPs in cancer is more complex than just ECM degradation. MMPs

can also promote cancer progression by regulating signaling pathways that control cell growth, apoptosis, inflammation and angiogenesis (Egeblad and Werb, 2002; Kessenbrock et al., 2010; Hua et al., 2011). Furthermore, MMP members have different roles in tumorigenesis. While the majority of MMPs promote cancer progression, some MMPs are inhibitory, possessing tumor-suppressing properties in a context-dependent manner (López-Otín and Matrisian, 2007; Decock et al., 2011).

1.5.2.3 Matrix metalloproteinases in angiogenesis

Angiogenesis is a process that is dependent on the BM and the remodeling of the ECM (Kalluri, 2003). For new blood vessels to sprout from existing vasculature, endothelial cells have to first break through the surrounding BM and ECM and migrate towards an angiogenic stimulus. This is followed by proliferation of the endothelial cells and the subsequent reorganization of the new outgrowth of endothelial cells into a tubular structure (Auerbach et al., 2003; Adams and Alitalo, 2007).

MMPs play an important role in angiogenesis and the contributions of several MMPs, specifically the gelatinases MMP-2 and MMP-9, and MT1-MMP have been extensively studied (Handsley and Edwards, 2005; van Hinsbergh et al., 2006).While proteolytic cleavage of the BM and ECM components by MMPs remove the physical constraint imposed on the sprouting vessels, the process may

also release growth factors such as VEGF-A, FGF-2 and transforming growth factor (TGF)-β that can promote angiogenesis, or generate bioactive fragments that inhibit angiogenesis (Rundhaug, 2005; van Hinsbergh and Koolwijk, 2008; Ribatti, 2009). For instance, angiogenesis can be promoted by MMP-9 and MMP-13 through mediating the release of sequestered VEGF-A from ECM (Bergers et al., 2000; Lederle et al., 2010). Cleavage of type XVII collagen by MMP-3, MMP-7, MMP-9, MMP-13 and MMP-20, and degradation of plasminogen by a range of MMPs including MMP-3, MMP-7, MMP-9 and MMP-12 can form endostatin and angiostatin respectively, products that suppress angiogenesis (Cornelius et al., 1998; Heljasvaara et al., 2005). In addition, MMPs can also increase the bioavailability of pro-angiogenic factors such as VEGF-A, whose expression is increased by MT1-MMP by forming a complex with VEGFR-2 and Src (Eisenach et al., 2010). On the other hand, MMPs can inhibit angiogenesis by restricting cell receptor signaling through the cleavage of FGF receptor (FGFR)-1 by MMP-2 (Levi et al., 1996). As a whole, MMPs are undeniably essential for angiogenesis. Due to the opposing effects of MMPs on angiogenesis, the outcome may be dependent on the spatial and temporal availability of specific MMPs and their substrates.

1.5.2.4 Matrix metalloproteinases in lymphangiogenesis

As lymphangiogenesis is a process fairly similar to angiogenesis, it is likely that MMPs also have a critical role in the sprouting and remodeling of the lymphatic vessels. While blood vessels are enclosed by a continuous BM, lymphatic vessels,

as described above, are only partially covered by BM and interact closely with the ECM through anchoring filaments (Paupert et al., 2011) (**Figure 1.1**). The resulting difference in the ECM and BM components that the two vasculatures associate with suggests that the MMPs involved in each sprouting process may be different.

The contributions of MMPs during lymphangiogenesis to date are not as well characterized compared to angiogenesis. Among the MMP members, MMP-2, MMP-9 and MT1-MMP are known to be produced by LECs (Nakamura et al., 2004; Bruyère et al., 2008), and the use of a broad-spectrum inhibitor that blocks the activities of MMP-1, MMP-2, MMP-3, MMP-9 and MMP-13 is able to inhibit lymphangiogenesis-related properties of LECs in culture, as well as lymph node metastasis (Nakamura et al., 2004). Several studies have also linked lymphangiogenesis and lymph node metastasis with elevated expression of MMPs such as MMP-2, MMP-3 and MMP-9 (Langenskiöld et al., 2005; Ueda et al., 2005; Işlekel et al., 2007; Yoo et al., 2011). The role of MMP-2 in lymphangiogenesis is highlighted in a study of lymphangiogenesis through a model of adult skin regeneration, where the increased expression of MMP-2 in the regenerating region correlates with the onset of lymphangiogenesis (Rutkowski et al., 2006). Furthermore, examination of lymphatic regeneration using transgenic mice lacking MMP-9 suggests that MMP-9 is not needed for lymphangiogenesis in the above model (Rutkowski et al., 2006). Blocking of MMP-2 by a synthetic inhibitor reduces the tube-forming ability of LECs in the tube formation assay

(Matsuo et al., 2007). Thoracic duct rings derived from MMP-2 but not MMP-9 deficient mice also showed impaired lymphangiogenesis in a three-dimensional lymphatic ring assay (Bruyère and Noël, 2010). More specifically, a recent study using various *in vitro* and *in vivo* models has demonstrated that MMP-2 participates in lymphangiogenesis during physiological and pathological conditions by degrading the interstitial collagen and regulating lymphatic vessel branching (Detry et al., 2012).

Studies so far have focused on the role of MMP-2 as a critical mediator of lymphangiogenesis. However, taking into account the complex role of MMPs in the modulation of angiogenesis, it is plausible that other MMP members may also contribute to lymphangiogenesis either positively or negatively. For instance, the regulation of VEGF-A bioavailability from the ECM by MMP-9 and MMP-13 may also promote lymphangiogenesis (Bergers et al., 2000; Lederle et al., 2010). Indeed, the role of MMPs in lymphangiogenesis is still at its infancy and further studies are needed to elucidate the aspects of lymphangiogenesis that are MMPs dependent.

1.6 Aims & rationale

Most studies to date on inflammatory lymphangiogenesis have been focused on the inflamed peripheral tissues. However, the remodeling of the lymphatic vessels in the LNs is an important question as the organization of the LN is important for

an efficient immune response as well as in the regulation of the inflammatory response. In this study, we investigated the role of B cells in the induction of LN lymphangiogensis, whether they regulate lymphangiogenesis directly or indirectly. Because B cells express $LT\alpha_1\beta_2$, we hypothesized that B cells may regulate lymphangiogenesis through LTβR signaling. Thus we also examined the involvement of LTβR signaling in the remodeling of the LN and lymphangiogenesis in response to an inflammatory stimulus, and how LTβR signaling is linked to B cells in driving lymphangiogenesis.

Chapter 2: Material & Methods

2.1 Mice

7-13-week old CD45.2, CD45.1, μMT and TNFαKO transgenic female mice on a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained under specific pathogen-free conditions with access to food and water within the National University of Singapore's satellite Animal Housing Unit (NUS). All experiments were performed under protocols approved by the National University of Singapore and Biological Resource Center Institutional Animal Care and Use Committee.

2.2 Induction of lymph node hypertrophy by immunization with complete Freund's adjuvant/keyhole limpet hemocyanin

Mice were anesthetized with intra-peritoneal injection of ketamine. The amount of ketamine used is determined by the weight of each mice. Equal volumes of complete Freund's adjuvant (CFA) and sterile PBS containing model antigen keyhole limpet hemocyanin (KLH) (2.5 mg/ml) were emulsified, and 20 μl of this emulsion was injected subcutaneously into the front and rear footpads of the mice that drain to the brachial, axillary and popliteal LNs respectively. PBS was used as a control. The mice were then sacrificed at various time points after immunization by CO_2 inhalation.

2.3 Inhibition of LTβR signaling in the mice

2.3.1 Preventive inhibition

Signaling by LTβR was blocked using the LTβRIg fusion protein (Biogen, a kind gift from Jeffrey L. Browning). After the mice were sedated with intra-peritoneal injection of ketamine, 15 μg of LTβRIg (15 μl in PBS) was injected subcutaneously into the front and rear footpads of the mice 1 day before immunization with CFA/KLH. Human IgG (huIgG) (Sigma) was used as a control.

2.3.2 Therapeutic inhibition

For short-term inhibition of LTβR signaling (3 days), 15 μg of LTβRIg was injected subcutaneously into the front and rear footpads of the mice 4 days after immunization with CFA/KLH. For long-term inhibition of LTβR signaling (>1 week), mice were given an intra-peritoneal injection of 100 μg of LTβRIg 4 days after CFA/KLH immunization. Likewise, huIgG was used as a control.

2.4 Stimulation of the LTβR signaling and the TNFR signaling pathways with LTβR agonist and TNFR agonist

Signaling by LTβR and TNFR were stimulated with anti-mouse LTβR antibody (eBioscience) and anti-mouse CD120a (TNFR type I/p55) antibody (BioLegend) respectively. When individual signaling pathway was stimulated, 20 μl of the respective antibody was injected subcutaneously into the front and rear footpads

of the mice twice with a 2 day interval. The mice were then sacrificed four days after the first administration of the agonist antibody. For priming of the LTβR by TNFR activation, 20 µl of the anti-mouse TNFR antibody was injected subcutaneously into the mice footpads a day before repeating the protocol to stimulate LTβR as described above. For activation of the LTβR signaling pathway in µMT mice along with CFA/KLH immunization, anti-mouse LTβR antibody was administered a day before and a day after immunization as described above. PBS was used as a control for these experiments.

2.5 Subcutaneous application of MMP-13 inhibitor

To block MMP-13 protease activity in the LNs, the MMP-13 inhibitor CL 82198 hydrochloride (Tocris Bioscience) was used. 10 µl of PBS was added to 10 µl of CL 82198 (75 mM in dimethyl sulfoxide (DMSO)) and 20 µl of this mixture was injected subcutaneously into the front and rear footpads of the mice after the mice were sedated with ketamine and before immunization with CFA/KLH as described above. For control, a mixture containing equal volumes of PBS and DMSO was used. Administration of CL 82198 was repeated daily until the mice was sacrificed according to the various time points of the study.

2.6 *In vivo*-labeling of mouse cells with 5-bromo-2'-deoxyuridine (BrdU)

In vivo BrdU labeling of mouse cells were carried out with the BD Pharmingen™ BrdU Flow Kit (BD Biosiences) according to the manufacturer's instructions. In

brief, mice were subjected to intra-peritoneal injection of 200 µl of BrdU solution (10 mg/ml) on the day of CFA/KLH immunization.

2.7 Transplantation of bone marrow cells

2.7.1 Generation of WT/WT and WT/µMT mice

Six-week-old recipient WT and µMT mice were lethally irradiated with 2 doses of 500 rad 2 hr apart and injected intravenously with a total of 10×10^6 bone marrow cells. Two experimental groups of five mice each were designed: WT that received WT BM (WT/WT) and µMT that received WT BM (WT/µMT).

2.7.2 Generation of WT/µMT and LTα/µMT mice

Six-week-old recipient µMT mice were lethally irradiated with 2 doses of 500 rad 2 hr apart and injected intravenously with a total of 10×10^6 BM cells. Two experimental groups of five mice each were designed: µMT that received a mix of 25% WT BM and 75% µMT BM (WT/µMT) or 25% LTαKO BM and 75% µMT BM (LTα/µMT). Six weeks after transplantation, mice were treated with CFA/KLH as described above. Control mice included non-transplanted WT and µMT mice.

2.8 Dendritic Cell migration assay

The area on either side of the dorsal skin of the mice that drain to the brachial and axillary LNs was shaved. Fluorescein-5-isothiocyanate (FITC) (Sigma) (8 mg/ml) was first dissolved in equal volumes of acetone (Sigma) and dibutyl phthalate (Sigma) and applied in 25 µl aliquots with a pipette tip onto the shaved skin 3 days after CFA/KLH injection. The mice were sacrificed 18 hr later and cell suspensions of the total LN cells were prepared as described above. Flow cytometry was then carried out. To quantify the migration of DCs induced by FITC application, the total number of $FITC^+CD11c^+$ DCs cells per LN was calculated by multiplying the percentage of $FITC^+CD11c^+$ cells by the total number of LN cells.

2.9 Cells isolation

2.9.1 Isolation of cells from lymph nodes

Isolation of cells from the LNs including LECs and BECs were performed by adopting a formerly described method (Halin et al., 2007). LNs were digested without any manipulations at 37 °C in 4 ml of Ca^{2+}/Mg^{2+} free Hank's Balanced Salt Solution (HBSS) (Gibco) containing collagenase IV (4 mg/ml) (Gibco) with gentle agitation. After 45 min, EDTA was added to the HBSS medium till a final concentration of 10 mM for another 5 min of incubation. The cell suspension was then collected after passing the digested tissue through a 70-µm mesh strainer 4 times. Total live cell number was evaluated with a hemacytometer after Trypan blue staining.

2.9.2 Isolation of dendritic cells from lymph nodes (Dendritic cell migration assay)

Cell suspensions of the total LN cells were prepared by incubating the pooled brachial and axillary LNs after teasing at 37° C in Ca^{2+}/Mg^{2+}-free HBSS containing 400 U/ml collagenase D (Roche Diagnostics) for 25 min, and another 5 min following the addition of 10 mM EDTA. Single cell suspensions were collected after gently pressing the digested tissue through a 70-µm mesh strainer. Assessment of the total live cell number was done using a hemacytometer after Trypan blue staining.

2.9.3 Isolation of B cells from lymph nodes

Digestion of the LNs was carried out with collagenase D as described above to obtain single cell suspensions. B cells were then isolated from single cell suspensions by magnetic-activated cell sorting (MACS) using CD19 MicroBeads (Miltenyi Biotec) magnetic labeling and LS Columns (Miltenyi Biotec) for magnetic separation. Isolation was done according to manufacturer's instructions. Two fractions were obtained after MACS: the positive B cell fraction and the negative fraction. The purity of the fractions was accessed by flow cytometry.

2.10 Flow cytometry

All the antibodies used in this study are listed in Appendix 1.

2.10.1 Immunofluorescence staining of cell surface antigens for flow cytometric analysis

Specific unconjugated or fluorochrome-conjugated primary antibodies and appropriate isotype control antibodies were used to stain the cells in fluorescence-activated cell sorting (FACS) buffer (1% normal mouse serum, 1% normal rat serum, 0.5% BSA, 2 mM EDTA in PBS, pH 7.4). Cells were incubated with antibodies for 20 min on ice. Appropriate flurochrome-conjugated secondary antibodies were added to the cells for staining where necessary and the cells were incubated for 20 min on ice. When fixation was required, 1% formalin was used to fix the cells.

2.10.2 Intracellular staining of cells for flow cytometric analysis of LEC proliferation

Mice were pulsed with BrdU as described above. The immunofluorescence staining of incorporated BrdU in the cells were performed with the BD Pharmingen™ BrdU Flow Kit according to the manufacturer's instructions. Briefly, cells were first stained for surface antigens as described above. BD Cytofix/Cytoperm Buffer were then added to the cells and incubated for 15 min at room temperature to fix and permeabilize the cells. The cells were washed with BD Perm/Wash Buffer and incubated with BD Cytoperm Plus Buffer for 10 min on ice before washing again with BD Perm/Wash Buffer. Re-fixation of the cells was carried out with the addition of BD Cytofix/Cytoperm Buffer and incubating for 5 min at room temperature. After washing the cells with BD Perm/Wash

Buffer, the cells were treated with DNase and subjected to incubation for 1 hr at 37 ^{o}C to expose incorporated BrdU. This was followed by a washing step with BD Perm/Wash Buffer. Staining of BrdU was carried out by resuspending the cells in BD Perm/Wash Buffer containing diluted APC-conjugated anti-BrdU antibody and incubated for 20 min at room temperature. The cells were then washed with BD Perm/Wash Buffer and resuspended for flow cytometric analysis.

2.11 Immunofluorescence analysis

All the antibodies used in this study are listed in Appendix 2.

LNs were either freshly embedded in Tissue-Tek optimum cutting temperature compound (Sakura) and frozen on dry ice immediately after extraction or fixed overnight in 2% paraformaldehyde/30% sucrose solution at 4°C. The paraformaldehyde-fixed LNs were then washed with 30% sucrose solution before embedding in the above-mentioned tissue freezing medium and snap frozen. Cryostat sections (6-8 μm) from the LNs were cut and mounted on non-coated polylysine-coated slides and stored at -20°C. Before carrying out immunofluorescence staining, the slides were allowed to thaw and dry at room temperature for at least 30 min. The LNs sections were then fixed in ice-cold acetone for 15 min. Prior to the actual staining, LN sections were blocked with 1% BSA in PBS for 10 min to minimize non-specific antibody binding. If biotinylated primary antibody was used in the staining, the Avidin/Biotin

Blocking Kit (Vector Laboratories) was employed according to the manufactuer's instructions before the addition of the primary antibody to inhibit the endogenous biotin, biotin receptors, and avidin binding sites present in the tissues. Primary antibodies diluted in PBS containing 10% normal mouse were added to the LN sections and incubated in a humidified chamber for 1 hr at room temperature or overnight at 4°C. The slides were then washed 3 times for 5 min each in PBS. LN sections were then incubated for 1 hr in a humidified chamber in the dark with secondary antibodies diluted in PBS containing 10% normal mouse. The slides were again washed 3 times for 5 min each in PBS. After the last wash, the slides were mounted with a cover slip using the Dako Fluorescent mounting medium (Dako Cytomation). Images were taken with the Carl Zeiss Axio Imager.Z1 and Axiocam HR microscope camera.

2.12 Polymerase chain reaction (PCR)

2.12.1 Total RNA extraction from mammalian cells and tissues

Total RNA was isolated from LNs, isolated B and T cells, monolayer SV-LECs or RAW 246.7 cells using a combination of TRIzol® Reagent (Invitrogen), MaXtract High Density tubes (Qiagen) and NucleoSpin® RNA II kit (Macherey-Nagel). Briefly, the homogenized samples were incubated in TRIzol® Reagent for 5 min at room temperature to allow complete dissociation of the nucleoprotein complex before chloroform (20% volume of TRIzol® Reagent) was added to the lysates and subjected to vigorous shaking. The contents were then transferred to the MaXtract High Density tubes and centrifuged for 12000 x g for 15 min at 4°C.

The separated aqueous phase was mixed with an equal volume of 70% ethanol and transferred to the NucleoSpin® RNA II Column from the NucleoSpin® RNA II kit for purification and DNA digestion according to the manufacturer's instructions. The concentration of the total RNA extracted was quantified using the NanoDrop™ 1000 Spectrophotometer (Thermo Scientific).

2.12.2 Reverse transcription

Reverse transcription was performed with SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. Briefly, 2 µg of total RNA was mixed with 1 µl of 50 µM oligo$(dT)_{20}$ and 1 µl of 50 ng random hexamers before topping up to 10 µl with DEPC-treated water. The mixture was incubated at 65°C for for 5 min then placed on ice for at least 1 min. 10 µl of cDNA Synthesis Mix, consisting of 2 µl of 10X RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNaseOUT™ (40 U/µl) and 1 µl of SuperScript™ III RT (200 U/µl), was added to each RNA/primer mixture to a total volume of 20 µl. The reaction was incubated for 10 min at 25°C followed by 50 min at 50°C and finally terminated at 85°C for 5 min and chilled on ice. 1 µl of RNase H was added to each tube and incubated for 20 min at 37°C. An aliquot of the reaction was used for PCR.

2.12.3 Semi-quantitative PCR

The resulting cDNA from the reverse transcription reaction (2 µl) was used in a 25 µl PCR reaction containing GoTaq Flexi DNA Polymerase (Promega) and 400 nM of primers. Primer squences are listed in Appendix 3. PCR amplification was performed by denaturation at 95°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min, for 30 or 35 cycles, on a 2720 Thermal Cycler (Applied Biosystems). 20 µl of each PCR products was separated by electrophoresis on a 1.5% (w/v) agarose gel supplemented with 1 µg/ml ethidium bromide in TAE buffer (40 mM Tris-acetate and 1mM EDTA). DNA bands were visualized under ultraviolet illumination. The integrity and input of CDNA was confirmed using β-actin.

2.12.4 Quantitative real-time PCR (qPCR)

qPCR was carried out on a 7500 Real-Time PCR System (Applied Biosystems). Reactions were performed in a 20 µl final volume containing 2 µl of cDNA, 200 nM of primers and iTaq^{TM} SYBR$^{®}$ Green Supermix With ROX (Bio-Rad). Primer sequences are listed in Appendix 4. Amplification and data collection were completed as per manufacturer's instruction. Briefly, 40 cycles of amplication were performed with a denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min. GAPDH was used as a housekeeping gene to normalize the amount of mRNA. The relative expression of each sample was caluculated by the $2^{-\Delta\Delta CT}$ method.

2.13 Protein expression & analysis

2.13.1 BCA protein assay

Protein concentration was determined by the BCA protein assay. 10 µl of each sample together with standard was pipetted into a 96-well flat-bottom plate with a working protein range of 25-2000 µg/ml. Preparation of the BCA working reagent was done by mixing BCA Reagent A with BCA Reagent B in a 50:1 ratio. 200 µl of the resulting BCA working reagent was then added to each well and mixed thoroughly on a plate shaker. Incubation of the plate was carried out at 37°C for 30 min. Absorbance was measured from 540-590 nm on a spectrophotometer plate reader (Biorad). A standard curve plotted with the standards (average corrected absorbance measurement of each standard against its concentration in µg/ml) was used to determine the protein concentration of the samples.

2.13.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were prepared by adding 2x Lammli's sample buffer (100mM Tris-HCl pH 6.8, 4% SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol and 10% (v/v) ß-mercaptoethanol) and heated at 95°C for 5 min before loading onto resolving gels of 10% (w/v) polyacrylamide and 5%(w/v) polyacrylamide stacking gels cast between 2 glass plates (Bio-rad). SDS-PAGE was carried out with the vertical slab gel unit, Mini-PROTEAN III Cell (Bi0-Rad). The PageRuler Plus Prestained Protein Ladder (Fermentas) was used as the protein marker. Gel electrophoresis was carried out in running buffer (25 mM Tris-HCl pH 8.3, 192

mM glycine and 0.1% SDS) at a constant voltage of 80-100 V for 2-3 hr at room temperature until the proteins were well separated.

2.13.3 Transfer of proteins

The separated proteins in the SDS-PAGE gels were electrophoretically transferred to nitrocellulose membranes (Bio-Rad) using the Mini Trans-Blot Cell (Bio-Rad). Transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine and 0.1% SDS) was cooled to 4°C and the gels were allowed to equilibrate for 5 min in the ice-cold buffer prior to the transfer. The transfer was performed overnight at 4°C and at a constant voltage of 20 V.

2.13.4 Immunoblotting

All the antibodies used in this study are listed in Appendix 5.

The membranes were blocked in blocking buffer (Tris-buffered saline (TBS) with 0.1% Tween 20 and 5% skim milk) at room temperature for 1 hr. After washing thrice with TBST (TBS with 0.1% Tween 20) at 10 min per wash, the membranes were incubated with the primary antibody of interest in blocking buffer overnight at 4°C. As above, the membranes were washed 3 times with TBST before incubating for 1 hour at room temperature with a horseradish peroxidase (HRP)-conjugated secondary antibody in TBST at the dilutions recommended by the

manufacturer. The membranes were again washed 3 times with TBST before they were subjected to chemiluminescence detection with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and visualization of the signal on ChemiDoc XRS (Bio-rad).

2.13.5 Zymography assay

Resolving gels of 10% (w/v) polyacrylamide and 1% (w/v) gelatin and 5%(w/v) polyacrylamide stacking gels were cast. Protein samples were prepared by adding 2X Lammli's sample buffer (100mM Tris-HCl pH 6.8, 4% SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol) without the addition of reducing agent such as ß-mercaptoethanol and without boiling. The PageRuler Plus Prestained Protein Ladder was used as the molecular weight standards. . Gel electrophoresis was performed in the Mini-PROTEAN III Cell with running buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine and 0.1% SDS) at a constant voltage of 125 V for ~2 hr at room temperature until the bromophenol blue tracking dye reached the bottom of the gel. The gel was then removed from the cassette and washed twice for 60 min in 100 ml of renaturing solution (2.5% Triton X-100 pH 8.0) at room temperature with gentle agitation. 100 ml of development buffer (0.05 M Tris–HCl pH 8.0, 5 mM $CaCl_2$) was added to the gel and agitated for 15 min at room temperature before transferring to a 37°C incubator for 16-20 hr. The gel was stained in staining solution (0.1% Coomassie Brilliant Blue R-250 (w/v) in fixing/destaining solution) for at least 4 hr until the gel was uniformly dark blue and destained in fixing/destaining solution (45% (v/v) methanol, 10% (v/v) acetic

acid and 45% (v/v) water) until the bands were clearly visible against the blue background. Quantification of the intensity of the proteinase bands was performed using ImageJ (version 1.43) (National Institute of Health [NIH]).

2.14 Cell Culture

2.14.1 SV-LEC

SV-LEC, a LEC line isolated from mesenteric adventitia from mice expressing temperature-sensitive SV40 large T antigen, was obtained from J. Steven Alexander (Louisiana State University Health Sciences Center) as a kind gift (Ando et al., 2005). High glucose (4500mg/L) Dulbecco's modified Eagle's medium (DMEM) (Gibco) enriched with 10% fetal bovine serum (FBS) (Hyclone) and 1% Penicillin-Streptomycin antibiotics (Invitrogen) was used as the culture medium to maintain the cells. SV-LECs were grown in a 37^{o}C incubator at 90% humidity level supplied with 5% CO_2. Confluent monolayers were sub-cultured weekly at 1:4 dilution. PBS was used to rinse the cells before a buffered, Ca^{2+}/Mg^{2+}-free salt solution containing 0.5% (w/v) trypsin and 0.2% (w/v) EDTA was used to dissociate adherent cells from tissue culture flasks. Dissociated cells were subsequently washed down by DMEM and collected by centrifugation at 300 x g for 5 min. Following re-suspension in fresh culture medium, SV-LECs were seeded in new culture flasks.

2.14.2 RAW 264.7 cells

The RAW 264.7, a macrophage-like, Abelson leukemia virus transformed cell line from BALB/c mice was obtained from the American Type Culture Collection (ATCC). Roswell Park Memorial Institute (RPMI)-1640 medium with L-glutamine and sodium bicarbonate (Sigma) enriched with 10% FBS and 1% Penicillin-Streptomycin antibiotics was used to maintain the cells. RAW 264.7 cells were grown in a 37^{o}C incubator at 90% humidity level supplied with 5% CO_2. Culture medium was replaced every 2 to 3 days. Subcultures of RAW 264.7 cells were prepared by scraping. Culture medium was removed except for a small amount (leave 10 ml of culture medium for a 75 cm^2 culture vessel) and cells were dislodged from the flask with a cell scraper. Appropriate aliquots of the suspension were then seeded into new culture vessels in a ratio of 1:4.

2.14.3 HMVEC-dLy cells

Primary human adult lymphatic microvascular endothelial cells (HMVEC-dLyAd-Der Lym Endo Cells) were obtained from Lonza Walkersville (Walkersville, MD). Endothelial growth medium 2 microvascular (EGM-2 MV) Bulletkit (Lonza) consisting of various growth supplements were used to maintain the cells. Thawing of the cryopreserved HMVEC-dLy cells, the initiation of the culture process and the general maintenance of the cells including subculturing were done according to the instructions provided by Lonza Walkersville. Briefly, HMVEC-dLy cells were seeded at the recommended seeding density of 5000 cells/cm^2 and incubated at 37^{o}C and 5% CO_2. Medium was changed every 2 days and HMVEC-

dLy were sub-cultured when they reached 80% confluency and then plated at a 1:4 ratio in fresh medium. HMVEC-dLy cells were used for a maximum of five passages.

2.15 Tube formation assay

The basement membrane extract used for the tube formation assay is BD Matrigel™ Basement Membrane Matrix, Growth Factor Reduced (BD Biosciences). A day before the start of the assay, cells were split using procedures as described above such that they would be ~80% confluent in 24 hr and matrigel, originally kept in the -20^{o}C freezer were allowed to thaw in ice in a refrigerator at 4^{o}C. For experiments using MMP-13 silenced SV-LECs, the cells were allowed to be transfected with siRNA for 72 hr. On the day of the assay, 100 µl of fully thawed Matrigel was loaded per well of a 96-well flat-bottom plate and centrifuged at 300 x g for 10 min at 4^{o}C to remove any air bubbles trapped in the wells. The plate was then transferred to a cell culture incubator and incubated at 37^{o}C for 30 min to allow the Matrigel to gel. The ~80% confluent cells were harvested as described above to make a single cell suspension and cell number was determined using a hemacytometer after Trypan blue staining. The cells were then resuspended in their respective basal culture media at a concentration of 3 x 10^{5} cells/ml. 1 ml of the single cell suspension was then loaded into various tubes corresponding to the number of conditions to be tested and centrifuged at 300 x g for 10 min. 1 ml of corresponding serum-free media containing basal media, MMP inhibitors, anti-mouse LTβR antibody (eBioscience) or recombinant TNFα

(R & D systems) and their respective control media (containing PBS or DMSO) were then added to the cell pellets to make single cell suspensions. The following MMP inhibitors were used and applied in the mentioned concentrations GM6001 (100 μM) (Calbiochem, Merck), MMP-9/MMP-13 inhibitor (100 μM) (Calbiochem, Merck) and CL 82198 (75 μM). 100 μl (3 x 10^4 cells) of the single cell suspensions was added gently to each well of the 96-well plate on top of the gelled Matrigel. The plate was then incubated at 37°C, 5% CO_2 in the cell culture incubator until the desired result was achieved (6-18 hr). Images were then taken using a Canon PowerShot G6 mounted on a Carl Zeiss Axiovert 40 CFL. For live-cell imaging of the tube formation process, images were taken for a period of 3 hr with a 90 s interval between each image using the Olympus Confocal Laser Scanning Biological Microscope FV1000 in a live-cell imaging chamber. Quantification of tube formation was performed in a blinded fashion using a commercial imaging service (Wimasis GmbH, Munich, Germany).

2.15 Scratch wound assay

SV-LECs were first allowed to grow to confluence on 6-well plates. Following starvation in serum-free DMEM overnight, a scratch was made across each full well diameter with a p200 pipette tip. Media were then replaced with DMEM (1% FBS) with CL 82198 (75 μM) or DMSO as control. The wounded monolayers were then photographed immediately after scratching before incubating at 37°C, 5% CO_2 in the cell culture incubator until the desired outcome was achieved (12 hr). Images were taken at 0 hr and 12 hr using a Canon PowerShot G6 mounted on

a Carl Zeiss Axiovert 40 CFL. Percentage of wounded area that was closed was determined using ImageJ.

2.16 RNA interference (RNAi)

siRNA targeting MMP-13, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (positive control) and the non-targeting negative control siRNA were all purchased from Dharmacon (Thermo Scientific) and shipped as dry pellets. Transfection of siRNA into SV-LECs grown to 60% confluence was performed using DharmaFECT 1 transfection reagent from Dharmacon (Thermo Scientific) according to the manufacturer's instructions. MMP-13 siRNA (ON-TARGETplus siRNA from Dharmacon) was used at a final concentration of 50 nM. Briefly, siRNA was diluted in serum-free medium and mixed with equal volume of the DharmaFECT 1 transfection reagent that was also diluted in serum-free medium and incubated for 20 min at room temperature. The amount of DharmaFECT 1 added as well as the total volume of the transfection mix was dependent on the size of the plate format used. For 6-well plate, 5 µl of DharmaFECT 1 was added to each well for transfection. Antibiotic-free complete medium was then added to the mix. Culture medium was then removed from the cells, and the transfection mix added to them. The transfection medium was replaced with complete medium after 24 hr in order to reduce cytotoxicity. Transfected cells are incubated at 37°C with 5% CO_2 for 48 hr (for mRNA analysis) or 72 hr (for protein analysis and tube formation assay).

2.17 Statistical analysis

Statistical comparisons were made using the Student's two-tailed t test. Data were expressed as mean ± standard deviation (SD). Analysis was performed with Prism 5 (Graph-Pad Software, Inc.).

Chapter 3: B cells mediate lymphangiogenesis in the lymph nodes through the expression of lymphotoxin α

3.1 Introduction

The lymphatic vasculature is indispensable in various physiological processes such as the maintenance of tissue fluid homeostasis, immune surveillance and fat absorption. Lymphangiogenesis, the process of forming new lymphatic vessels from pre-existing vasculatures, occurs spontaneously during embryogenesis. However, lymphangiogenesis and the remodeling of the lymphatic vessels in adulthood is mainly involved in pathologic processes such as inflammation, tissue injury and tumor cell metastasis (Cueni and Detmar, 2008; Alitalo, 2011).

Lymphangiogenesis has been known to occur in various forms of inflammation, and the remodeling of the lymphatic vessels is usually due to the accumulation of inflammatory cells and the inflammatory mediators that these cells secrete such as TNFα, VEGF-A and VEGF-C (Ji and Ji, 2007; Huggenberger et al., 2011a). In our previous study, the expansion of the lymphatic vessel network in activated LNs of immunized mice has been shown to require the recruitment of B cells within the LNs (Angeli et al., 2006). Since then, macrophages (Kataru et al., 2009), DCs (Webster et al., 2006), FRCs (Chyou et al., 2008) and T cells (Furtado et al., 2007) have all been shown to be involved in lymphangiogenesis in the lymphoid structures. The molecular mechanisms and signaling pathways underlying inflammatory LN lymphangiogenesis are not as well characterized compared to the growth of lymphatic vasculature during embryonic development. This study is focused on the signaling pathway behind the B-cells mediated LN lymphangiogenesis.

In this chapter, we delve into the role of B cells in driving LN lymphangiogenesis in our CFA/KLH model of immunization. Taking into account the important function of FDC networks in the formation of germinal centers and the regulation of B cell functions in immunity (Klaus et al., 1980; Tew et al., 1997), we also examined the possible involvement of FDCs in lymphangiogenesis.

3.2 Results

3.2.1 Induction of LN lymphangiogenesis by CFA/KLH immunization

We began by investigating the effects of immunization with CFA/KLH on LN lymphangiogenesis. Immunohistochemistry together with flow cytometry were performed in order to examine the modifications in the lymphatic vessel network, together with the extent of lymphangiogenesis by quantifying LEC number in the non-immunized and immunized LNs. CFA/KLH, an immunization model that triggers robust LN expansion (Angeli et al., 2006), was used to immunize the mice. Explicitly, subcutaneously injections of CFA/KLH (controls were injected with PBS) into the footpads of the mice were carried out, and the brachial and axillary LNs that drain the front footpads were extracted after 4 days for the flow cytometric analysis, while the popliteal LNs that drain the rear footpads were obtained for immunostaining (Figure 3.1). The time point was chosen because a robust inflammatory response could be detected in the LNs 4 days after immunization. Immunostaining of the LNs revealed that lymphatic vessels, recognized specifically by LYVE-1 immunoreactivity, were mainly located in the subcapsular sinus of the non-immunized control LNs, whereas the $B220^+$ B cells were detected in the cortex of the LNs (Figure 3.1A). As expected, the size of the LN and the B cell compartment, as well as the organization of the lymphatic vessel network, all showed significant differences after CFA/KLH immunization. Immunized LNs expanded in size along with the B cell compartments, and the lymphatic vessels were seen to be more enlarged in the subcapsular space. Intriguingly, lymphatic vessels were also observed in the cortex of the LNs in close association with the B cells. The lymphatic vessels growing into the cortex

are in vicinity to B cells, frequently seen to be around the B cell follicles (Figure 3.1A).

After presenting the expansion of the lymphatic vessel network by immunohistochemistry, we went on to quantify the number of LECs by flow cytometry. The LN cells were stained for CD45 (a marker for hematopoietic cells), podoplanin and CD31. As mentioned previously, podoplanin is a marker of LECs, while CD31 is a marker for endothelial cells, staining both LECs and BECs. Representative flow cytometry plots pre-gated on live cells illustrated the isolation of the non-hematopoietic LECs, which are $CD45^{low}Podoplanin^{+}CD31^{+}$ (Figure 3.1B). Stromal cells, consisting of FRCs and FDCs, along with BECs are also represented on the plot by $CD45^{low}Podoplanin^{+}CD31^{-}$ and $CD45^{low}Podoplanin^{-}CD31^{+}$ respectively (Figure 3.1B). Expansion of the LN was observed 4 days after immunization, resulting in about 6-fold increase in LN cellularity as compared to non-immunized LNs (Figure 3.1C). Similarly, there was also approximately a 5-fold increase in the number of LECs in the LNs after CFA/KLH immunization (Figure 3.1C), reinforcing the expansion of the lymphatic vessel network observed by immunostaining. Taken together, immunohistochemistry and flow cytometry allowed the qualitative and quantitative analysis of lymphangiogenesis in LNs following immunization. Our results revealed that inflammation-associated immunization with CFA/KLH induced a marked remodeling of the lymphatic vessel network within the LNs.

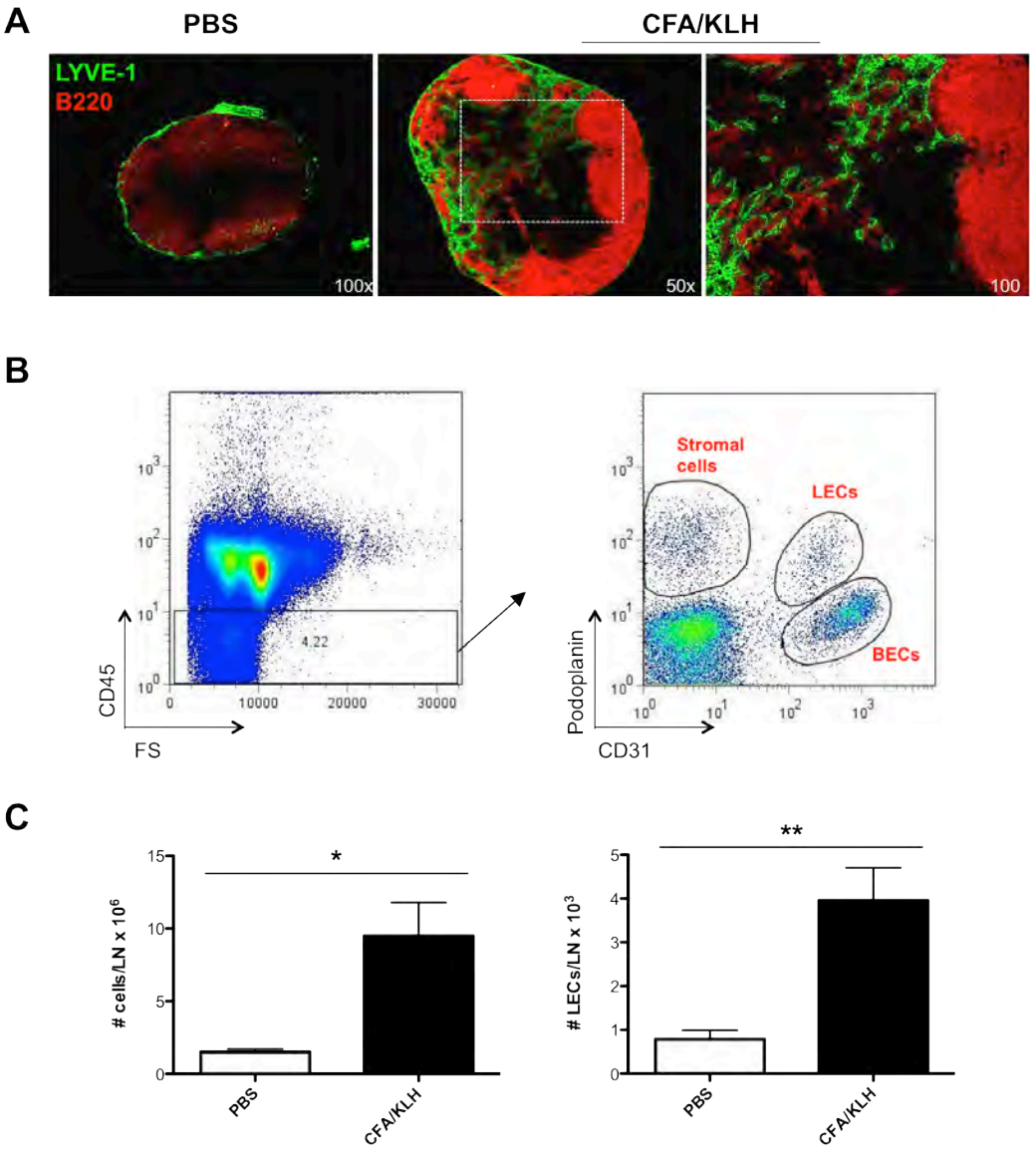


Figure 3.1: Expanded LNs and lymphangiogenesis 4 days after CFA/KLH immunization. (A) LN sections from control (PBS) and immunized (CFA/KLH) mice were stained for LYVE-1 and B220, markers for lymphatic vessels and B cells respectively. (B) Representative flow cytometry plot showing the gating of LECs ($CD45^{low}Podoplanin^{+}CD31^{+}$), BECs ($CD45^{low}Podoplanin^{-}CD31^{+}$) and stromal cells ($CD45^{low}Podoplanin^{+}CD31^{-}$). (C) LN cellularity and number of LECs in LNs were evaluated 4 days after CFA/KLH immunization. Images and flow cytometry results are representative of 5 independent experiments (n=3). Significant differences are designated by *$p < 0.05$ and **$p < 0.01$.

3.2.2 Requirement of B cells in the expansion of the LNs and LN lymphangiogenesis in response to immunization

Since the lymphocytes are the main constituents of the LNs, the enlargement of the LNs upon immunization requires an expansion in the B and T cell sections of the LN as well. As the B and T lymphocytes are also known to play a role in LN lymphangiogenesis, we next investigated the growth in the B and T cell compartments of the LN 4 days after CFA/KLH immunization. The representative flow cytometry plot showed how the $B220^+$ B cells and $CD3^+$ T cells were gated (Figure 3.2A). Correspondingly, LN cellularity showed approximately 5-fold increase in the total cell count after immunization (Figure 3.2B). While there was an expected increase in the lymphocytes count upon CFA/KLH administration, B cells increased proportionally more than T cells. B cells increased by more than 5-fold after immunization, whereas there was only about a 3-fold increase in T cell count (Figure 3.2B). These observations together with the expansion of the lymphatic vessel network in the B cell follicles led us to hypothesize that B cells might be essential for the increase in LN cellularity as well as LN lymphangiogenesis.

In order to investigate the role of B cells in LN lymphangiogenesis, we evaluated the effect of CFA/KLH immunization on μMT mice that lack B cells. LNs from WT and μMT mice were stained with both LYVE-1 and B220 to visualize the lymphatic vessel network and the presence of B cells. Accordingly, B cells were absent in the LNs of μMT mice, while in the LNs of WT mice, B cells can be

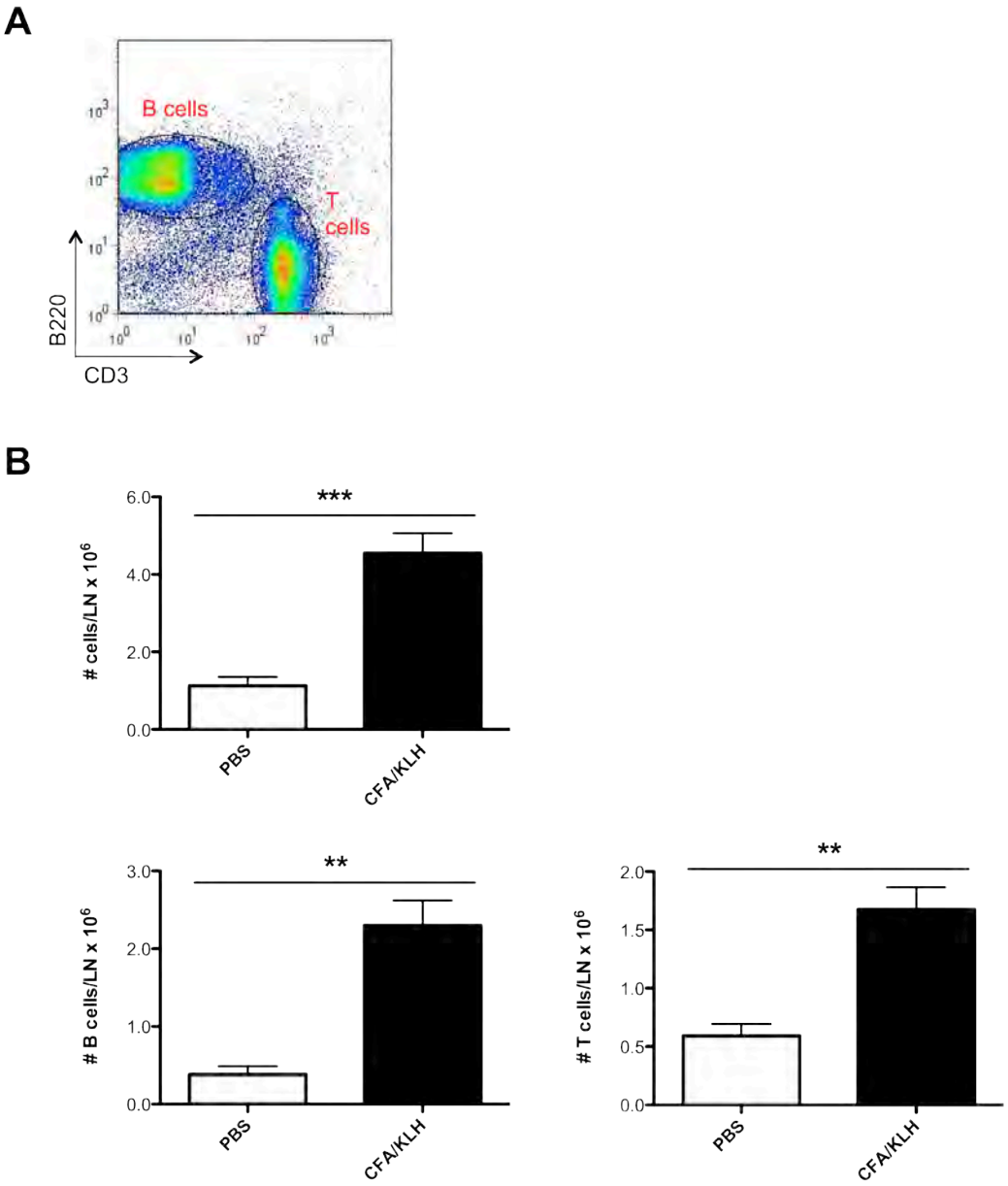


Figure 3.2: Expansion of the B and T cells population in the LNs after immunization. (A) Representative flow cytometry plot showing B cells ($B220^+$) and T cells ($CD3^+$) in the LNs. (B) LN cellularity and number of B and T cells in LNs were evaluated 4 days after CFA/KLN immunization. Data shown are representative of 5 independent experiments with 3 mice per group in each experiment and significant differences are designated by $^{**}p < 0.01$ and $^{***}p < 0.001$.

observed in the cortex region (Figure 3.3A). Immunization on the WT mice footpad induced the enlargement of the draining LNs together with the expansion of the lymphatic vessel network, but these effects on the LNs of μMT mice were greatly reduced (Figure 3.3A). While lymphatic vessels can be observed in the cortex region of the B cells follicles in the LNs of WT mice, LYVE-1 staining of the μMT mice was mainly localized around the subcapsular space of the LNs (Figure 3.3A). The observations made in the immunohistochemistry study were reinforced by flow cytometry analysis. LN expansion was smaller in response to CFA/KLH immunization in μMT mice, having only a 3-fold increase in LN cellularity compared to the 5-fold increase seen in WT mice (Figure 3.3B). In the absence of B cells, the immunized LNs of the μMT mice were also strikingly smaller than the immunized LNs of the WT mice having only about 9 million cells per LN in the μMT mice compared to the 16 million cells per LN seen in the WT mice (Figure 3.3B). Total LECs count in the the μMT mice was also remarkably reduced compared to WT mice. The LNs of non-immunized WT mice have 5 times as much LECs as the LNs of non-immunized μMT mice (Figure 3.3B). Following immunization, the expansion of the lymphatic vessel network can be indicated by the 4-fold increase in the number of LECs in the LNs of WT mice (Figure 3.3B). On the other hand, the number of LECs in the LNs of μMT mice only increased by less than 2-fold in response to CFA/KLH (Figure 3.3B). These results from the μMT mice suggested that B cells may indeed play a critical role in LN lymphangiogenesis.

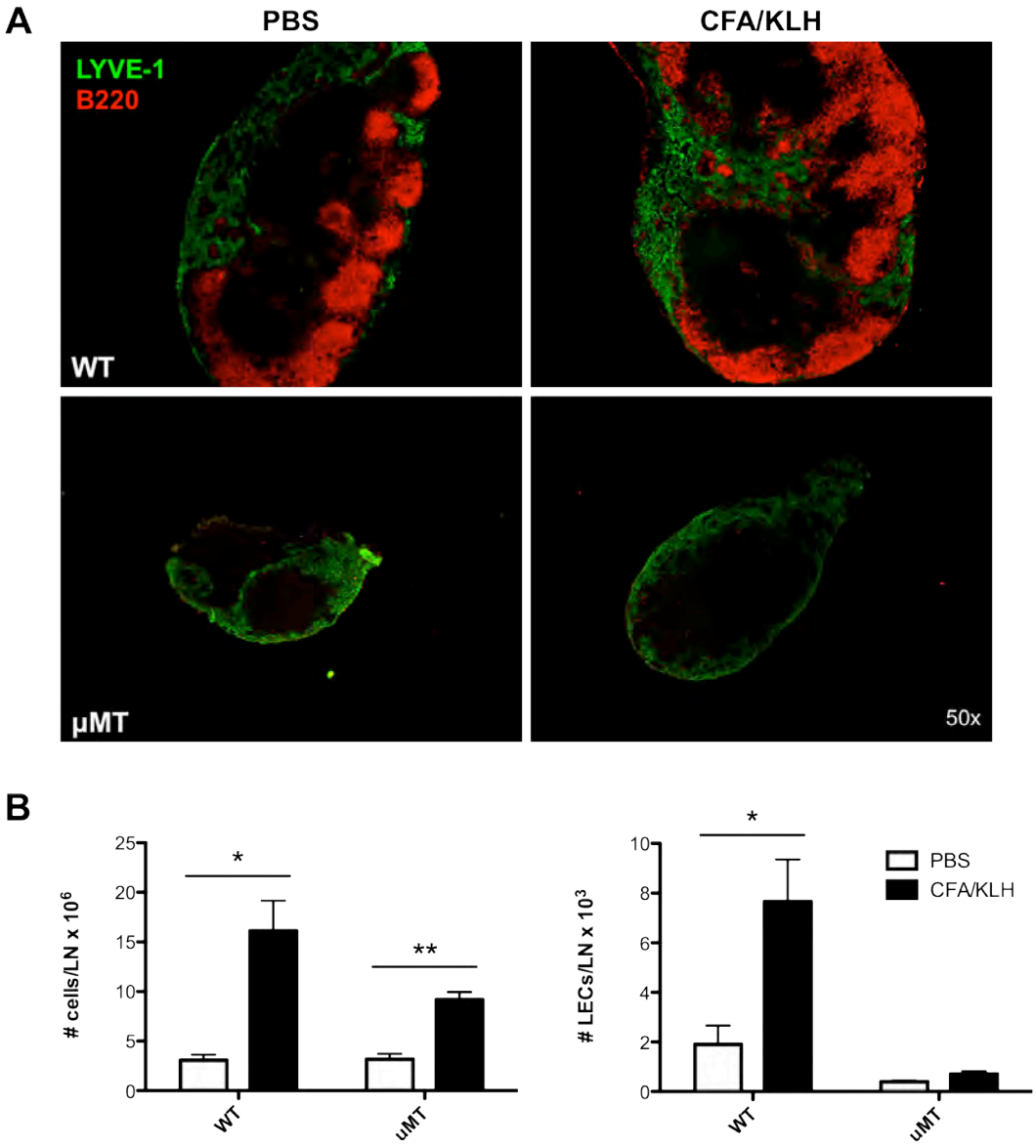


Figure 3.3: Absence of lymphangiogenesis in µMT mice. (A) LN sections from control (PBS) and immunized (CFA/KLH) WT and µMT mice were immunostained for LYVE-1 and B220. (B) LN cellularity and number of LECs in the LNs of WT and µMT mice were analyzed 4 days after immunization with CFA/KLH. Images and flow cytometry results are representative of 3 independent experiments (n=3). Significant differences are designated by *$p < 0.05$ and **$p < 0.01$.

Having established that the LNs of μMT mice which do not have B cells react much less drastically upon CFA/KLH immunization in terms of LN expansion and LN lymphangiogenesis, we speculated that the lack of B cells in the μMT mice may be the main cause for our observations. To address this issue, we generated chimeric mice where μMT recipient mice were transplanted with BM from WT mice to increase the number of B cells in these mice (WT/μMT). As control, WT recipient mice received BM transplantation from WT mice (WT/WT). From the flow cytometry analysis, the non-immunized LNs of the WT/μMT were slightly smaller than the WT/WT control and they expanded by 5-fold upon CFA/KLH immunization compared to the 8-fold seen in the LNs of the control mice (Figure 3.4A). Parallel to μMT mice, the LNs of the chimeric WT/μMT mice have a much lower total LECs count compared to their WT/WT mice counterpart, having almost 5 times less LECs as the LNs of the control mice (Figure 3.4A). The total LEC count of the immunized WT/μMT mice LNs was 6-fold less than that of the WT/WT control mice LNs (Figure 3.4A). However, the fold change of the LEC count in the LNs after immunization was about the same in the LNs of both the WT/μMT mice and the WT/WT mice (Figure 3.4B). The effect of CFA/KLH immunization was able to increase the number of LECs in the LNs by 8-fold, indicating the expansion of the lymphatic vessel network in both group of mice (Figure 3.4B). Although the number of LECs in the LNs of the chimeric WT/μMT mice was much lower than in the control group, comparable to the μMT mice, our results indicated that the increase in number of B cells in μMT mice was able to restore lymphangiogenesis in the LNs after CFA/KLH

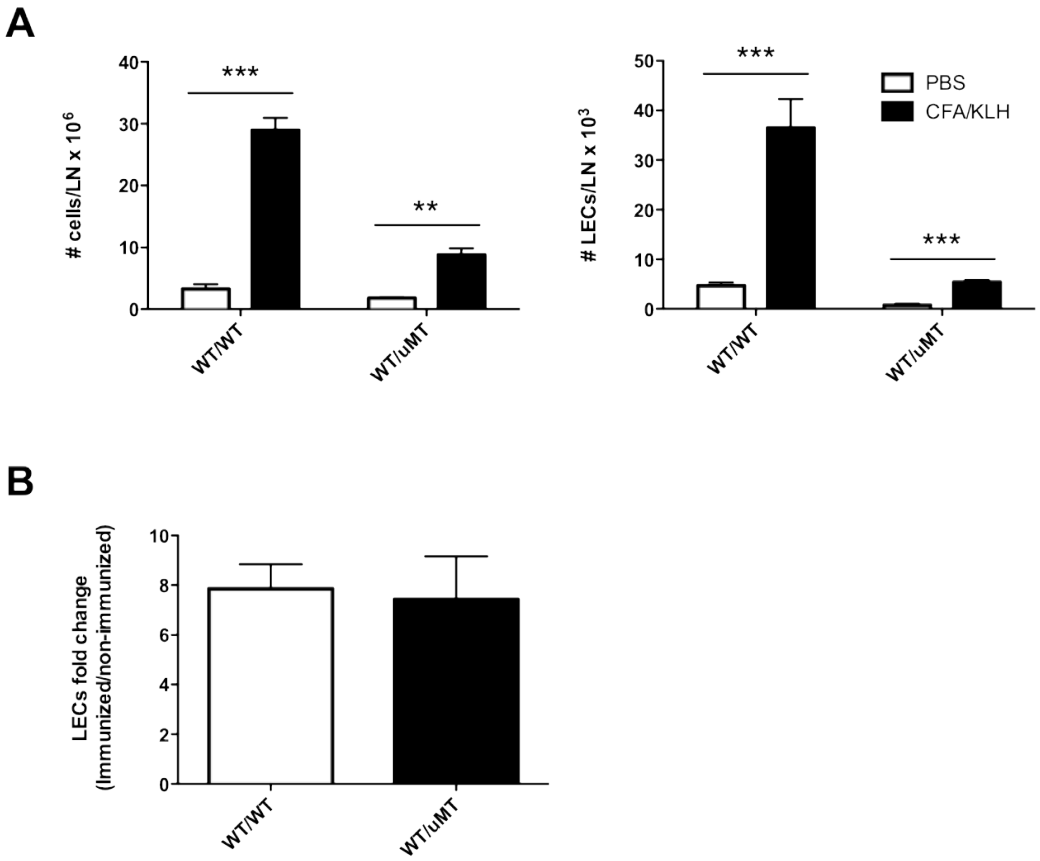


Figure 3.4: Transplantation of B cells in μMT mice induced LN lymphangiogenesis by immunization. (A) LN cellularity and number of LECs in LNs of WT/WT & WT/μMT mice were evaluated 4 days after CFA/KLH immunization. (B) Fold change in the number of LECs in LNs of WT/WT & WT/μMT mice after immunization. Data shown are representative of 3 independent experiments with 3 mice per group in each experiment and significant differences are designated by **$p < 0.01$ and ***$p < 0.001$.

immunization, reinforcing the importance of B cells in LN lymphangiogenesis.

3.2.3 Expression of LTα by B cells critical for LN lymphangiogenesis induced by immunization

After showing that LN lymphangiogenesis induced by immunization is dependent on B cells, we decided to investigate the signaling pathway involved. As LTα has been described to promote lymphangiogenesis (Mounzer et al., 2010), and B cells are known to express LTα, we hypothesized that the B cells-induced lymphangiogenesis may be dependent on LTα. To test this hypothesis, we again generated chimeric mice from μMT hosts through BM transplantation to generate mice with B cells that were deficient for LTα. These mice were reconstituted with a mix of 25% LTαKO BM and 75% μMT BM {Angeli:2006gb}, and as a result, the leukocytes present in these mice were mainly $LT\alpha^{+}$, and only B cells were $LT\alpha^{-}$ (Table 3.1). Since LTα is required in the generation of the LTβR ligand, $LT\alpha_1\beta_2$, these chimeric mice also cannot form the $LT\alpha_1\beta_2$ heterotrimer on the B cell surface. A second group of chimeric mice reconstituted with 25% WT BM and 75% μMT BM, was generated as control. From the immunohistochemistry results, the LNs of the control WT/μMT mice revealed a fairly healthy population of B cells similar to those of the WT mice (Figure 3.5). As expected from the flow cytometry data of our previous experiment, reconstitution of WT BM in μMT mice fully enabled the LNs to expand and lymphangiogenesis to occur upon immunization (Figure 3.5). Interestingly, the LNs from the LTαKO/μMT mice

Table 3.1: B cells population in the different mice group

Mice	B cell population
WT	Normal
μMT	Absent
WT/μMT	Normai
LTαKO/μMT	No LTα

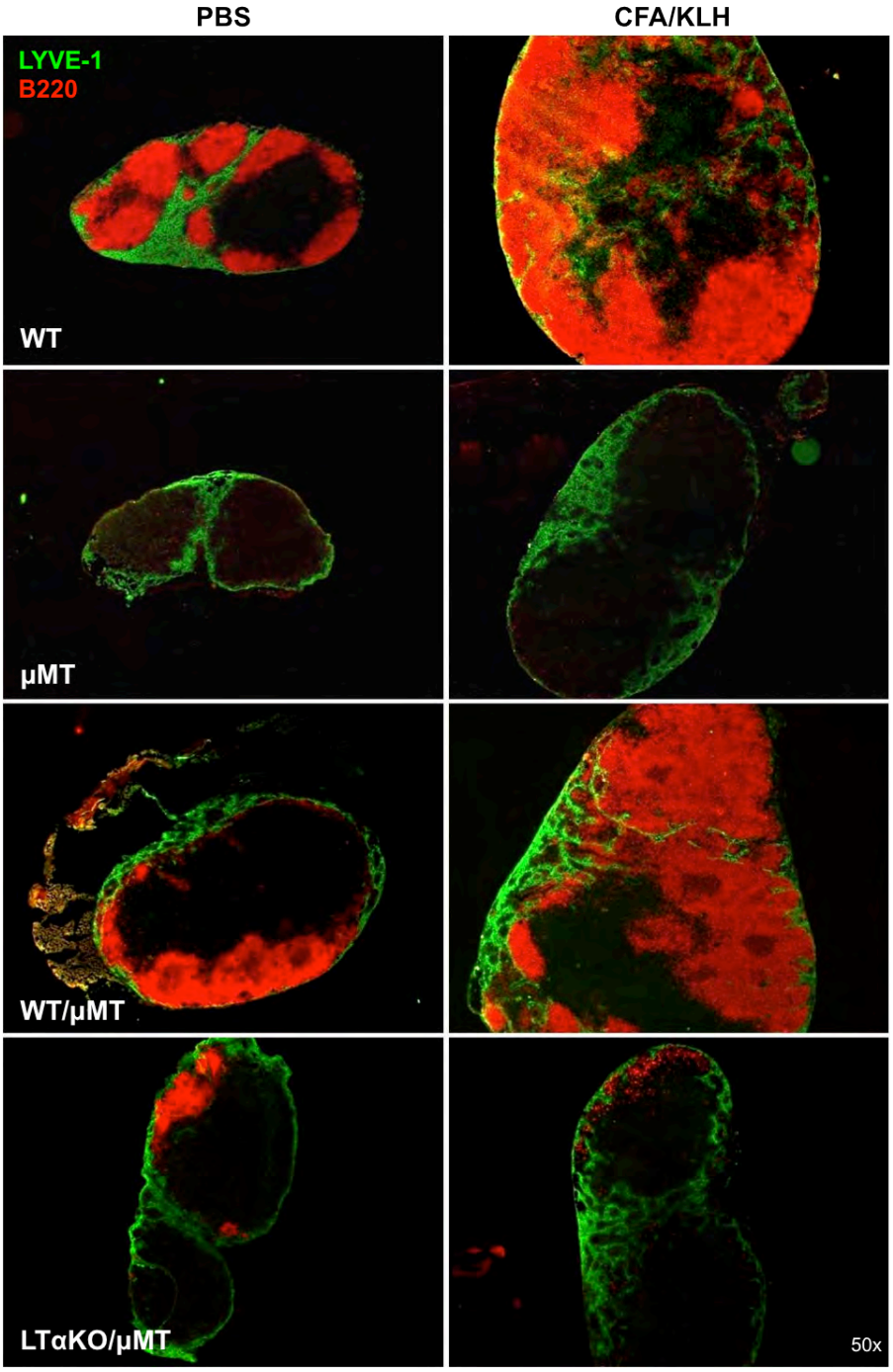


Figure 3.5: Examination of the LV network in the LNs of the various chimeric mice following immunization. Non-immunized and immunized LN sections from control and chimeric mice were immunostained for LYVE-1 and B220. Images are representative of 2 independent experiments consisting of 5 - 6 mice per group in each experiment.

had a lower number of B cells in the LNs at steady state, suggesting that when LTα expression was selectively removed {Mackay:1998ka}, B cells either do not move into the LNs or they are not retained in the LNs (Figure 3.5). In response to CFA/KLH, the LNs of the LTαKO/μMT chimeric mice were able to increase slightly in size, but lymphangiogenesis was not detected (Figure 3.5). These observations from the LTαKO/μMT chimeric mice were similar to those from the μMT mice (Figure 3.5). Taken together, these data suggested that the expression of LTα on B cells in the LNs is necessary for optimal LN expansion and lymphangiogenesis after CFA/KLH immunization.

3.2.4 Implication of FDCs in the induction of LN lymphangiogenesis in response to immunization

The importance of FDCs in B cell functions and the formation of germinal centers led us to investigate the role of FDCs in LN lymphangiogenesis. As FDCs are maintained by the presence of the LTβR signaling (Tumanov et al., 2003), we wondered if the lack of LN lymphangiogenesis observed in the mice lacking B cells or mice with $LT\alpha^{-}$ B cells is due to the absence of FDCs in the LNs. Therefore, we assessed the presence of FDCs by staining for FDCM2 in the LNs of these mice. We first analyzed LNs from WT mice where FDC clusters were clearly observable in the region of the B cells follicles in both the non-immunized and immunized LNs (Figure 3.6A and B). On the other hand, in the absence of B cells in the μMT mice, FDCs were not detected at both steady state and inflamed LNs (Figure 3.6A and B). Predictably, LNs of the WT/μMT mice that were

A
WT
μMT
WT/μMT
LTαKO/μMT
Lyve-1
FDCM2
PBS
CFA/KLH
50x
B
Lyve-1
FDCM2
PBS
CFA/KLH
400x

Figure 3.6: Examination of the FDC network in the LNs of the various chimeric mice. (A) Non-immunized and immunized LN sections from control and chimeric mice were immunostained for LYVE-1 and FDCM2. (B) High magnification images of the boxed regions in (A). Images are representative of 2 independent experiments consisting of 5-6 mice per group in each experiment.

occupied with B cells were also populated with FDCs in both non-immunized and immunized LNs similar to WT mice (Figure 3.6A and B). Results from LTαKO/μMT chimeric mice were comparable to μMT mice whereby FDCs were not seen in the LNs (Figure 3.6A and B). These results indicated that the expression of LTα on B cells, and likely the resultant expression of $LT\alpha_1\beta_2$, was critical for the localization of FDCs in the LNs. Furthermore, these data also suggest that the presence of FDCs might be involved in LN lymphangiogenesis upon immunization.

To further inspect the role of FDCs in the LN lymphangiogenesis, we made use of TNFα-deficient mice with unorganized B cell follicles and absence of FDC networks in the LNs (Pasparakis et al., 1996; 1997). TNFαKO mice were immunized with CFA/KLH and the extent of LN expansion and lymphangiogenesis assessed by flow cytometry. Comparing the LN cellularity of the WT and TNFα deficient mice by flow cytometry prior to immunization showed that the LNs from the TNFαKO mice were smaller than those found in the WT mice (Figure 3.7). After CFA/KLH immunization, total LN cellularity of the TNFαKO mice were expectedly lower than WT mice, however, there was still a significant increase in the total cell count of about 9-fold comparable to WT mice (Figure 3.7), suggesting that despite the lack of TNFα in the mice, there were compensatory pathways present to make up for its absence in mediating inflammation. The LECs count in the LNs of TNFαKO mice were lower than that of the WT mice, but after immunization, the number of LECs increased by more

than 10-fold to be on par with the number of LECs in the immunized WT LNs (Figure 3.7), clearly demonstrating that LN lymphaniogenesis is not impaired in these TNFαKO mice. Following that, we also compared the number of lymphocytes, DCs and macrophages in the LNs (Figure 3.7). In our flow cytometry analysis, we defined DCs by the expression of CD11c and macrophages by CD11b. Expectedly from the enlargement of the LN, there were significant increases in the amount of B and T cells in the TNFαKO mice LNs upon immunization like the WT mice (Figure 3.7). The number of DCs and macrophages also increased significantly in the LNs of TNFαKO mice after CFA/KLH treatment (Figure 3.7). Although the DC count in the immunized LNs of TNFKO mice was only 60% of the total amount of DCs compared to the immunized LNs of WT mice, the 10-fold increase in the number of DCs from the non-immunized TNFαKO mice LNs was proportional to those of the WT mice (Figure 3.7). In the same way, the amount of macrophages in both the non-immunized LNs and immunized LNs of the WT mice was more than twice the number of macrophages found in the respective LNs of the TNFαKO mice, but the relative 7-fold increase in the sum of macrophages from non-immunized to immunized LNs was analogous in both group of mice (Figure 3.7). Taken together, these results revealed that the deprivation of TNF in the TNFαKO was inconsequential to LN expansion and lymphangiogenesis in response to immunization. That LN lymphangiogenesis was able to occur in the absence of FDCs in the TNαFKO mice also suggested that the FDCs may be dispensable in the process.

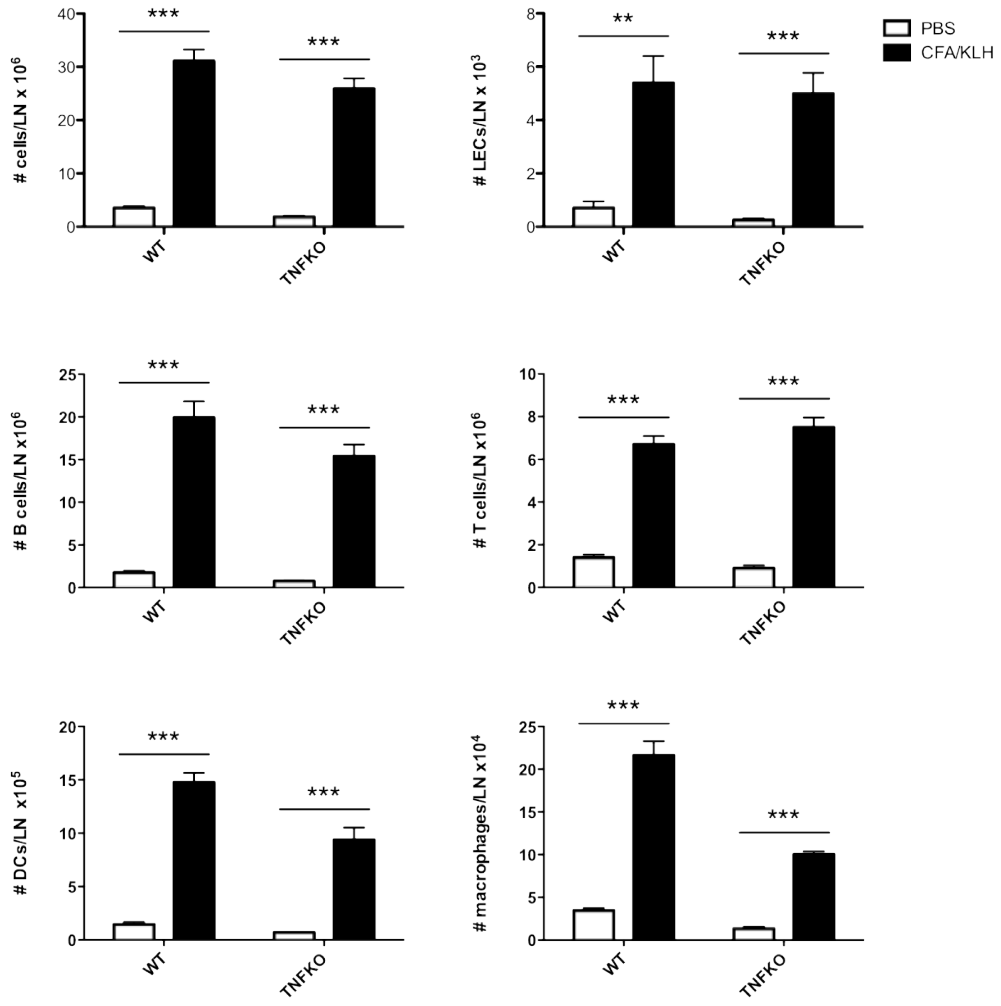


Figure 3.7: Effects of CFA/KLH immunization on TNFαKO mice. LN cellularity, number of LECs, B and T cells, DCs and macrophages in LNs of WT and TNFKO mice were evaluated 4 days after CFA/KLH immunization. Data shown are representative of 3 independent experiments with 4 mice per group in each experiment and significant differences are designated by **$p < 0.01$ and ***$p < 0.001$.

3.3 Discussion

In our study, we qualitatively and quantitatively analyzed lymphangiogenesis by immunohistochemistry and flow cytometry, respectively. We demonstrated that immunization in mice footpad with CFA/KLH was able to induce a distinct remodeling of the lymphatic vessel network within the LNs. The fact that the newly formed lymphatic vessels in the expanded LNs after immunization were in close contact with the B cells, and that the B cells population expanded more than T cells following immunization, led us to hypothesize the implication of B cells in regulating lymphatic vessel growth in the LNs.

Using μMT mice that lack B cells, we were able to show the importance of B cells in mediating lymphangiogenesis as μMT mice do not display any expansion in the lymphatic vessel network after immunization. Our data indicated that reconstitution of μMT mice with WT B cells was able to restore lymphatic vessel growth, further illustrating the central role of B cells in LN lymphangiogenesis.

While B cells have been reported to drive lymphangiogenesis through a mechanism dependent on VEGF-A (Angeli et al., 2006), we now show that the expression of LTα on B cells is critical for modulating the growth of new lymphatic vessels after immunization. Intriguingly, the B cell follicles of LTαKO/μMT mice were not as organized compared to control mice. This was most likely due to the absence of FDC networks in these mice as a result of the

lack of LTβR signaling from B cells of these mice. Investigation of the FDC networks in the LNs of chimeric mice suggested that they might be vital for lymphangiogenesis as the absence of FDC networks correlates with deficiency in the lymphatic vessel network expansion.

However, our results with the TNFαKO mice suggested that LN lymphangiogenesis can still occur in the absence of the FDC networks. This exaggerated growth in lymphatic vessels after immunization in TNFαKO mice is in line with a previous study (Baluk et al., 2009), and showed that either TNFα and/or FDCs are not required for lymphangiogenesis, or compensatory pathways are involved in the TNFαKO mice. Nevertheless, our study illustrated the importance of B cells in regulating lymphangiogenesis, as well as linking this regulation to the LTα and LTβR signaling.

Chapter 4: Regulation of lymph node lymphangiogenesis by lymphotoxin β receptor signaling

4.1 Introduction

The LTβR signaling pathway is important in the development of secondary lymphoid organs as well as in maintaining the organization of these microenvironments (Fu and Chaplin, 1999; Mebius, 2003; Drayton et al., 2006; van de Pavert and Mebius, 2010). Unlike the role of LTβR in the control of splenic architecture, the aspects of the LN organization in mice that are LTβR-dependent are less defined, especially in the remodeling of LN during inflammation. Studies on LTβR signaling in the LNs have focused on the maintenance and regulation of the homeostasis and function of lymphatic and blood vessels (Browning et al., 2005; Liao and Ruddle, 2006; Chyou et al., 2008). Interestingly, LTβR signaling has been shown to be required for *de novo* inflammatory lymphangiogenesis in tertiary lymphoid structures (Furtado et al., 2007).

In the previous chapter, we demonstrated that the expression of LTα on B cells is critical in the regulation of lymphangiogensis after immunization. In this chapter, we explored the role of LTβR signaling in lymphangiogenesis and LN remodeling. We made use of a LTβR inhibitor, the fusion protein LTβRIg, in our study (Browning, 2008). LTβRIg acts as a soluble decoy receptor that inhibits both LTβR ligands: $LT\alpha_1\beta_2$ and LIGHT. We also sought to examine the expression of LTβR by LECs, as well as investigate the possible source of the LTβR ligand, $LT\alpha_1\beta_2$, in the regulation of lymphangiogenesis.

4.2 Results

4.2.1 LTβR signaling in the regulation of LN expansion and lymphangiogenesis following immunization

With the aim of studying the role of LTβR signaling in LN expansion and lymphangiogenesis induced by our model of CFA/KLH immunization, we used LTβRIg to inhibit the LTβR ligands. This inhibitor was injected subcutaneously into the mice footpads a day before immunization with CFA/KLH, and we chose the same timepoint of 4 days after immunization to examine the ensuing LN expansion and lymphangiogenesis by both flow cytometry and immunohistochemistry. In our flow cytometry experiment, we added a group of untreated mice alongside the control group that were treated with huIgG to test the effect of huIgG on the mice (Figure 4.1). Treating the mice with either huIgG or LTβRIg did not lead to any changes to the baseline LN cellularity as compared to the non-treated mice (Figure 4.1A). As expected, mice administered with huIgG showed a significant 5-fold increase in LN cellularity after immunization, similar to the non-treated mice (Figure 4.1A). In contrast, the treatment of LTβRIg was able to block the expansion of the LNs in response to CFA/KLH, as the LNs of immunized LTβRIg-treated mice did not result in any significant increase in LN cellularity, with only less than a 2-fold increase (Figure 4.1A). Having revealed the effects of LTβR signaling on LN expansion, we proceeded to explore its influence on lymphangiogenesis. By the same comparison, huIgG and LTβRIg did not alter the sum of LECs in non-immunized LNs (Figure 4.1A). Likewise, immunized LNs of non-treated mice and huIgG-treated mice showed a 7-fold and 10-fold increase in LN LECs number respectively (Figure 4.1A). The treatment of

huIgG on mice was shown not to have any negative influence on the LNs in terms of total LN cellularity and LEC count in response to immunization, and thus, huIgG-treated mice were used as control in subsequent experiments. However, LTβRIg-treated mice only resulted in a less than 4-fold increase in LEC number after CFA/KLH immunization (Figure 4.1A), illustrating that blocking LTβR signaling in the LN impedes lymphangiogenesis. The examination of the LN and lymphatic vessel network expansion in LNs of immunized mice by immunostaining further strengthened our findings that LTβRIg impairs the growth of LN and lymphangiogenesis in response to CFA/KLH (Figure 4.1B). Control LNs treated with huIgG exhibited the increase in the LN size and expansion of the lymphatic vessel network in the subcapsular sinus as well as in the cortex after immunization as expected from our previous data (Figure 4.1B). Conversely, LNs from mice treated with LTβRIg only presented a minimal increase in size and did not show any significant difference in the architecture of the lymphatic vessel network before and after immunization (Figure 4.1B). The wide-spread growth of lymphatic vessels in the cortex region of the LNs that characterized immunized LNs were not observed in the LTβRIg-treated LNs (Figure 4.1B). These results, together with the quantitative flow cytometry analysis of the total LN cell count and LECs number, were in line with our hypothesis that LTβR signaling has a vital role in lymphangiogenesis upon immunization.

While we have shown that LN lymphangiogenesis induced by immunization with CFA/KLH is mediated by LTβR signaling, the identity of the LTβR-expressing

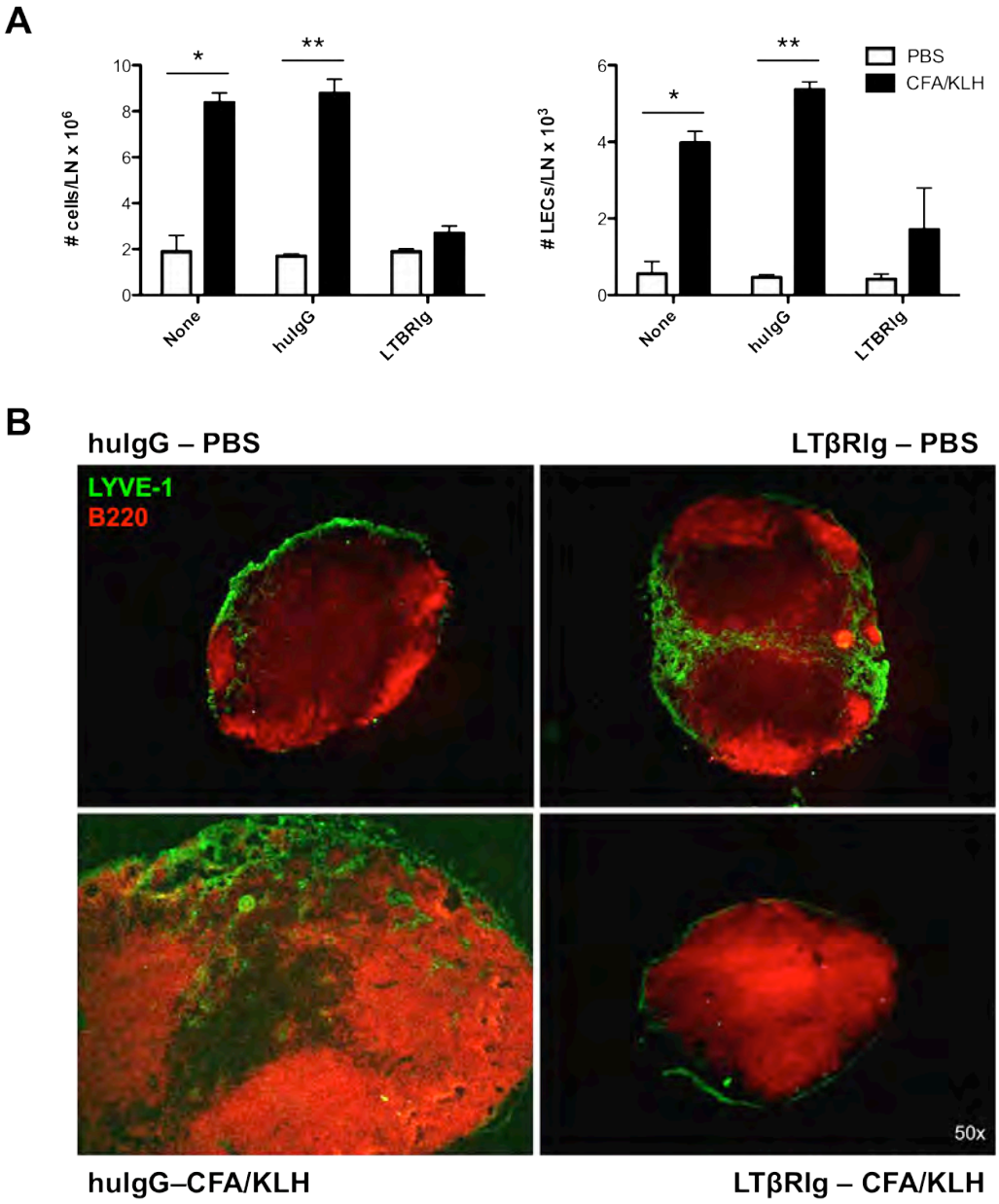


Figure 4.1: Blocking the LTβR signaling pathway inhibits LN expansion and lymphangiogenesis following immunization. (A) LN cellularity and number of LECs were evaluated in LNs of non-, huIgG- and LTβRIg-treated mice 4 days after immunization. (B) LN sections from non-immunized and immunized mice treated with huIgG (control) or LTβRIg were stained for LYVE-1 and B220. Images and flow cytometry results are representative of 5 independent experiments (n=3). Significant differences are designated by *$p < 0.05$ and **$p < 0.01$.

cells involved in the signaling in our model has not been defined. Therefore, we examined the expression of LTβR on LECs, taking into consideration the likelihood that the LTβR signaling pathway could promote lymphangiogenesis directly through LECs. Our study of the expression of LTβR was established by immunohistochemistry of LN sections together with flow cytometry analysis of LECs. We first determined by immunohistochemistry in both non-immunized and immunized LNs if the LTβR expression could be detected on lymphatic vessels, and secondly, if there are any differences in the expression in response to CFA/KLH. Figure 4.2A reveals the high and low magnification microscopy images of these LNs respectively. As stated previously, CD31 is a marker for endothelial cells. Lymphatic vessels were represented by the double positive staining of LYVE-1 and CD31, although the expression of CD31 was shown to be quite low (arrowheads in Figure 4.2A). The structures expressing a high level of CD31 but negative for LYVE-1 staining were the blood vessels, likely HEVs considering the structure of the cells (arrows in Figure 4.2A). Furthermore, these blood vessels stained brightly for LTβR, consistent with previous studies on the marked expression of LTβR on HEVs (Gommerman and Browning, 2003). Lymphatic vessels were also observed to express LTβR, although the expression level was lower compared to blood vessels as shown by the intensity of the staining (Figure 4.2A). Comparison of the non-immunized LNs with the immunized LNs revealed that there was no apparent difference in the expression level of LTβR on both vessels (Figure 4.2A), suggesting that neither LECs nor BECs upregulate the expression of LTβR in response to CFA/KLH. We then used flow cytometry to examine LTβR expression on LECs to corroborate our findings

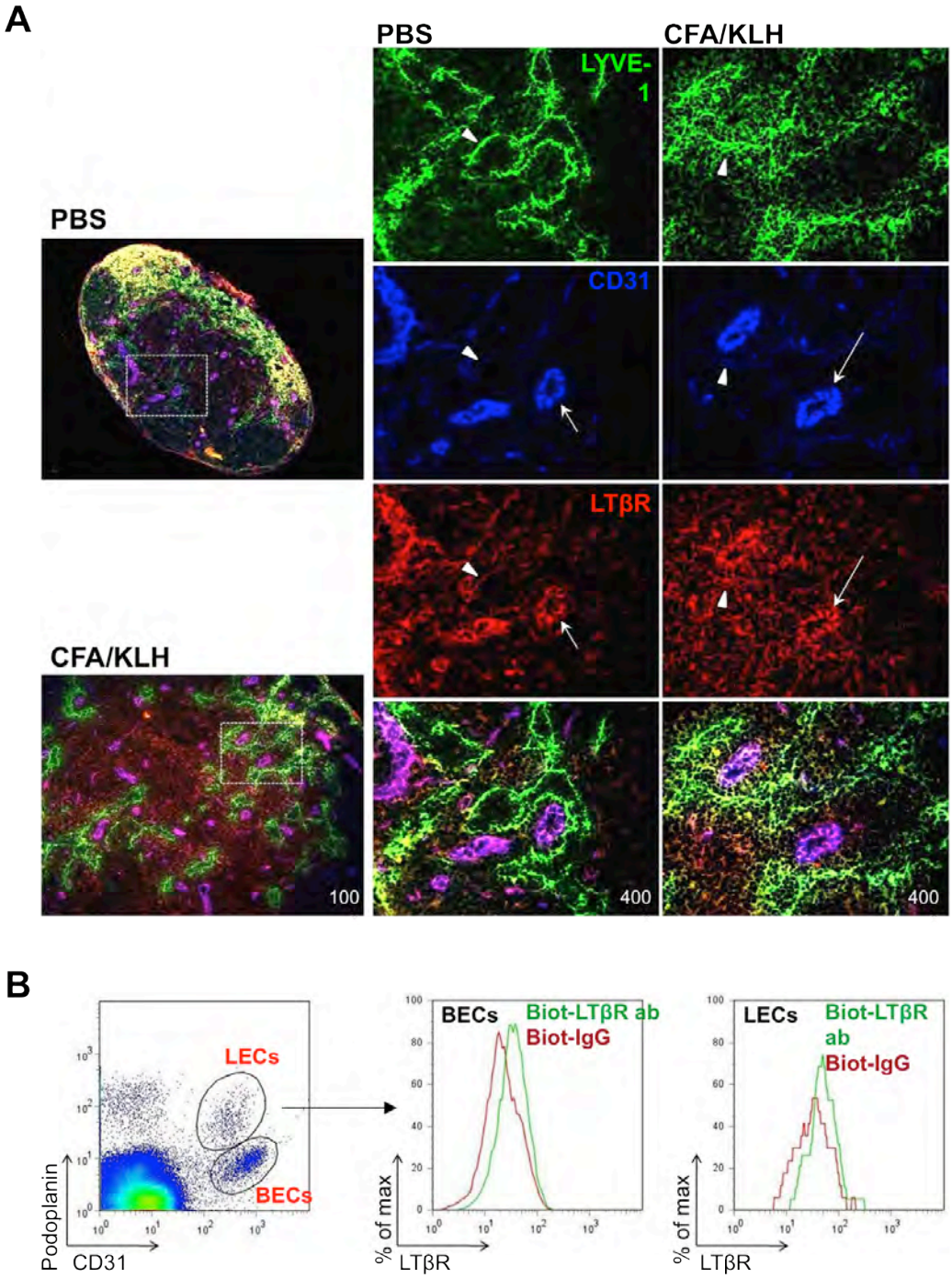


Figure 4.2: Expression of LTβR by LECs. (A) LN sections from non-immunized and immunized mice were stained for LYVE-1, CD31 and LTβR. LYVE-1^{+}CD31^{low} lymphatic vessels (arrowhead) and LYVE-1^{-}CD31^{+} blood vessels (likely HEVs) (arrow) were examined for coexpression of LTβR. (B) FACs analysis of the expression of LTβR on LECs with BECs as a postive control. Data shown are representative of 3 independent experiments with 3 mice per group in each experiment.

from the immunostaining results. The representative flow cytometry dot plot was pre-gated on live, $CD45^{low}$ cells as described earlier on (Figure 4.2B). As in the immunohistochemistry analysis, $CD45^{low}Podoplanin^{-}CD31^{+}$ BECs were employed as the positive control in our study and as anticipated, both BECs and $CD45^{low}Podoplanin^{+}CD31^{+}$ LECs exhibited expression of LTβR (Figure 4.2B). Similarly, we also could not detect any difference in expression of LTβR in LECs of non-immunized and immunized LNs. Collectively, these outcomes illustrated the positive expression of LTβR on LECs, and this expression suggested that regulation of LN lymphangiogenesis following immunization by LTβR signaling might be through a direct interaction on the LTβR expressed on LECs.

Previously we have shown the unexpected amplified LN lymphangiogenesis in TNFαKO mice. Here, we examined if this uncharacteristic expansion in the lymphatic vessel network in TNFα deficient mice is under the regulation of the LTβR signaling pathway. TNFαKO mice were treated with either LTβRIg or with huIgG as control before CFA/KLH immunization. From our results, it appeared that at baseline level without immunization, blocking LTβR signaling resulted in a slight decrease in LN cellularity of about 30% when compared to the control (Figure 4.3A). After immunization, the total cell count of LTβRIg treated LNs from TNFαKO mice had only about 60% of the total cell count of the control TNFαKO mice LNs (Figure 4.3A). However, while treatment of LTβRIg restricted LN expansion of TNFαKO mice after immunization, blocking LTβR did not appear to have any effect on lymphangiogenesis. Comparing the number of

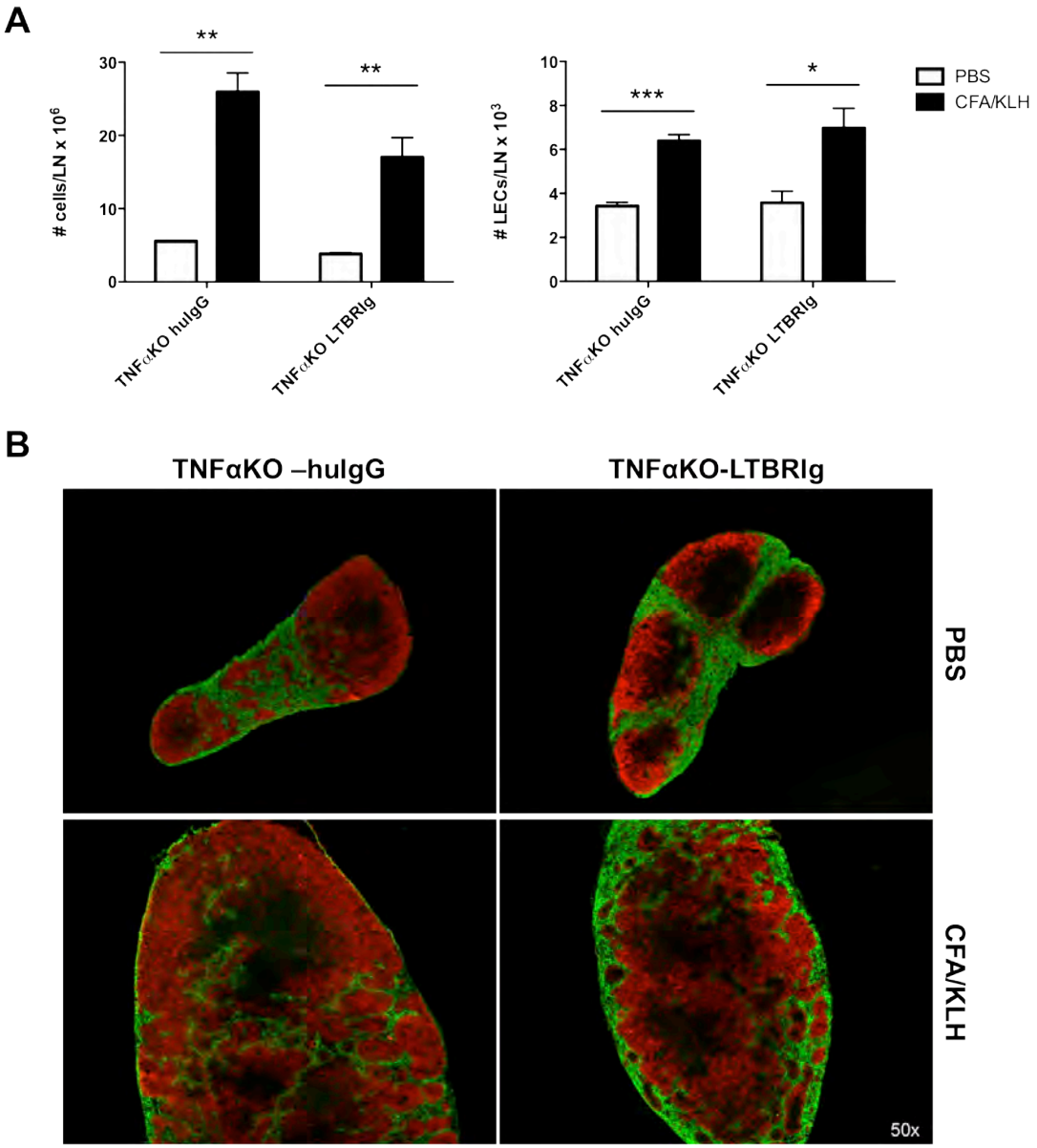


Figure 4.3: Unexpected LN lymphangiogenesis in TNFαKO mice not regulated by LTβR. (A) LN cellularity and number of LECs were evaluated in LNs of huIgG- and LTβRIg-treated TNFαKO mice 4 days after immunization. (B) LN sections from non-immunized and immunized mice TNFαKO treated with or LTβRIg were stained for LYVE-1 and B220. Images and flow cytometry results are representative of 5 independent experiments (n=3). Significant differences are designated by *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

LECs in control and LTβRIg treated LNs of TNFαKO mice before and after immunization showed no significant differences in lymphatic vessel growth at both baseline and inflamed states (Figure 4.3A). Immunohistochemistry analysis of LN sections from TNFαKO mice reinforced our observations from our flow cytometry results. Immunized TNFαKO LNs treated with LTβRIg looked smaller compared to the control LNs (Figure 4.3B). Expanded lymphatic vessel network could also be observed in both the control and treatment LNs (Figure 4.3B). Furthermore, confirming what was previously described (Pasparakis et al., 1997), the B cell follicles of these LNs were disorganized due to the absence of FDC networks (Figure 4.3B). These observations suggested that LTβR signaling is involved in mediating LN expansion in the TNFαKO mice but not lymphangiogenesis in response to immunization.

4.2.2 Blocking lymphangiogenesis through the LTβR signaling pathway hampers the enhancement of DC migration induced by immunization

The enhancement of DC migration to the LNs induced by CFA/KLH immunization was supported by the growth of the LN along with LN lymphangiogenesis (Angeli et al., 2006). As we have shown that inhibiting the LTβR signaling impeded LN expansion and lymphangiogenesis, we wondered if blocking the same signaling pathway would have any effect on the enhanced DC migration following immunization. To address this issue, FITC painting assay was carried out, where DC migration was stimulated by an epicutaneous application of FITC in a contact sensitizing solution on the scapular area of the skin that drains

to the same brachial and axillary LNs as the front footpads of the mice. Because administration of CFA/KLH is known to result in a strong inflammation reaction at the site of injection, a separate location was selected for the FITC painting so as not to subject the DCs to this inflammation. This is important since we want to show that any variations in DC migration is only due to the activation of the draining LNs. As before, LTβRIg was administered subcutaneously into the front footpads of the mice a day before CFA/KLH was injected into the same footpads.

Gating of the $CD11c^{+}FITC^{+}$ DCs that migrated from the skin into the LNs was shown in the representative flow cytometry plot (Figure 4.4A). The expected increase in the number of $CD11c^{+}FITC^{+}$ DCs in the LNs following immunization was a result of the increase in the migration of DCs from the periphery as reported previously (Angeli et al., 2006). We first looked at LN cellularity to confirm that effects of the treatment of LTβRIg in the LNs and similar to the experiment before, the increase in the cellularity of the LNs after immunization was opposed by the inhibition of the LTβR signaling in the mice treated with LTβRIg (Figure 4.4B). Evaluation of the number of migrated DCs in the LNs indicated a 3-fold rise in the sum of $CD11c^{+}FITC^{+}$ DCs in the LNs of control mice after immunization (Figure 4.4B). Interestingly, the number of $CD11c^{+}FITC^{+}$ DCs was reduced in the immunized LNs of LTβRIg-treated mice (Figure 4.4B), likely a result of the reduced lymphangiogenesis in the LNs. An analysis was also done on the B and T cell count of the LNs to evaluate the influence of blocking LTβR signaling on lymphocytes in the LNs in response to immunization. Parallel to our

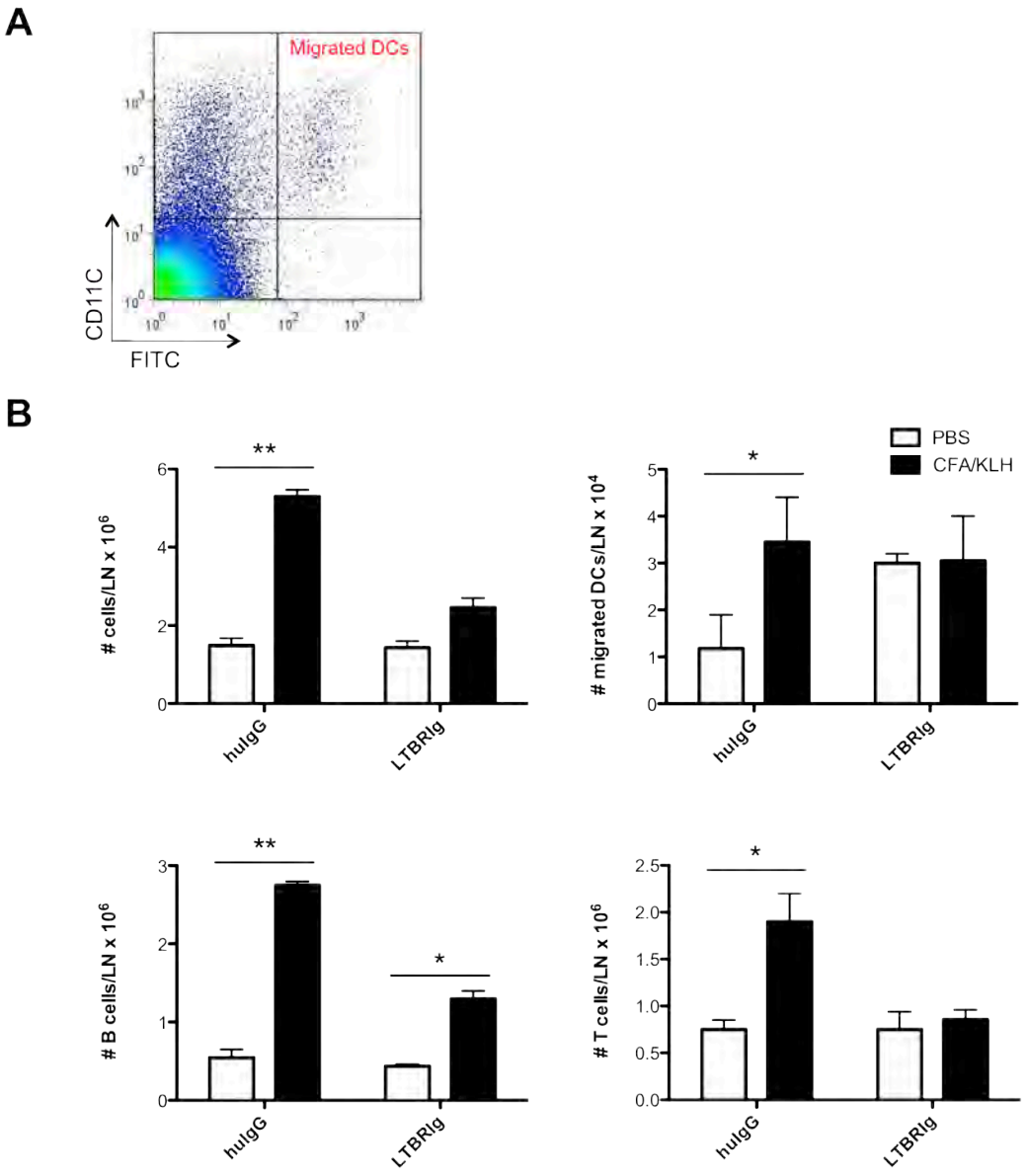


Figure 4.4: Blocking lymphangiogenesis through the LTβR signaling pathway hampers the enhancement of DC migration induced by immunization. (A) Non-immunized and immunized mice were subjected to FITC painting on the skin and update of FITC by DCs was analyzed. A representative flow cytometry plot of $CD11c^{+}FITC^{+}$ DCs is shown. (B) LN cellularity, number of migrated DCs and B and T cells in non-immunized and immunized LNs treated with huIgG and LTβRIg were evaluated 18 hr after FITC application. Data shown are representative of 3 independent experiments with 3 mice per group in each experiment and significant differences are designated by *$p < 0.05$ and **$p < 0.01$.

earlier study, the LNs of the huIgG-treated control mice displayed a 6-fold increase in the amount of B cells upon immunization, while T cells only showed a 3-fold increase in number (Figure 4.4B). As projected by the observations on LN cellularity, LTβRIg treatment also impeded the expansion of the lymphocytes compartments in the immunized LNs (Figure 4.4B). Following the blocking of LTβR signaling, the increase in B cells number were restricted to only 3-fold, whereas there was no significant increase in the amount of T cells in response to immunization (Figure 4.4B). Summing up the data from the experiments thus far indicated that LTβR signaling is critical in LN lymphangiogenesis induced by immunization. The reduced growth of lymphatic vessels in inflamed LNs due to the blocking of LTβR signaling can then inhibit the increase in DC migration typically detected in response to immunization.

4.2.3 Immunization induces the expression of LTα in B cells

After verifying the expression of LTβR on LECs, we set out to identify the source of the LTβR ligands involved in the signaling pathway. B and T cells are the main $LT\alpha_1\beta_2$-expressing cells found in the LNs (Gommerman and Browning, 2003). As we have shown that lymphangiogenesis in the LN is promoted by B cells, and based on our LTαKO/μMT mice data, where chimeric mice with B cells that were deficient for LTα suffered from the inability to induce LN lymphangiogenesis upon immunization, we hypothesized that rather than T cells, LTβR signaling implicated in lymphangiogenesis is activated through the ligands produced by B cells. Furthermore, since we did not observe any upregulation of LTβR expression

on both BECs and LECs upon immunization, we speculated that the effects of LTβR signaling on lymphangiogenesis may be due to an increase in the LTβR ligands in response to CFA/KLH. To prove these assumptions, we isolated B and T cells from the LNs and measured the mRNA levels of the LTβR ligands in these cells at various time points after CFA/KLH immunization. In view of the fact that we have based our analysis of the LNs on the 4^{th} day after immunization, where distinct modifications in lymphatic vessel network of the LNs could be detected, we opted to evaluate the mRNA expression levels of the LTβR ligands at earlier time points: 1 day, 2 days and 3 days after immunization.

$CD19^+$ magnetic beads (MACS) were used to isolate the B cells from the LNs. The negative fraction of the LN cell suspension after the separation of B cells was thought to consist mainly of T cells. An examination of the purity of the two fractions by flow cytometry was carried after the isolation process as represented by Figure 4.5A. The average purity of the positive selection of B cells was shown to be above 80%, while the negative fraction was indeed confirmed to be an accurate representation of the T cell fraction, consistently comprising of above 85% of T cells (Figure 4.5A). mRNA was then extracted from these two fractions for the subsequent PCR analysis.

From the PCR results, LTα was only weakly expressed in B cells at Day 0 of immunization (non-immunized) (Figure 4.5B). Interestingly, the expression of LTα increased in B cells along with the number of days after immunization

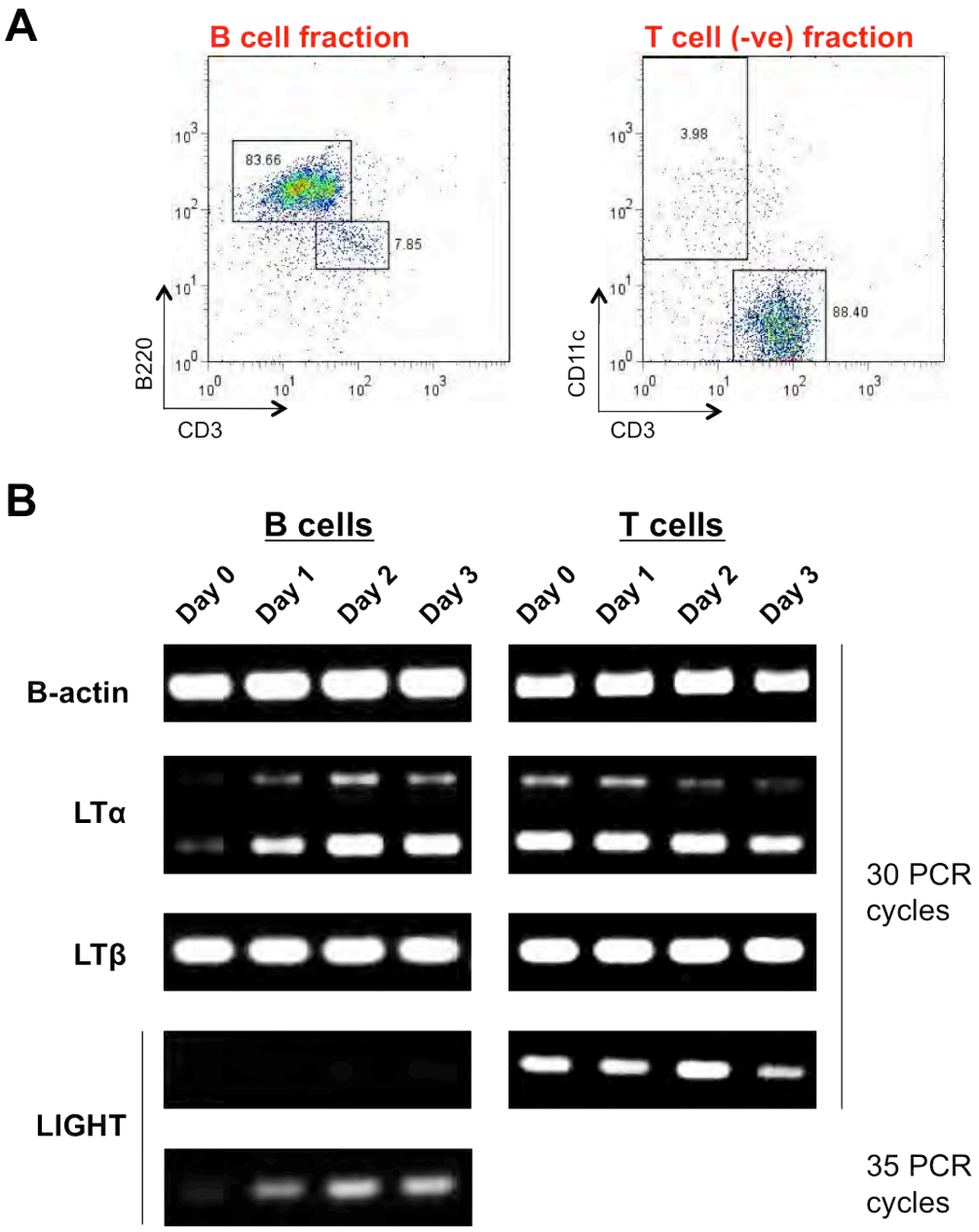


Figure 4.5: Differential expression of LTβR ligands by B and T cells upon immunization. (A) Purity of the B cell and T cell (negative) fractions were evaluated after MACS by flow cytometry. (B) Expression of LTβR ligands by B and T cells in the LNs at different time points after CFA/KLH immunization. Data shown are representative of 5 independent experiments with 3 mice per group in each experiment.

(Figure 4.5B). The expression of LTβ in the B cells on the other hand, was consistent throughout the four time points that we observed (Figure 4.5B). While LIGHT was originally not detected in our first PCR run at 30 cycles of amplification, subsequent PCR runs with increased cycles of amplification was able to reveal weak expression of LIGHT by B cells that increased from Day 0 to Day 3 of immunization (Figure 4.5B). In contrast, there was no upregulation of any mRNA levels in T cells (Figure 4.5B). The expression of LTβ in T cells was fairly uniform throughout the experiment, mirroring that of B cells (Figure 4.5B). Contrary to that in B cells, LTα and LIGHT expression in T cells was moderately even across the various time points, with LTα seemingly having a lower expression at Day 3 compared to Day 0 (Figure 4.5B). Remarkably, there was a presence of an additional band for LTα in both B and T cells (Figure 4.5B). An additional set of primers was used for verification and it showed similar results, suggesting that the extra band corresponded to the inclusion of an intron. Together with our earlier results, the upregulation of LTα by B cells suggested that B cells stimulate lymphangiogenesis in the LNs through activation of the LTβR signaling pathway by increasing production of LTβR ligands, particularly $LT\alpha_1\beta_2$, in response to CFA/KLH immunization.

4.2.4 Activation of the LTβR signaling pathway in the absence of immunization is insufficient to trigger lymphangiogenesis

The fact that blocking the LTβR signaling pathway is capable of preventing LN lymphangiogenesis from taking place after immunization indicates that LTβR

signaling is a fundamental requirement for lymphatic vessel growth. However, we do not know if activation of the LTβR signaling alone is able to initiate the lymphangiogenic process. Therefore, to further explore the role of LTβR signaling in LN lymphangiogenesis, we decided to activate LTβR in the LNs without any immunizing agent and investigate if stimulation of the LTβR signaling alone is sufficient to trigger lymphangiogenesis or LN expansion. For this experiment, we administered the receptor agonist, LTβRag, subcutaneously into the mice footpads twice with a 2 days interval in between, hoping to have a sustained activation of the signaling pathway, before analyzing the results on flow cytometry and immunohistochemistry 4 days after the first administration of the agonist. Regrettably, the LN cellularity of both the control and the LTβRag-treated LNs did not show any significant difference in number (Figure 4.6A). While the treatment of LTβRag seemed to result in a small rise in the amount of LECs compared to the number in the control LNS, this increase was statistically not significant (Figure 4.6A). Next, we proceed on to study the LN sections by immunostaining, thinking that even if there was no overall significant increase in LECs number by the application of LTβRag, there might be differences in the overall lymphatic vessel network due to remodeling of the vessels by the activation of the LTβR signaling. However, we could not distinguish any observable differences in the architecture of the lymphatic vessel network as well as the B cell compartment of the LNs that we have stained for (Figure 4.6B). These data indicated that activation of the LTβR by itself is not adequate to trigger the growth of the lymphatic vessels.

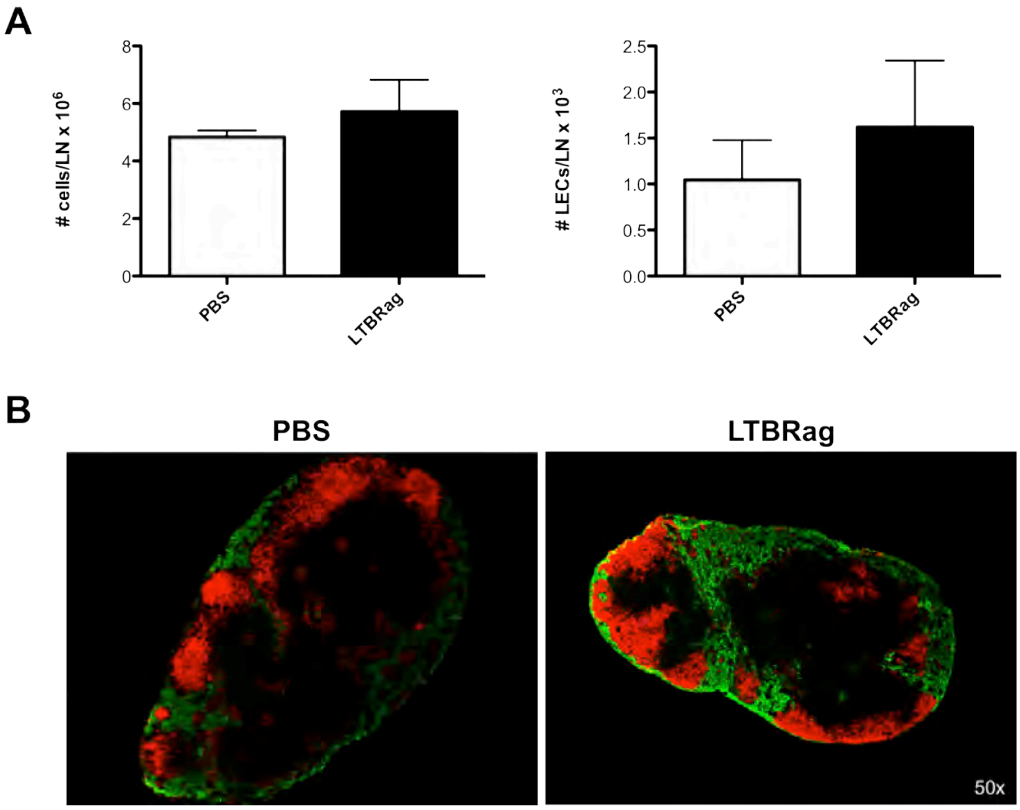


Figure 4.6: Activation of the LTβR signaling pathway in the absence of immunization is insufficient to trigger lymphangiogenesis. (A) LN cellularity and number of LECs were evaluated in LNs after treatment with LTβRag for 4 days. (B) LN sections from PBS control and immunized LTβRag-treated mice were stained for LYVE-1 and B220. Images and flow cytometry results are representative of 3 independent experiments (n=3).

Results from our previous experiment with LTβRag suggested that the induction of lymphangiogenesis in the LNs is not dependent on LTβR signaling only, but also on additional inflammatory signals brought about by the immunizing agent CFA/KLH during our immunization process. We also wondered if the activation of the NF-κB pathway by LTβR alone is a strong enough signal to trigger lymphangiogenesis, as both the canonical and non-canonical pathways of NF-κB have been shown to regulate cellular responses in a cooperative manner (Tully et al., 2012). To test this conjucture, we decided to activate TNFR, which triggers the canonical NF-κB pathway, along with LTβR activation. First, we used agonist for TNFR, TNFRag, and LTβRag to stimulate fibroblasts separately and together and measure the expression of CXCL-13 expression. CXCL-13 is chosen in our test as the gene expression is known to be controlled by NF-κB signaling pathway. We found that while fibroblasts stimulated with either LTβRag or TNFRag showed a 2-fold increase in gene expression, combined stimulation of fibroblasts with LTβRag and TNFRag showed a 3-fold increase in gene expression (Figure 4.7A). We then injected TNFRag subcutaneously into the footpads of the mice in the same way as the previous experiment: twice 2 days apart, and sacrificing the mice for examination 4 days after the first administration of TNFRag. Analogous to the LTβRag experiment, the treatment of TNFRag did not result in any significant expansion of the LNs, as well as any increase of the lymphatic vessels as seen by the number of LECs from our flow cytometry analysis (Figure 4.7B). Again, our data pointed out that stimulation of the TNFR alone in the LNs was not sufficient to trigger lymphangiogenesis in the absence of immunization. Next, we injected TNFRag subcutaneously into the mice footpads

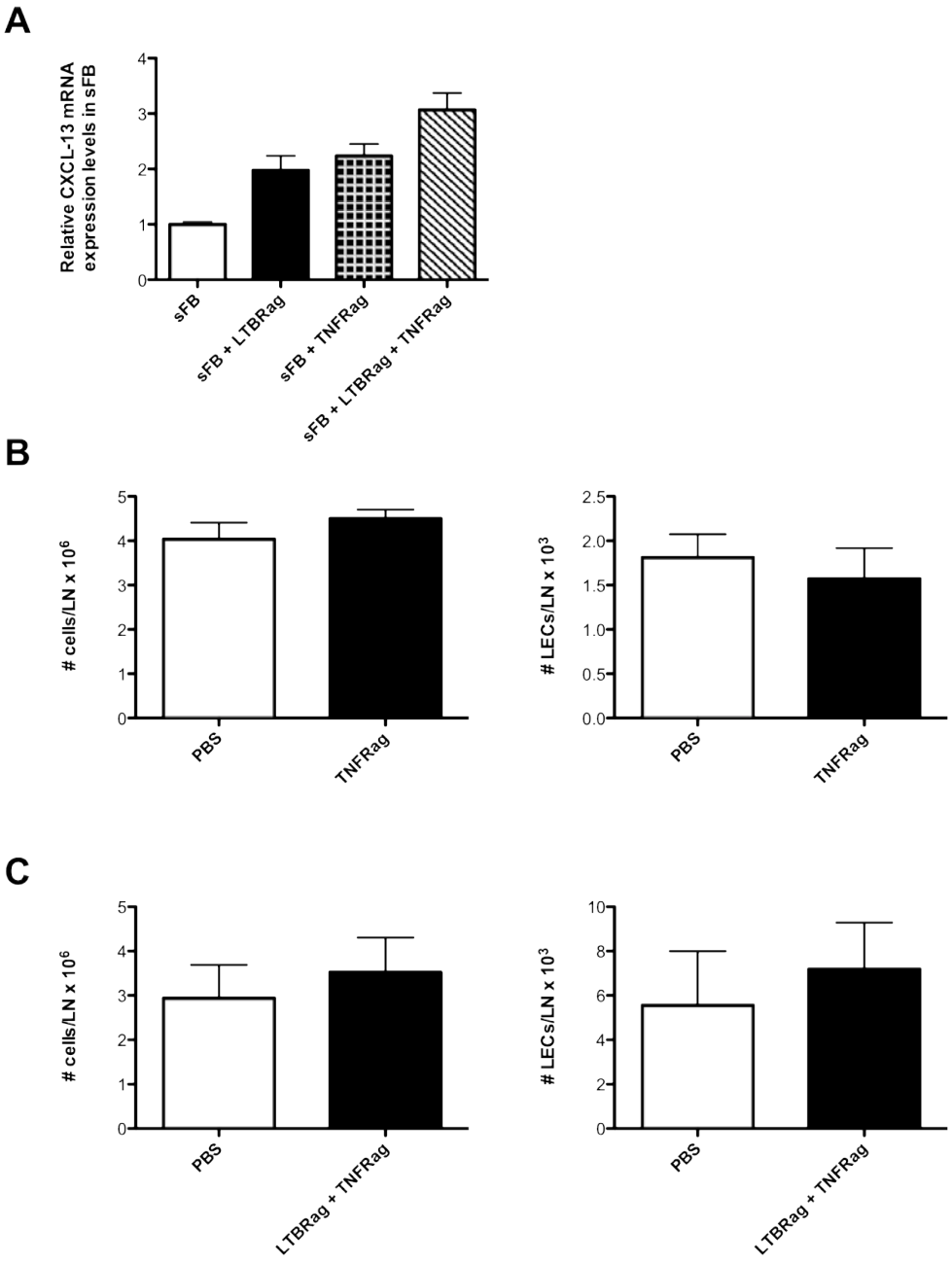


Figure 4.7: Triggering both the canonical and non-canonical NF-κB pathways by activation of TNFR and LTβR is not adequate to induce LN lymphangiogenesis. (A) Fibroblasts treated with either TNFR agonist, LTβR agonist or both for 12 hr were collected and CXCL-13 mRNA levels were quantified by qPCR and normalized to GAPDH mRNA levels. Data shown are representative of more than 3 independent experiments. (B) LN cellularity and number of LECs were evaluated in LNs after treatment with TNFRag for 4 days. (C) LN cellularity and number of LECs were evaluated in LNs after activation of both TNFR and LTβR. Data shown are representative of 3 independent experiments with 3 mice per group in each experiment.

a day before repeating the exact same experimental protocol as we did in the LTβRag study. Our analysis revealed the same outcome as the above two experiments, that is, there was no significant difference in both the LN cellularity and the total number of LECs in the LNs (Figure 4.7C). Together, our results showed that while LTβR signaling is essential for the induction of lymphangiogenesis in the LNs, activation of LTβR alone orafter priming by TNFR activation is not enough to trigger the growth of lymphatic vessels.

We have shown that in the absence of immunizing agents, activation of the LTβR signaling pathway alone is not sufficient for inducing LN lymphangiogenesis. Next, we wondered if the stimulation of LTβR signaling can initiate the growth of lymphatic vessels in a system whereby LN lymphangiogenesis is impaired despite the presence of immunizing agents. The μMT mice would be a suitable model as we have verified that there is diminished lymphangiogenesis in the LNs following CFA/KLH immunization due to the lack of B cells. In our experiment, we have included three groups of mice in our experiment: WT mice as a positive control, μMT mice as a negative control, and the treatment group was μMT mice administered with LTβRag. Mice were injected subcutaneously into the footpads with either PBS, for controls, or LTβRag a day before and a day after immunization with CFA/KLH. Flow cytometry and immunohistochemistry studies were done on the LNs 4 days after immunization. LN cellularity analysis from non-immunized mice showed that μMT mice LNs were slightly smaller than those of WT mice and, like treatment of LTβRag in WT mice, there was no

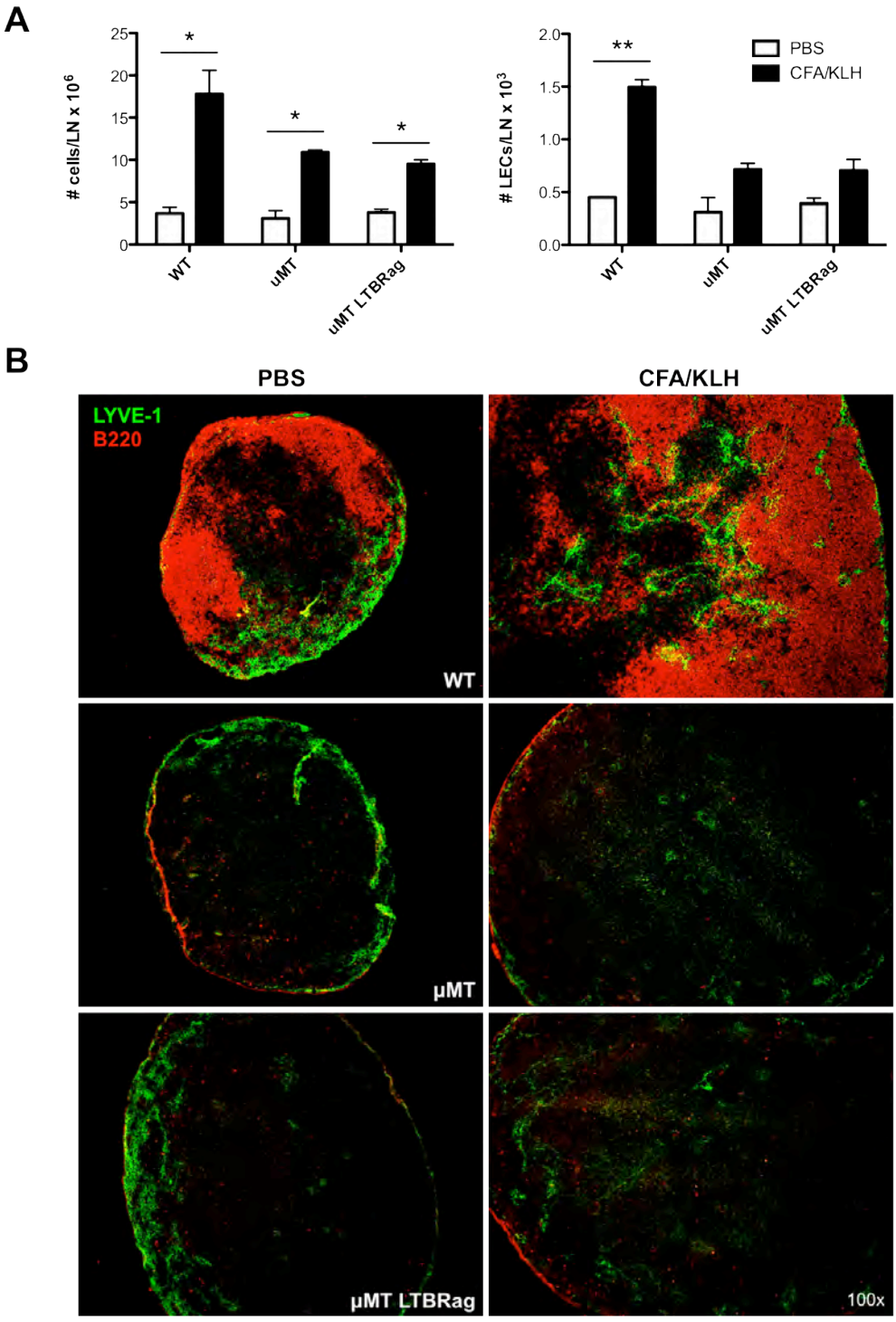


Figure 4.8: Stimulation of LTβR along with immunization is not enough to initate LN lymphangiogenesis in µMT mice. (A) LN cellularity and number of LECs were evaluated in LNs of WT, µMT and µMT mice treated with LTβRag 4 days after immunization. (B) LN sections from WT, µMT and µMT mice treated with LTβRag were stained for LYVE-1 and B220 after immunization. Images and flow cytometry results are representative of 3 independent experiments (n=3). Significant differences are designated by *$p < 0.05$ and **$p < 0.01$.

significant increase in the total cell number in the LNs of the LTβRag-treated μMT mice compared to the control μMT mice (Figure 4.8A). Activation of LTβR signaling in the LNs of μMT mice did not lead to further expansion of the LNs following immunization, displaying a 3-fold increase in LN cellularity analogous to the control μMT mice, while the WT mice LNs expanded by more than 5-fold (Figure 4.8A). Examination of the LECs number in the LNs indicated similar results as above. LECs count in the non-immunized LNs of μMT mice was lower than that of WT mice LNs and, although injection of LTβRag resulted in a slight increase in the amount of LECs in the LNs of μMT mice, the difference was not statistically significant (Figure 4.8A). Upon immunization, the number of LECs in the WT mice LNs was more than twice the amount of LECs in the LNS of μMT mice in both the LTβRag-treated and control group (Figure 4.8A). There was no substantial difference in the number of LECs in the μMT mice LNs with or without activation of LTβR signaling by the receptor agonist (Figure 4.8A). The flow cytometry data were substantiated by examining the LN sections of these mice (Figure 4.8B). While the expansion of the lymphatic vessels in the cortex region of the LNs could clearly be seen in the WT LNs, such modifications in the lymphatic vessel network in the LNs of μMT mice were largely absent (Figure 4.8B). The treatment of LTβRag did not bring about any noteworthy differences in the μMT mice LNs (Figure 4.8B). Thus, these findings indicate that, even with immunization, LTβR signaling is insufficient by itself to support lymphangiogenesis in LNs that are lacking B cells.

4.2.5 Therapeutic inhibition of the LTβR signaling does not affect LN lymphangiogenesis

Previously in our experiments with blocking LTβR signaling, we administered the inhibitor LTβRIg before immunizing the mice. This "preventive" method of impeding the LTβR signaling pathway was shown to be effective in preventing LN expansion and lymphaniogenesis. While we have then proceeded on to prove that activation of LTβR by itself is not adequate to promote LN lymphangiogenesis, the next question is whether a sustained LTβR signaling is necessary to maintain the growth of the lymphatic vessels, exploring the therapeutic potential of inhibiting LTβR signaling in limiting lymphangiogenesis. To answer this question, we decided to inhibit the LTβR signaling after lymphangiogenesis has taken place in the LN and investigate if this would lead to the regression of lymphatic growth. Since lymphangiogenesis in the LNs on the 4^{th} day after CFA/KLH immunization has been well-documented in our studies, for our current "therapeutic" protocol, we only began to block LTβR signaling in the mice 4 days after immunization by injecting LTβRIg subcutaneously into their footpads. Flow cytometry study on the effects of "therapeutic" blocking of LTβR signaling on LN lymphangiogenesis was done 3 days after administration of LTβRIg. First we looked at the effects of immunization for 7 days on the control LNs. LNs that had been immunized for 7 days displayed a greater than 9-fold dramatic increase in cell count, indicative of continued expansion of the LNs from 4 days to 7 days after immunization (Figure 4.9). Reflecting the enlargement of the LNs, there was a comparable large increase in the number of LECs of more than 7-fold in the LNs after immunization (Figure 4.9). These figures signaled that

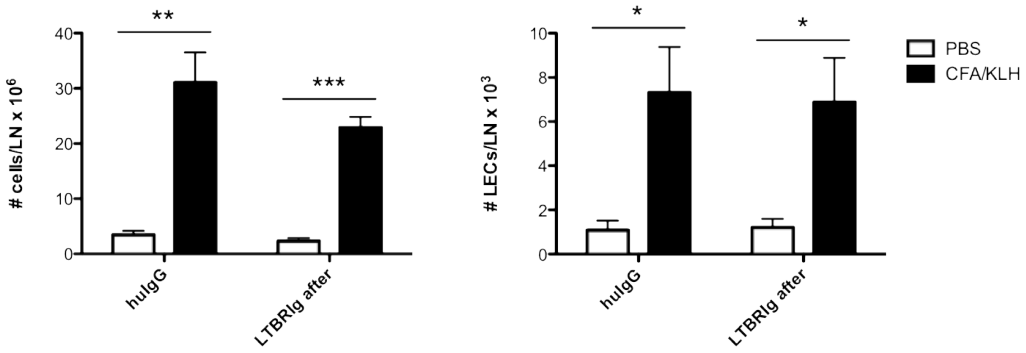


Figure 4.9: Effects of therapeutic inhibition of LTβR signaling for 3 days after immunization on LN cellularity and lymphangiogenesis. LN cellularity and number of LECs were evaluated in LNs mice treated with huIgG or LTβRIg for 3 days after 4 days of CFA/KLH immunization. Data shown are representative of 3 independent experiments with 3 mice per group in each experiment and significant differences are designated by *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

the LNs endured sustained growth and lymphangiogenesis 7 days after CFA/KLH immunization. Next we evaluated the influences of blocking LTβR signaling after the LNs had been allowed time for expansion and lymphangiogenesis to take place. There was no significant difference in the LN cell count of the non-immunized LTβRIg-treated LNs compared with the LNs of the control mice (Figure 4.9). However, the treatment of LTβRIg resulted in a less robust expansion of the LNs upon immunization (Figure 4.9). LNs of mice with LTβR signaling inhibited had a 7-fold increase in LN celllularity, and only about 75% of the total cell count of the control mice LNs (Figure 4.9). Similarly, there was no significant difference in the sum of LECs in the non-immunized LNs of the mice with or without LTβRIg treatment (Figure 4.9). Although we expected the amount of LECs in the LNs of LTβRIg-treated mice to be less than that of the control mice due to inactivation of LTβR after immunization, the flow cytometry analysis revealed that there was no substantial variation in the number of LECs when compared with the control group (Figure 4.9). LECs in the LNs treated with LTβRIg exhibited a 7-fold increase in number that was equivalent to the control mice (Figure 4.9). Therefore after the expansion of the size and lymphatic vessel network in the LNs had transpired, blocking LTβR signaling for 3 days could only moderate the expansion of the LNs and not regulate lymphatic vessel growth.

Inhibition of the LTβR signaling for 3 days after immunization of the LNs did not result in reduced LN lymphagiogenesis. We wondered if this was due to the inadequate time allowed for the blocking of LTβR signaling to have a significant

effect on LN lymphangiogenesis, as a study on LTβR signaling had revealed that it requires one or two weeks for the effects of LTβRIg treatment to be fully established (Browning et al., 2005). Before we repeated the experiment to allow for a longer duration of LTβR inhibiton, we decided to first study the effects of prolonged immunization on LN lymphangiogenesis. We have shown that swelling of the LNs and lymphangiogenesis could be detected a week after immunization. Thus, we chose to look at LN lymphangiogenesis at later time points, namely, at day 15, 20 and 25 after immunization. As seen from our analysis on LN cellularity, massive expansion of the LNs could be seen for all three time points (Figure 4.10). However, for the three time points that we had selected, the largest cell count was detected at day 15, and the number gradually decreased as the days of immunization increased (Figure 4.10). Analysis of LEC count in the LNs by quantitative flow cytometry revealed that lymphangiogenesis was present in the LNs throughout the three time points selected (Figure 4.10). Although a similar trend in the number of LECs could be observed on the immunized LNs, the differences were not significant (Figure 4.10). These data indicated that the effects of CFA/KLH immunization on the expansion of the LNs and lymphangiogenesis could still be detected up to 25 days after immunization. While the decrease in LN cellularity from day 20 onwards hinted at the resolution of inflammation in the LNs, there was no accompanying regression of the lymphatic vessels at these time points.

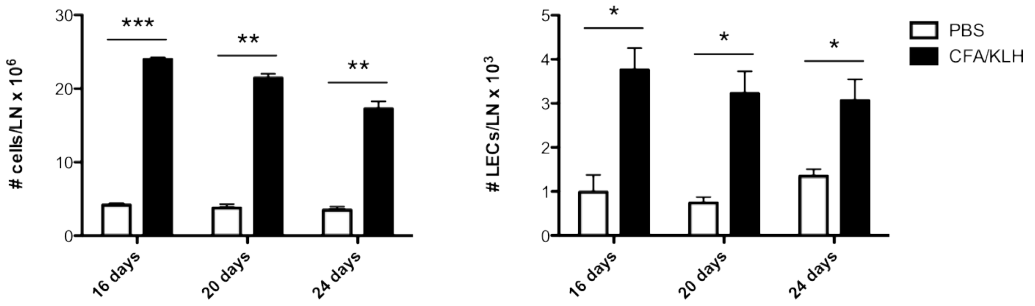


Figure 4.10: Prolonged LN enlargement and lymphangiogenesis after immunization. LN cellularity and number of LECs in LNs were evaluated 16, 20 and 24 days after CFA/KLH immunization. Data shown are representative of 3 independent experiments with 3 mice per group in each experiment and significant differences are designated by *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Having confirmed that the effect of prolonged CFA/KLH immunization on LN cellularity and lymphangiogenesis, we went on to investigate the consequences of extended LTβRIg treatment on LN lymphangiogenesis. In the same way, we first immunized the mice for 4 days before administering LTβRIg via intraperitoneal injection. For this experiment, LTβR signaling was inhibited for a week to allow for its effects to manifest prior to examining the outcomes by flow cytometry and immunohistochemistry. We began by looking at the effects on non-immunized LNs from administering the mice with LTβRIg for a week. Like our earlier experiments where we treated the mice with LTβRIg for a shorter duration, extended treatment of LTβRIg on the mice via intraperitoneal injections did not result in any significant changes in the LN cellularity and numbers of LECs in the LNs at baseline level (Figure 4.11A). For our flow cytometry analysis, we also delved into the effects of blocking LTβR signaling for 1 week on the hematopoietic cellular components of the LNs, namely the B and T cells, DCs and macrophages. As expected from our examination of the LN cellularity, there was also no substantial variations in the above mentioned cell types in the LNs after LTβRIg treatment (Figure 4.11A). After immunization, the therapeutic treatment of LTβRIg reduced the cellularity of the inflamed LNs by about 40% (Figure 4.11A). However, the exact same blocking of LTβR signaling did not reduce lymphangiogenesis. Instead, the amount of LECs for the LTβRIg-treated LNs increased as compared to the untreated immunized LNs (Figure 4.11A). Analysis of the B and T cells revealed a comparable decrease in numbers as the total LN cellularity of the LTβRIg-treated immunized LNs (Figure 4.11A). Similarly, LTβRIg treatment also led to a reduction in the number of DCs in the LNs after

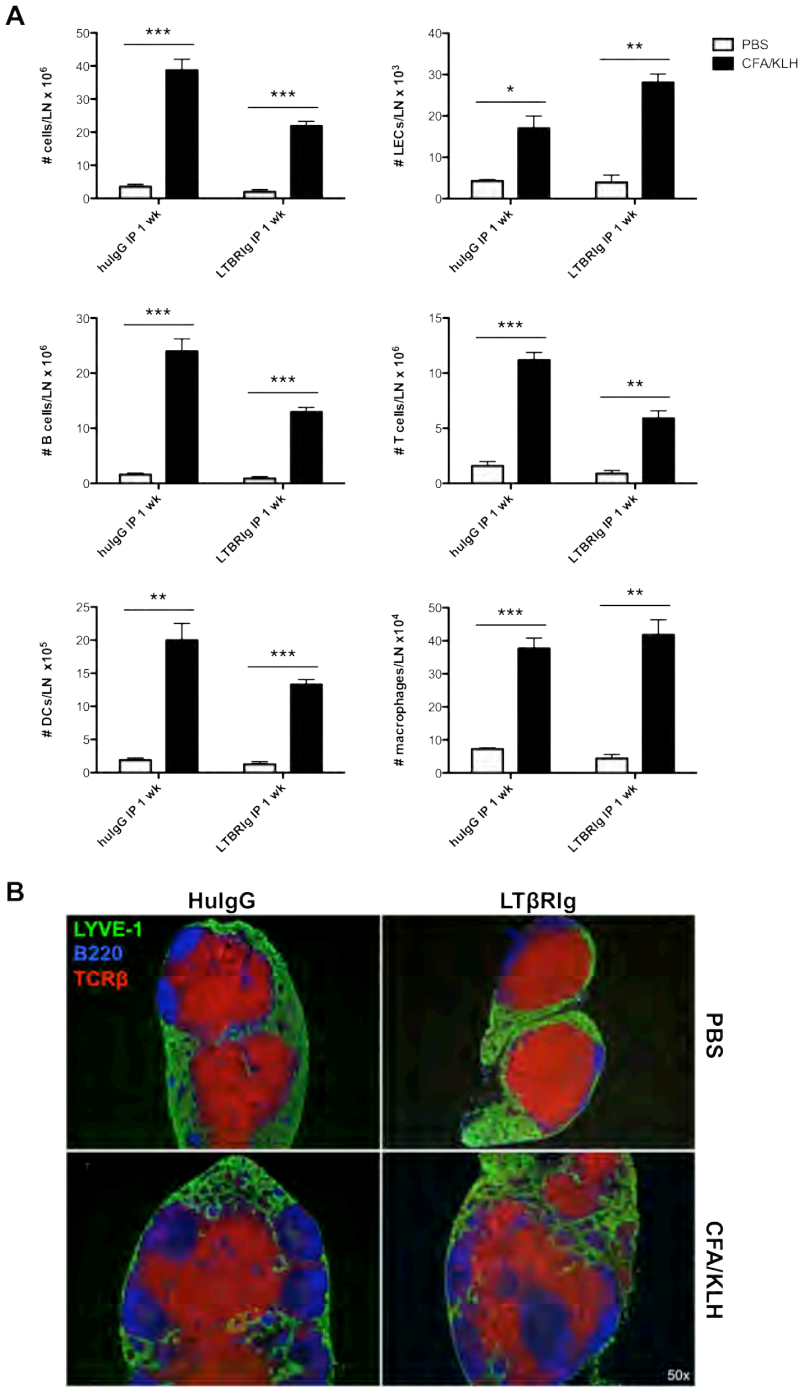


Figure 4.11: Effects of prolonged therapeutic inhibition of LTβR signaling for 1 week after immunization on the LNs. (A) LN cellularity, number of LECs, B and T cells, DCs and macrophages were evaluated in LNs mice treated with huIgG or LTβRIg for 1 week after 4 days of CFA/KLH immunization. (B) LN sections from non-immunized and immunized mice treated with huIgG or LTβRIg were stained for LYVE-1, B220 and TCRβ. Images and flow cytometry results are representative of 3 independent experiments (n=3). Significant differences are designated by *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

immunization (Figure 4.11A). Surprisingly though, the number of macrophages in the inflamed LTβRIg-treated LNs was about the same as that of the inflamed control LNs, and did not follow a proportional diminution in numbers due to the reduced size of the LNs (Figure 4.11A). Immunohistochemical analysis on the LNs revealed the expansion of the lymphatic vessel network, along with the growth of new lymphatic vessels around the B cells follicles in the control immunized mice (Figure 4.11B). These modifications of the lymphatic vessel network could be observed in the inflamed LNs of LTβRIg-treated mice as well (Figure 4.11B), confirming that blocking LTβRIg for a week four days after immunization did not have any effect on lessening lymphangiogenesis in the LNs.

To reinforce our findings that LN lymphangiogenesis, once it has taken place, does not require LTβR signaling to sustain itself, we repeated our extended therapeutic treatment of LTβRIg in the mice for 2 weeks. Our experimental protocol was largely the same as before, except that we injected the mice twice with LTβRIg a week apart for a total of 2 weeks of LTβR signaling inhibition and 17 days of immunization. Our results illustrated that treatment of LTβRIg for 2 weeks produced similar outcomes as blocking LTβR signaling for a week (Figure 4.12). There were no significant differences in the LN cellularity, LECs number, as well as the cell count of the B and T cells, DCs and macrophages that we examined in the LNs of the non-immunized control and LTβRIg treated mice (Figure 4.12). On immunized mice, the LTβRIg treatment led to smaller LNs with about half the total cell count of the control LNs (Figure 4.12). Again, there was

an increase in the number of LECs in the LNs from the resulting LTβR signaling inhibition as we have seen in the one-week blocking (Figure 4.12). Examination of the B and T cells count displayed proportional decrease in numbers, while there was no significant difference in the number of DCs in the inflamed LNs after LTβRIg treatment (Figure 4.12). This time round, the additional week of LTβR signaling blocking gave rise to an increase of almost twice the amount of macrophages in the LNs weighed against control mice (Figure 4.12). These results suggest that lymphangiogenesis, once it has taken place, does not require LTβR signaling to sustain itself.

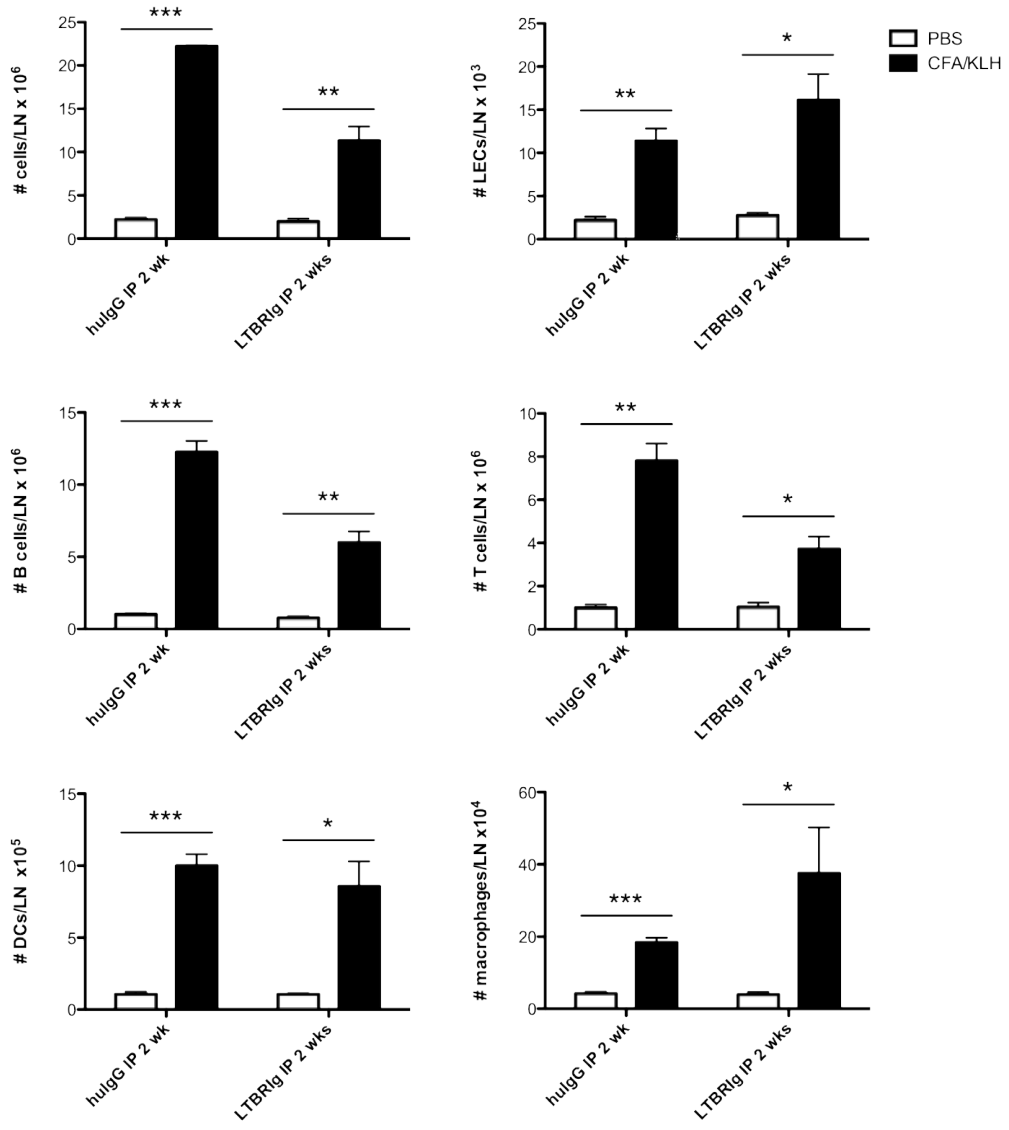


Figure 4.12: Effects of prolonged therapeutic inhibition of LTβR signaling for 2 weeks. LN cellularity, number of LECs, B and T cells, DCs and macrophages were evaluated in LNs mice treated with huIgG or LTβRIg for 2 weeks after 4 days of CFA/KLH immunization. Flow cytometry results are representative of 3 independent experiments (n=3), and significant differences are designated by *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

4.3 Discussion

Our earlier study established the requirement of the expression of LTα by B cells for the induction of lymphangiogenesis after immunization. Here, we highlighted the regulation of lymphangiogenesis by LTβR signaling. Using a soluble inhibitor of the LTβR, we demonstrated that inhibition of the LTβR signaling prevented the expansion of the LN and lymphangiogenesis following immunization. Impeding lymphangiogenesis by blocking LTβR signaling could also mediate secondary responses in the LNs brought about by the expansion of the lymphatic vessel network, such as the enhancement of DC migration.

We also showed that LECs express LTβR, opening up the possibility that lymphangiogenesis could be induced directly through the activation of the LTβR signaling on LECs. This is interesting because we have presented earlier that the newly formed lymphatic vessels in the cortex of immunized LNs are in vicinity to B cells, which are known to express the LTβR ligand, $LT\alpha_1\beta_2$. Our findings that B cells, but not T cells, upregulate the expression of LTα upon immunization further strengthened our notion that B cells regulate lymphangiogenesis through activation of the LTβR signaling.

To further investigate the role of LTβR signaling in LN lymphangiogenesis, we made use of a LTβR agonist to stimulate LTβR signaling. The fact that activation of the LTβR, and together with TNFR, in the absence of immunization did not

result in LN lymphangiogenesis suggested that additional signaling brought about by immunization is required for LN lymphangiogenesis. Triggering the LTβR along with immunization is again insufficient to initate LN lymphangiogenesis in μMT mice shows that B cells are strictly required for the growth of lymphatic vessel and the effect of LTβR signaling is not directly on LECs. The absence of FDCs in the μMT mice might also be a factor in the lack of lymphangiogenesis.

Interestingly, our therapeutic treatment of LTβRIg on LNs after immunization has taken place did not result in any reduction in lymphangiogenesis in the LNs. These findings suggested that once lymphangiogenesis has been initated in the LN, the process does not require further LTβR signaling to maintain the the growth of new lymphatic vessels. More importantly, it indicates that the involvement of LTβR signaling in lymphangiogenesis is at the early stages of the process.

Chapter 5: Blocking lymphotoxin β receptor signaling reveals the role of matrix metalloproteinase-13 in lymph node lymphangiogenesis

5.1 Introduction

Our findings, that inhibition of lymphangiogenesis by blocking LTβR signaling was only observed when the mice were treated with LTβRIg prior to CFA/KLH immunization, give rise to the hypothesis that the LTβR signaling may exert its regulatory role in the early stages of lymphangiogenesis. Since one of the intital steps of lymphangiogenesis involves the degradation of the ECM and BM by MMPs, and that the NF-κB signaling pathway is involved in the regulation of MMP gene expression, we propose that LTβR signaling may control the expression of MMPs in the LNs

The lack of a complete coverage of the lymphatic capillaries by BM means that the sprouting of new lymphatic vessels may be mainly through the degradation of ECM comprising mainly of the fibrillar type I collagen (Wiig et al., 2010; Paupert et al., 2011). We focused our study on four members of the MMP family: MMP-2, MMP-9, MMP-13 and MT1-MMP. We chose these MMPs based on their substrate specificities as well as their involvement in the degradation of the ECM. MMP-2, MMP-9, and MT1-MMP have been considered to be among the most involved MMP members in angiogenesis. (Handsley and Edwards, 2005; van Hinsbergh et al., 2006). The membrane bound MT1-MMP, one of the most important enzymes in pericellular proteolytic activities, is capable of acting as an interstitial collagenase (Barbolina and Stack, 2008). Gelatinases, MMP-2 and MMP-9, are known to actively degrade type IV collagen, a major component of the BM (Pytliak et al., 2012). Interestingly, research to date on the role of MMPs

in lymphangiogenesis has centered on MMP-2 (Matsuo et al., 2007; Bruyère and Noël, 2010; Detry et al., 2012). MMP-13, also known as collagenase 3, despite belonging to the collagenase subfamily of MMPs, has an active site sequence similar to the gelatinases (Knäuper et al., 1996a). Therefore MMP-13 is also an interesting candidate as it is able to cleave a wide range of substrates, including various components of the ECM, together with displaying high collagenolytic and gelatinolytic activity (Knäuper et al., 1996a; Lemaître and D'Armiento, 2006).

Here, we examined the mRNA expression as well as the proteolytic activities of the MMPs in the LNs upon immunization and their regulation by LTβR signaling. We also studied the compartmentalization of the MMPs in the LNs as well as their possible involvement in the lymphangiogenesis process.

5.2 Results

5.2.1 LTβR signaling regulates the expression of MMP-13 in LNs

In order to investigate the relationship between MMPs and LN lymphangiogenesis, we first examined the mRNA expression levels of the selected MMP-2, MMP-9, MMP-13 and MT1-MMP in the LNs after CFA/KLH immunization. With the purpose of studying the regulation of MMPs by LTβR signaling, again, we exploited the fusion protein LTβRIg. We selected time points of 1 day, 2 days and 4 days after immunization to look at the mRNA expression levels of the various MMPs in the LNs as lymphangiogenesis progresses. Following our preventive blocking protocol, LTβRIg was administered subcutaneously into the footpads of the mice a day before CFA/KLH immunization. Figure 5.1A shows the starting amount of each MMPs in the LNs. For MMP-2, MMP-9 and MT1-MMP, there were no significant differences in their expression with or without LTβRIg treatment (Figure 5.1B). mRNA expression of these three MMPs followed a similar trend whereby their expression decreased as the days after immunization increased (Figure 5.1B). Of the four MMPs studied, only MMP-13 showed a significant 8-fold increase in mRNA expression level 1 day after immunization, and decreased to a 4-fold increase in mRNA expression 2 days after immunization and finally dropping to baseline level at 4 days after immunization in control mice (Figure 5.1B). Increased expression of MMP-13 could also be detected in mice with LTβR signaling inhibited (Figure 5.1A). However, intriguingly, the increase in MMP-13 mRNA expression in control mice was significantly higher than mice treated with LTβRIg1day before immunization (Figure 5.1B). As we noticed that the

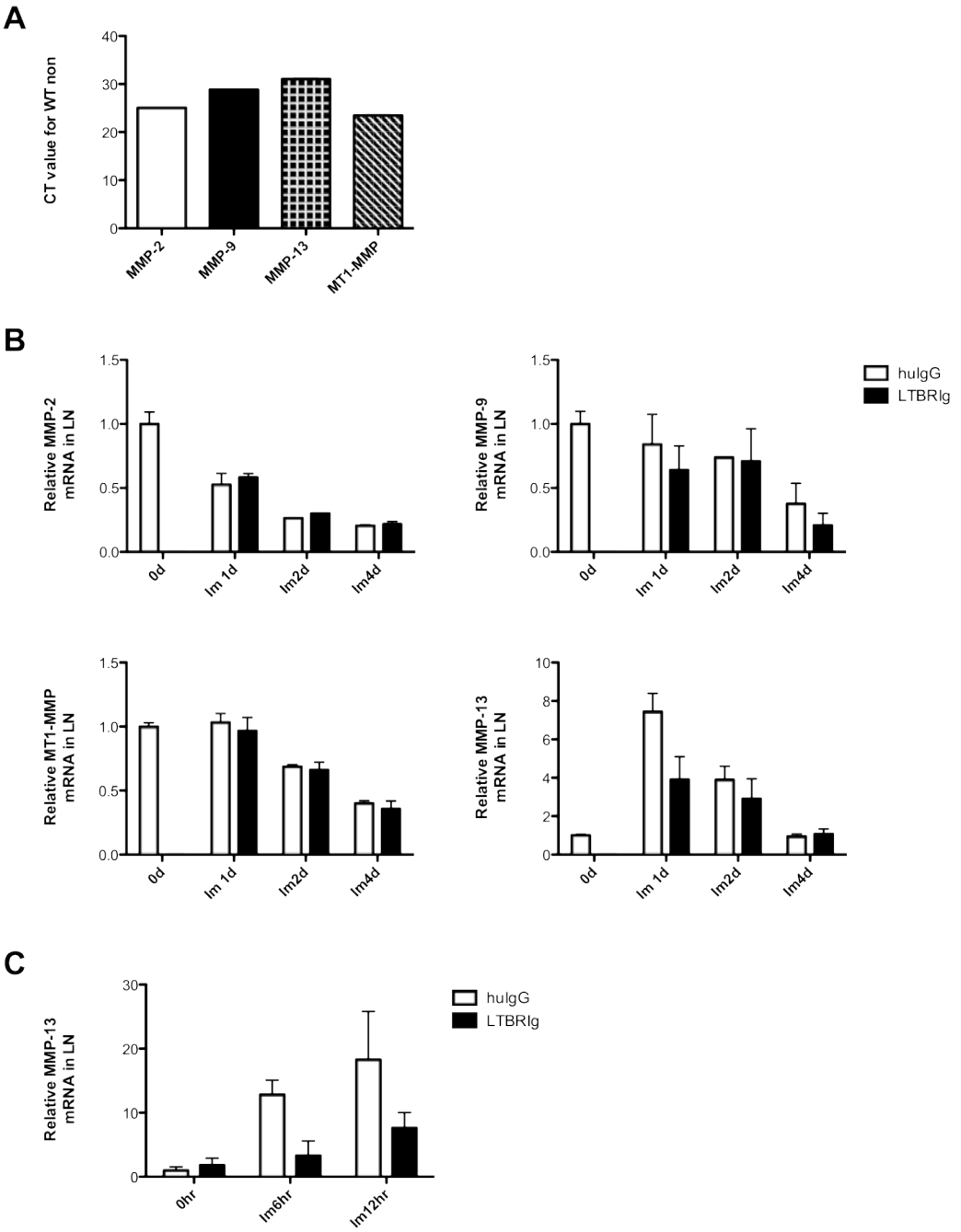


Figure 5.1: Expression of MMP-2, MMP-9, MMP-13 and MT-MMP in LNs. (A) Starting CT value (absolute mRNA level) of each MMP in the LNs. (B) qPCR analysis of MMP-2, MMP-9, MT1-MMP and MMP-13 expression in LNs at 1, 2 and 4 days after immunization. (C) qPCR analysis of MMP-13 expression in LNs at 6 and 12 hr after immunization. Data shown are representative of 3 independent experiments with 3-4 mice in each group.

expression level of MMP-13 was highest on the first day after immunization and dropped off at subsequent time points, we wondered if MMP-13 was expressed at higher levels at even earlier time points. Thus we continued to determine the expression of MMP-13 at 6 hours and 12 hours after immunization. We found that the MMP-13 was expressed at much higher levels in these earlier time points with a 13-fold and a 18-fold increase in mRNA expression levels at 6 hours and 12 hours respectively in the LNs of control mice (Figure 5.1C). Similarly, the blocking of the LTβR signaling pathway resulted in a significant reduction in the expression of MMP-13 (Figure 5.1C). Our results indicated that there is upregulation of MMP-13 expression in response to CFA/KLH immunization, and this expression is controlled by LTβR signaling.

The increased mRNA expression of MMP-13 in the LNs within the first day after immunization suggested that the protease might have an important role in the regulation of the early stages of LN lymphangiogenesis. To test our hypothesis, we ran a gelatin zymography assay with the whole cell lysate from the LNs of the mice. The point of running a zymography assay is that the MMPs with various molecular weights could be identified and quantified on a single gel based on the degradation of the substrate. As we are measuring the protease activity, we collected the LNs from the mice at an interval of 12 hours from the start of immunization to 36 hours after immunization. The mice were treated with either HuIgG as control or LTβRIg to assess the effects of LTβR signaling blocking. Although gelatin is known to be a preferred substrate for both MMP-2 and MMP-

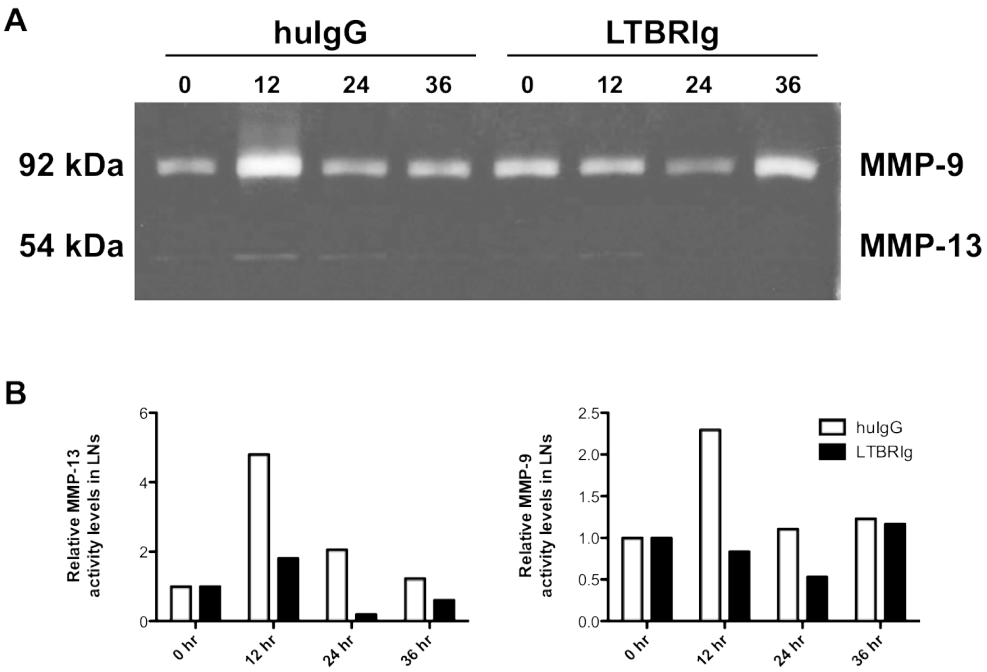


Figure 5.2: Proteolytic activites of MMP-9 and MMP-13 in the LNs. (A) A representative zymography showing MMP-9 and MMP-13 levels in the LNs at 0, 12, 24 and 36 hr after immunization. (B) MMP levels in zymography were determined by band densitometry and are shown as fold increase relative to corresponding MMPs in control LNs at 0 hr. Data shown are representative of 3 independent experiments with 3 mice in each group.

9, only MMP-9 gelatinase activity as well as MMP-13 could be detected on the gel (Figure 5.2A). Quantification of the bands on the gel by ImageJ revealed that the activity of MMP-13 in the LNs was increased by almost 5-fold at 12 hours after CFA/KLH injection compared to non-immunized control mice (Figure 5.2B). MMP-13 activity remained at 2-fold of baseline activity at 24 hours before dropping to a comparable level with non-immunized mice at 36 hours after immunization (Figure 5.2B). As expected, the activity of MMP-13 in the LNs was significantly reduced by the treatment of LTβRIg (Figure 5.2B). While we did not detect any increase in MMP-9 mRNA expression by qPCR, quantification of the zymography assay by densitometry showed that MMP-9 activity in the LNs briefly increased by more than 2-fold at 12 hours after immunization in the control mice before reverting back to baseline levels at subsequent time points (Figure 5.2B). Similar to MMP-13, blocking LTβR signaling in the LNs of the mice prevented the increase in MMP-9 activity seen in control mice (Figure 5.2B). Our data indicated that the increase in activities of MMP-13 and MMP-9 in response to immunization was regulated by LTβR signaling.

5.2.2 Localization of MMP-13 and MMP-9 in the LNs

We have demonstrated the increase in MMP-13 expression and activity in the LNs upon immunization through our mRNA analysis and zymography studies respectively. However, due to the nature of the above experiments, we do not know the localization of MMP-13. This is an important question because compartmentalization of MMPs in tissues, which is the binding of MMPs to

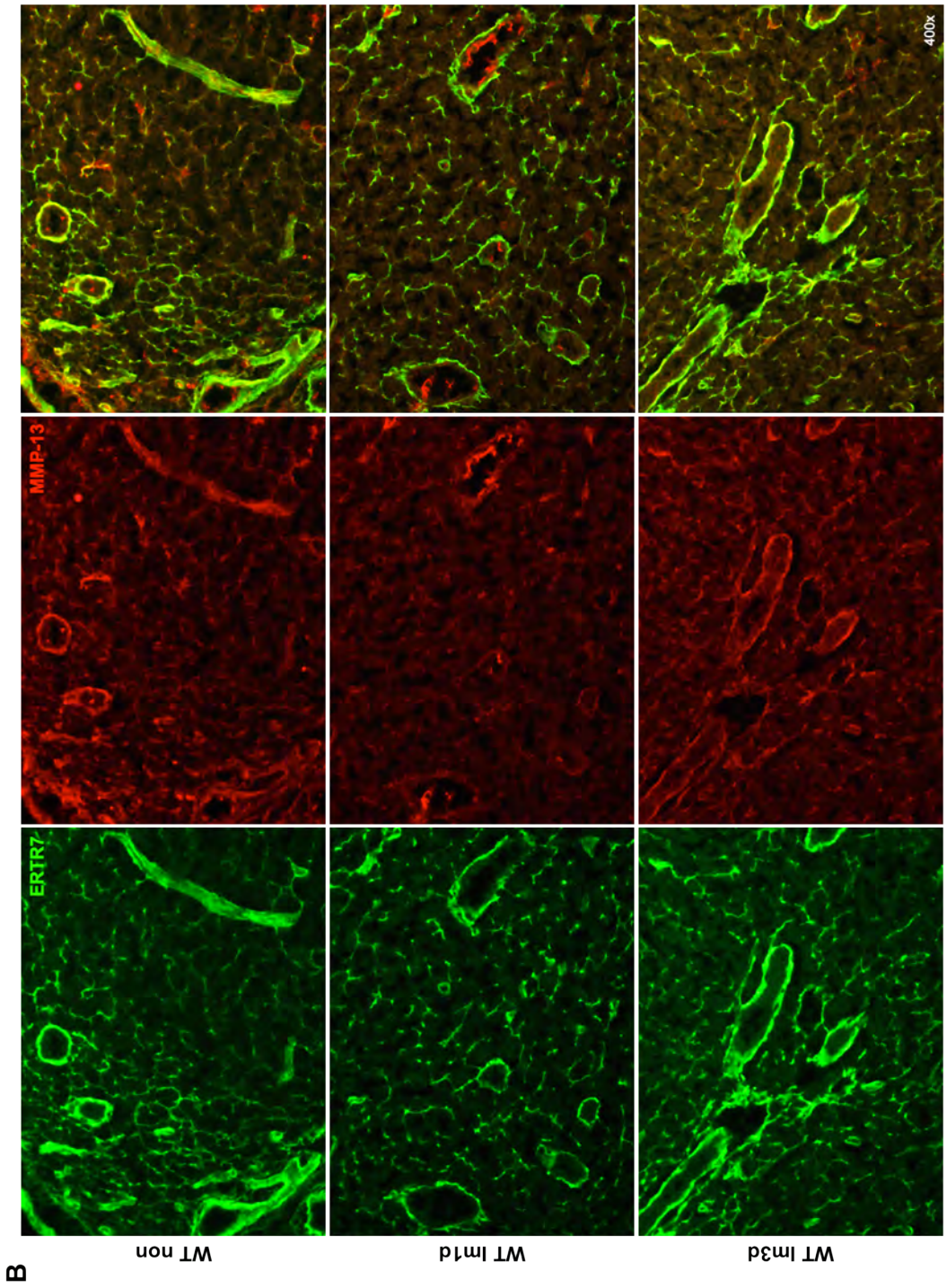
specific regions in the pericellular environment, is an critical aspect of MMP activity regulation. Knowing the location of MMP-13 in the LNs could also provide a hint on its role in the regulation of LN lymphangiogenesis. Therefore, we proceeded on to determine the localization of MMP-13 by immunohistochemistry. We looked at the expression of MMP-13 in non-immunized LNs as well as LNs after 1 day and 3 days of immunization. The LN sections were also stained for LYVE-1, ERTR7 and CD11b to examine the localization of MMP-13 with respect to the lymphatics, the FRCs and the macrophages correspondingly.

First we stained for LYVE-1 together with MMP-13 (Figure 5.3A). Both before and after immunization, MMP-13 staining could be seen to co-localize with LYVE-1 staining under high magnification (400x) (Figure 5.3A). Interestingly, some segments of the lymphatic vessels in the non-immunized LNs exhibited decreased LYVE-1 expression (Figure 5.3A). The downregulation of LYVE-1 expression on lymphatic segments was more prominent and was observed more frequently after immunization (Arrows in Figure 5.3A). Comparing the LYVE-1 and MMP-13 staining of the same lymphatic vessel, as pointed out by the arrows in the LN after 1 day of immunization, antibodies for LYVE-1 only covered approximately half the lymphatic vessel, whereas MMP-13 stained for the entire lymphatic vessel (Figure 5.3A). A similar staining pattern was observed in LNs after 3 days of immunization (Arrows in Figure 5.3A). Curiously, we noticed that the parts of the lymphatic vessels that were not stained for LYVE-1 typically

stained for MMP-13 with a higher intensity in contrast to the other regions that were LYVE-1^+ (Arrows in Figure 5.3A). Besides being observed to co-localize with LYVE-1, MMP-13 was also seen to stain for LYVE-1^- vessel-like structures (Arrowheads in Figure 5.3A). These structures were most likely to be blood vessels. Next, we examined the localization of MMP-13 together with ERTR7 (Figure 5.3B). FRCs, as revealed by ERTR7 staining, were found mainly in the T cell zone (paracortex) as well as surrounding the blood and lymphatic vessels in the LNs (Figure 5.3B). From our immunohistochemistry results, MMP-13 was generally not found to be co-stained with the FRCS in the paracortex region of the LNs but was instead observed only around the vessels in the LNs (Figure 5.3B). There were no discernible differences in the staining pattern of MMP-13 with respect to ERTR7 in LNs of both non-immunized and immunized mice (Figure 5.3B). Because macrophages have been reported to be a major cellular source of MMP-13 (Fallowfield et al., 2007), we stained for CD11b along with MMP-13 (Figure 5.3C). In the LN sections of non-immunized mice, $CD11b^+$ cells could be observed to stain weakly for MMP-13 (Figure 5.3C). The staining of MMP-13 on the $CD11b^+$ cells increased in intensity slightly at 1 day and 3 days after immunization, indicating the presence of higher levels of MMP-13 in these cells in response to CFA/KLH (Figure 5.3C).

The increase in the levels of activity of MMP-9 in the LNs indicated that MMP-9 might also be crucial in the degradation processes in the LNs in response to immunization. Hence, after investigating the localization of MMP-13 in the LNs,

A
LYVE-1
MMP-13
WT non
WT lm1d
WT lm3d
400x


B
WT non
WT Im1d
WT Im3d
ERTR7
MMP-13
400x

C
CD11b
MMP-13
WT non
WT lm1d
WT lm3d
400x

Figure 5.3: Localization of MMP-13 in the LNs. LN sections from non-immunized and immunized mice at day 1 and 3 were stained for MMP-13 and (A) LYVE-1, (B) ERTR7 and (C) CD11b. (A) LYVE-1^{+} lymphatic vessels (arrows) and structures resembling HEVs (arrowheads) were examined for costaining of MMP-13. Images are representative of 4 independent experiments consisting of 3 mice per group in each experiment.

we next examined the localization of MMP-9 in the LNs. Similar to our previous immunohistochemistry study on MMP-13, we stained for LYVE-1, ERTR7 and CD11b together with MMP-9 on resting LNs and LNs following 1 day and 3 days of immunization. Looking at the staining of MMP-9 together with LYVE-1, we observed that the localization of MMP-9 largely followed that of MMP-13 with respect to LYVE-1 staining (Figure 5.4A). MMP-9 was detected to co-localize with LYVE-1 staining in non-immunized LNs as well as in LNs after immunization (Arrows in Figure 5.4A). Other than appearing on LYVE-1^+ lymphatic vessels, MMP-9 also localize on LYVE-1^- blood vessels (Arrowheads in Figure 5.4A). We also noticed the broken LYVE-1 staining of the lymphatic vessels and similar to the staining of MMP-13, segments of the lymphatic vessels that were not covered by LYVE-1 generally appeared to stain brighter for MMP-9 compared to the other parts of the vessels that were LYVE-1^+ (Arrows in Figure 5.4A). Next we stained for ERTR7 alongside MMP-9 (Figure 5.4B). Different from MMP-13, MMP-9 was seen to co-localize with ERTR7 staining both around the vessels of the LNs as well as the FRCs in the T cell zone of the non-immunized LNs (Figure 5.4B). Following the immunization of the mice, the effects of CFA/KLH did not seem to result in any significant changes in the staining of MMP-9 with respect to ERTR7 (Figure 5.4B). We also did not observe any changes in the intensity of MMP-9 staining (Figure 5.4B). Finally, we examined the localization of MMP-9 alongside CD11b staining (Figure 5.4C). The staining of MMP-9 could hardly be seen in CD11b^+ cells in the LNs of non-immunized mice (Figure 5.4C). The presence of MMP-9 in the CD11b^+ cells appeared to increase after 1 day of immunization as indicated by the increase in

the intensity of the MMP-9 staining on the $CD11b^+$ cells (Figure 5.4C). However, this increment in MMP-9 seemed to be transient as the brightness of the MMP-9 staining in the $CD11b^+$ cells dropped to similar levels as those in the non-immunized LNs after 3 days of immunization (Figure 5.4C).

The compartmentalization of MMP-13 and MMP-9 around the lymphatic vessels suggested that the degradation of the ECM and/or basement membrane by MMPs might have been an important first step in the lymphangiogenesis process. Types I and IV collagen, known to be among the main ECM and BM components, are recognized substrates of MMP-13 and MMP-9, respectively. Thus, we decided to investigate the expression and localization of types I and IV collagen by immunohistochemistry, with respect to MMP-13 and MMP-9, LYVE-1 and CD31. Non-immunized LNs along with LNs after 3 days of immunization were used in this study to compare the effects of immunization on the collagen distribution in the LNs.

We first studied the localization of types I and IV collagen in non-immunized LNs under high magnification (400x) (Figure 5.5A). As before, blood vessels were identified by the expression of high levels of CD31 and the absence of LYVE-1 staining. Unlike lymphatic vessels, where MMP-13 and MMP-9 were revealed to co-localize with $LYVE\text{-}1^+$ lymphatic vessels as previously described, MMP-13 and MMP-9 were observed to surround the $CD31^+$ blood vessels (Figure 5.5A).

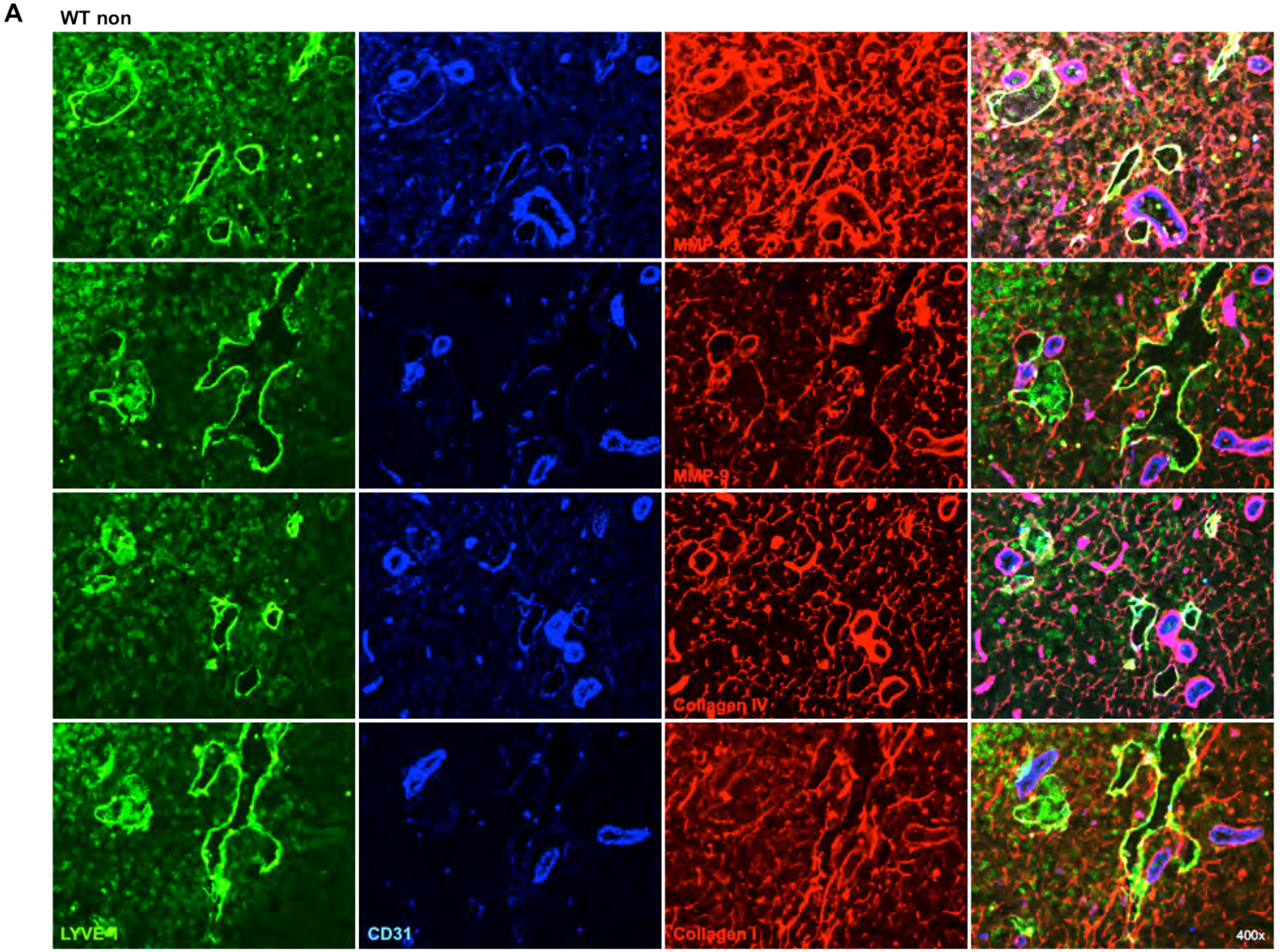
A
LYVE-1
MMP-9
WT non
WT lm1d
WT lm3d
400x

B
ERTR7
MMP-9
WT non
WT lm1d
WT lm3d
400x

C
CD11b
MMP-9
WT non
WT lm1d
WT lm3d
400x

Figure 5.4: Localization of MMP-9 in the LNs. LN sections from non-immunized and immunized mice at day 1 and 3 were stained for MMP-9 and (A) LYVE-1, (B) ERTR7 and (C) CD11b. (A) LYVE-1^{+} lymphatic vessels (arrows) and structures resembling HEVs (arrowheads) were examined for costaining of MMP-9. Images are representative of 4 independent experiments consisting of 3 mice per group in each experiment.

The staining of type IV collagen appeared to follow similar patterns to both MMP-13 and MMP-9, where collagen IV could be detected to co-localize with LYVE-1 staining, and around $CD31^+$ structures (Figure 5.5A). The staining of collagen IV around the blood vessels appeared to be thicker and usually of a higher intensity compared to the weak and "punctuated" staining on the lymphatic vessels (Figure 5.5A). For type I collagen, the staining was also similar to both MMPs as well as type IV collagen. Collagen I staining was observed on the LYVE-1^+ lymphatic vessels and encircling $CD31^+$ blood vessels, however, the intensity of the collagen I staining was about the same for both types of vessels and not especially brighter on blood vessels (Figure 5.5A). Although both collagen IV and I could be seen to stain for the reticular fibers in the LNs, type IV collagen staining appeared to be stronger around the vessels while type I collagen staining was more evenly distributed among the reticular fibers and the vessels (Figure 5.5A). Disrupted staining of LYVE-1 on lymphatic vessels could again be detected on sections of LNs after 3 days of immunization (Figure 5.5B). However, unlike for LYVE-1 expression, it was observed that the CD31 expression on the lymphatic vessels was uniform (Figure 5.5B). MMP-13 and MMP-9 staining were similarly observed to co-stain with LYVE-1 and the staining of the MMPs was particularly brighter at parts of the lymphatic vessels not stained with LYVE-1 (Figure 5.5B). The staining pattern and intensity of both types IV and I collagen were comparable to non-immunized LNs, suggesting that immunization did not influence the main collagen make-up of the ECM and BM (Figure 5.5B). We also looked at LNs that were immunized for1day and found the staining were similar to LNs after 3 days of immunization (data not shown).


A
WT non
MMP-9
MMP-9
Collagen IV
LYVE-1
CD31
Collagen I
400x

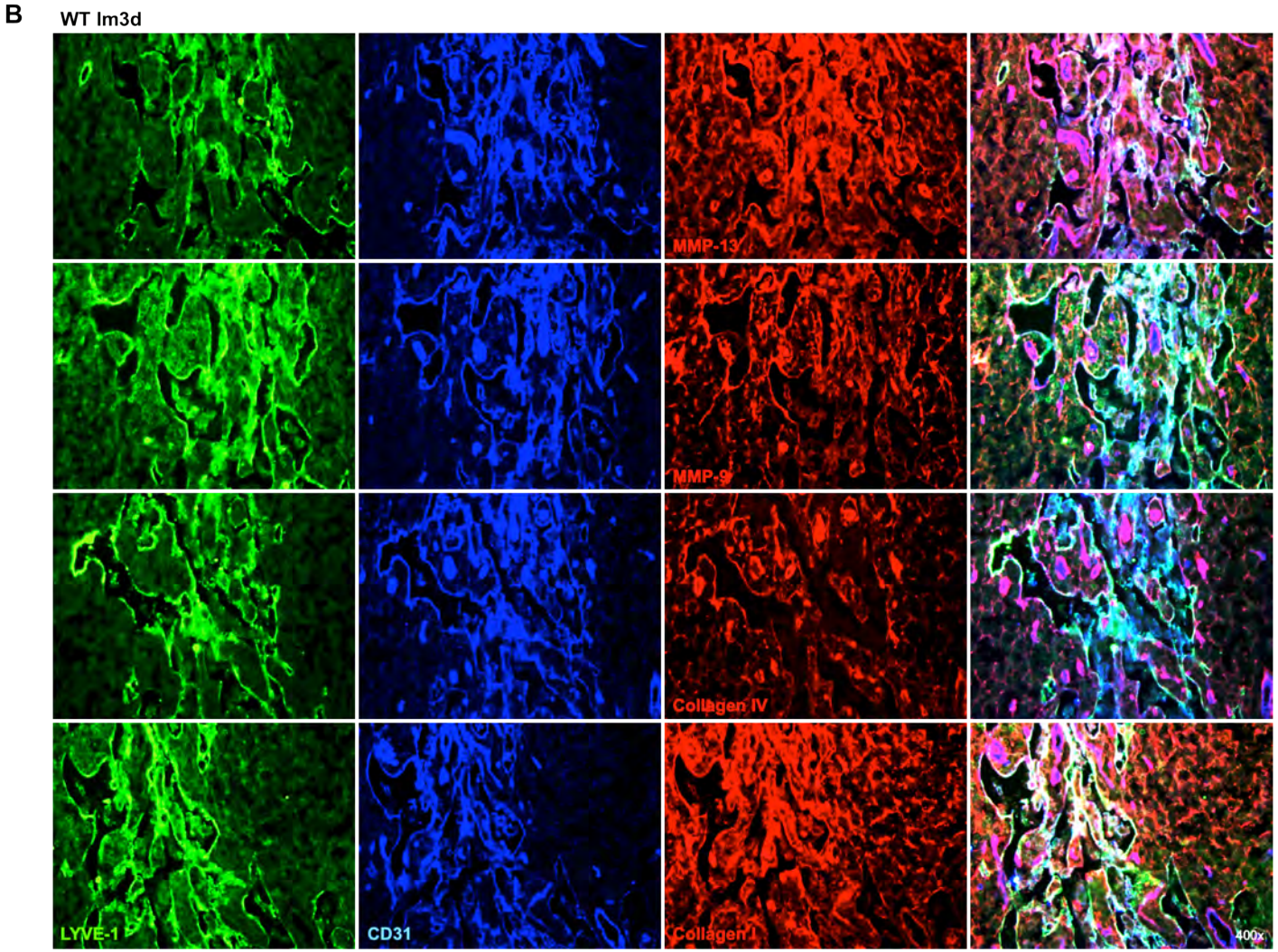
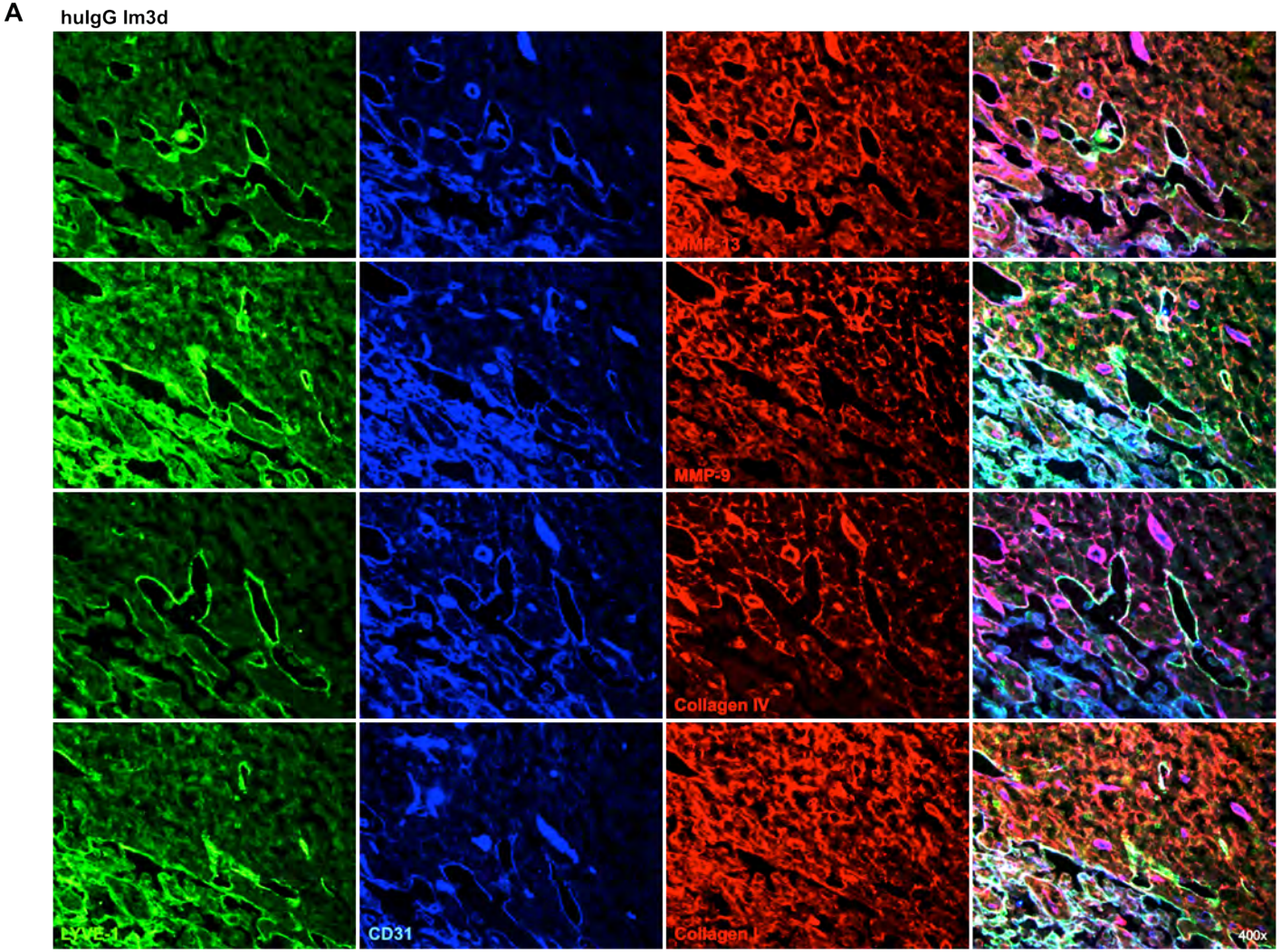

B
WT lm3d
MMP-13
MMP-9
Collagen IV
LYVE-1
CD31
Collagen I
400x

Figure 5.5: Localization of types I and IV collagen in the LNs with respect to compartmentalization of MMP-9 and -13. LN sections from (A) non-immunized and (B) immunized mice at day 3 were stained for LYVE-1, CD31 and MMP-13, MMP-9, type IV or type I collagens. Images are representative of 4 independent experiments consisting of 3 mice per group in each experiment.

We then investigated the effects of blocking LTβR signaling on the localization and distribution of both MMP-13 and MMP-9, and both types IV and I collagen in relation to lymphatic vessels in the LNs after immunization. We compared LN sections after 3 days of immunization whereby the modifications of the lymphatic vessels brought about by the effects of immunization were readily detectable. The staining of LYVE-1 on the lymphatic vessels of LTβRIg treated immunized LNs (Figure 5.6B) appeared to be more weakly stained compared to control immunized LNs (Figure 5.6A) when viewed under high magnification (400x). Furthermore, the downregulation of LYVE-1 on lymphatic segments seemed to be more frequent and cover a larger portion of the lymphatic vessels after LTβRIg treatment on the LNs (Figure 5.6B). LTβRIg treatment did not seem to affect the staining of both MMP-13 and MMP-9 (Figure 5.6B). MMP-13 staining could still be detected around the vessels and are usually brighter on the parts of the lymphatic vessels not stained for LYVE-1 (Figure 5.6B). Similarly, no differences in the immunofluroscent staining of both type IV and I collagen were detactable on the LTβRIg treated LNs compared to the control (Figure 5.6B).

To sum it up, study of the compartmentalization of MMP-13 by immunohistochemistry indicated that MMP-13 was found mainly in the LNs surrounding both the lymphatic vessels and the blood vessels, as well as the $CD11b^{+}$ macrophages. On the other hand, the main distribution of MMP-9 in the non-immunized LNs was found around the lymphatic vessels, the blood vessels and the FRCs. After immunization, a transient increase in the amount of MMP-9

could also be detected in the $CD11b^+$ macrophages at 1 day after immunization. Staining for the collagen in the LNs indicates that BM, as identified by collagen IV in this study, is present around lymphatic vessels in the LNs, although it is discontinuous and much thinner compared to the blood vessels. On the other side, type I collagen, one of the main components of the ECM, is more evenly spread out in the LNs.


A
huIgG Im3d
MMP-13
MMP-9
Collagen IV
LYVE-1
CD31
Collagen I
400x

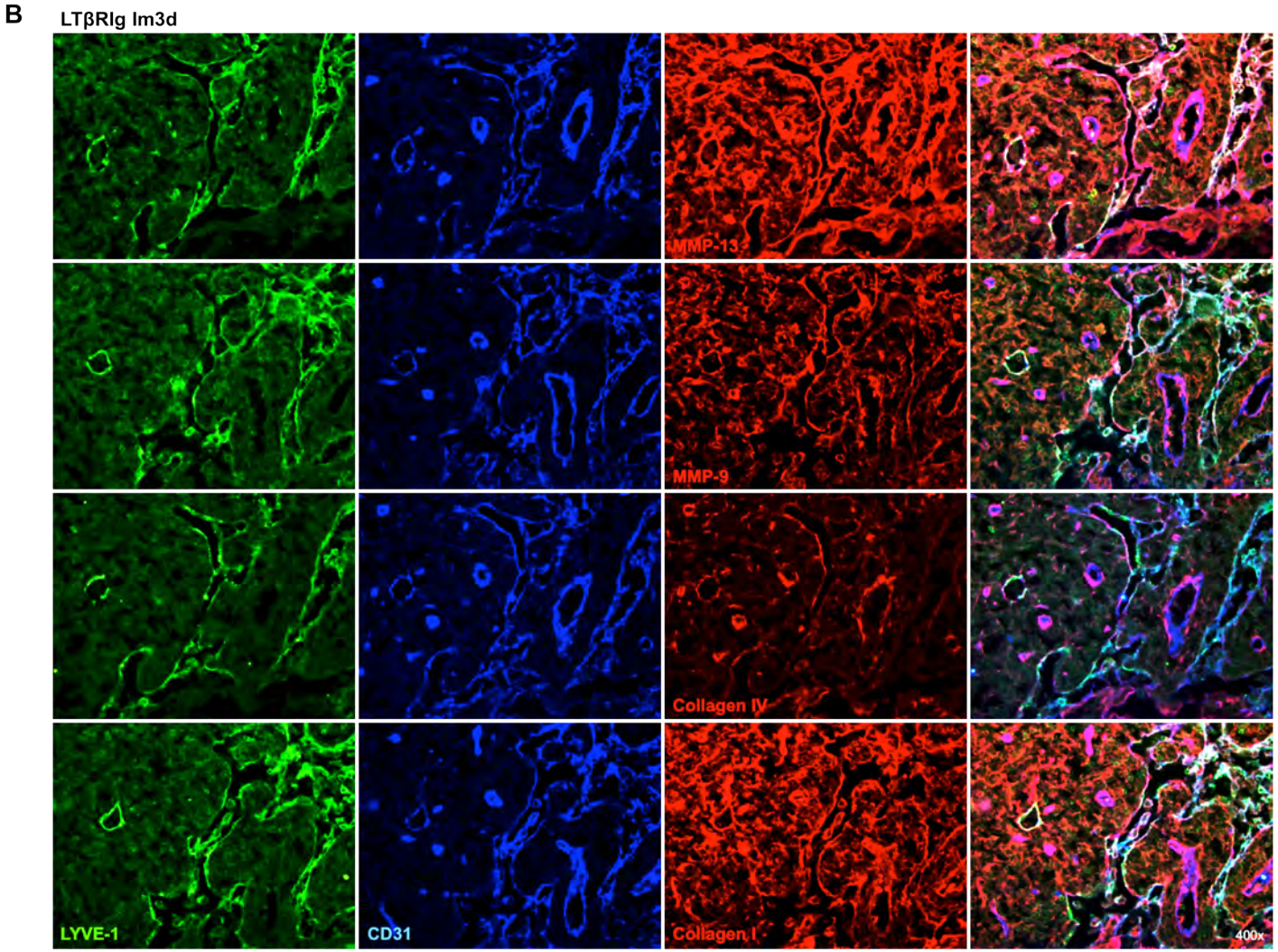

B
LTβRIg Im3d
MMP-13
MMP-9
Collagen IV
LYVE-1
CD31
Collagen I
400x

Figure 5.6: Effect of blocking LTβR signaling on MMP-9 and -13 and type I and IV collagens in the LNs. LN sections from immunized mice at day 3 treated with (A) huIgG and (B) LTβRIg were stained for LYVE-1, CD31 and MMP-13, MMP-9, type IV or type I collagens. Images are representative of 4 independent experiments consisting of 3 mice per group in each experiment.

5.3 Discussion

Our data show for the first time the regulation of the expression of MMPs by the LTβR signaling, as well as the involvement of MMP-13 in lymphangiogenesis. Of the four MMPs analyzed, we demonstrated that upregulation of MMP-13 mRNA expression levels could be detected as early as 6 hours after immunization. Using a gelatin zymography assay, we were able to confirm the upregulation of MMP-13 upon immunization through its proteinase activity, along with detecting an increase in MMP-9 activity. Interestingly, both the increase in mRNA expression levels and proteinase activity of MMP-13, and proteolytic activity of MMP-9 can be moderated by blocking LTβR signaling, suggesting that the regulatory role of LTβR signaling in the early stages of LN lymphangiogenesis may be in the control of MMP-13 expression.

Studying the compartmentalization of MMP-13 and MMP-9 in the LNs, we revealed that they localize mainly around lymphatic vessels, blood vessels and FRCs. As the localization of the MMPs is critical in determining the specificity of proteolysis, we also looked at the expression of types I and IV collagen which are the main components of the ECM and BM, respectively. These types of collagens are also the main substrates of MMP-13 and MMP-9. While lymphatic vessels are known for the absence or at most partial covering by BM (Wiig et al., 2010; Paupert et al., 2011), we do detect collagen IV around the lymphatic vessels, although the amount of collagen IV surrounding the lymphatic vessels is much lower and discontinuous compared to the HEVs. These observations indicate that

for sprouting lymphangiogenesis to occur, degradation of collagen IV in addition to collagen I may be required. In addition, effects, if any, brought about by blocking LTβR signaling on the localization of the MMPs and the main collagen constituents of the ECM and BM could not be detected by the fluorescent microscope.

In conclusion, the close association of the MMPs and collagens imply that the role of MMP-13, and perhaps MMP-9 as well, in lymphangiogenesis may be in the degradation of the ECM and BM.

Chapter 6: Matrix metalloproteinase-13 regulates lymphangiogenesis through proteolytic degradation of extracellular matrix and basement membrane

6.1 Introduction

In the previous chapters, we identified the regulatory role of LTβR signaling in the initiation of lymphangiogenesis through the expression of MMP-13. MMP-13 has been widely recognized as the main collagenase in mice involved in the remodeling of fibrillar collagen due to the restricted expression of MMP-1 in the murine system (Henriet et al., 1992). In addition, MMP-13 has been associated with numerous types of cancer, and high expression of the protease is often linked to the malignancy of the tumors {Balbin:1999ww, Klein:2011cv}. Due to its ability to cleave collagen II, MMP-13 is also critical in bone development and disease {Lemaitre:2006ga}. Besides being an effective proteinase against a broad spectrum of ECM components, MMP-13 is a major player in the MMP activation cascade (Leeman et al., 2002). For instance, MMP-13 is involved in the activation of proMMP-9 (Knäuper et al., 1997), and proMMP-13 can be activated by MT1-MMP and MMP-2 (Knäuper et al., 1996b).

Having established the link between MMP-13 expression and lymphangiogenesis, we sought to determine the aspect of the growth of lymphatic vessels that is regulated by MMP-13. We hypothesized that MMP-13 is important in controlling the steps in the lymphangiogenesis process that requires proteolytic activity such as LEC adhesion or migration. Using a stable mouse LEC line from mesenteric adventitial tissue (SV-LEC) (Ando et al., 2005), we showed that LECs are capable of producing MMP-13 upon stimulation. Through an *in vitro* model of

lymphangiogenesis, we demonstrated that indeed, MMP-13 mediates lymphangiogenesis through its proteolytic activities.

6.2 Results

6.2.1 SV-LECs upregulate the expression of MMP-13 upon stimulation

Endothelial cells have been widely known to express various members of the MMP family (Newby, 2012) and, specifically, MMP-2, MMP-9 and MT1-MMP have been shown to be produced by LECs (Nakamura et al., 2004). Using the stable mouse LEC line, SV-LEC for our study of LECs *in vitro*, we set out to evaluate the expression of the various MMPs, MMP-2, MMP-9, MT1-MMP and MMP-13 by LECs. As TNFα is a known stimulator of MMPs expression, we used recombinant TNFα to induce the expression of the MMPs by the SV-LECs. We then measured the mRNA expression levels of the MMPs at 2, 6, 12, and 30 hours after TNFα stimulation.

SV-LECs were found to express all four MMPs at baseline (Figure 6.1A) Upon stimulation with TNFα, the mRNA expression of MMP-2 increased by 1.2-fold after 2 hours compared to resting SV-LECs, but its expression dropped as the duration of TNFα stimulation increased, settling at about 0.4-fold of the baseline level after 30 hours of stimulation (Figure 6.1B). On the other hand, the mRNA expression of MMP-9 fluctuated throughout the various time points reaching 1.5-fold of baseline expression at 30 hours of TNFα stimulation (Figure 6.1B). For MT1-MMP, its mRNA expression in the SV-LECs slowly declined with time after TNFα stimulation to 0.4-fold of the level expressed by resting SV-LECs at the last time point that we measured (Figure 6.1B). Unlike the other three MMPs

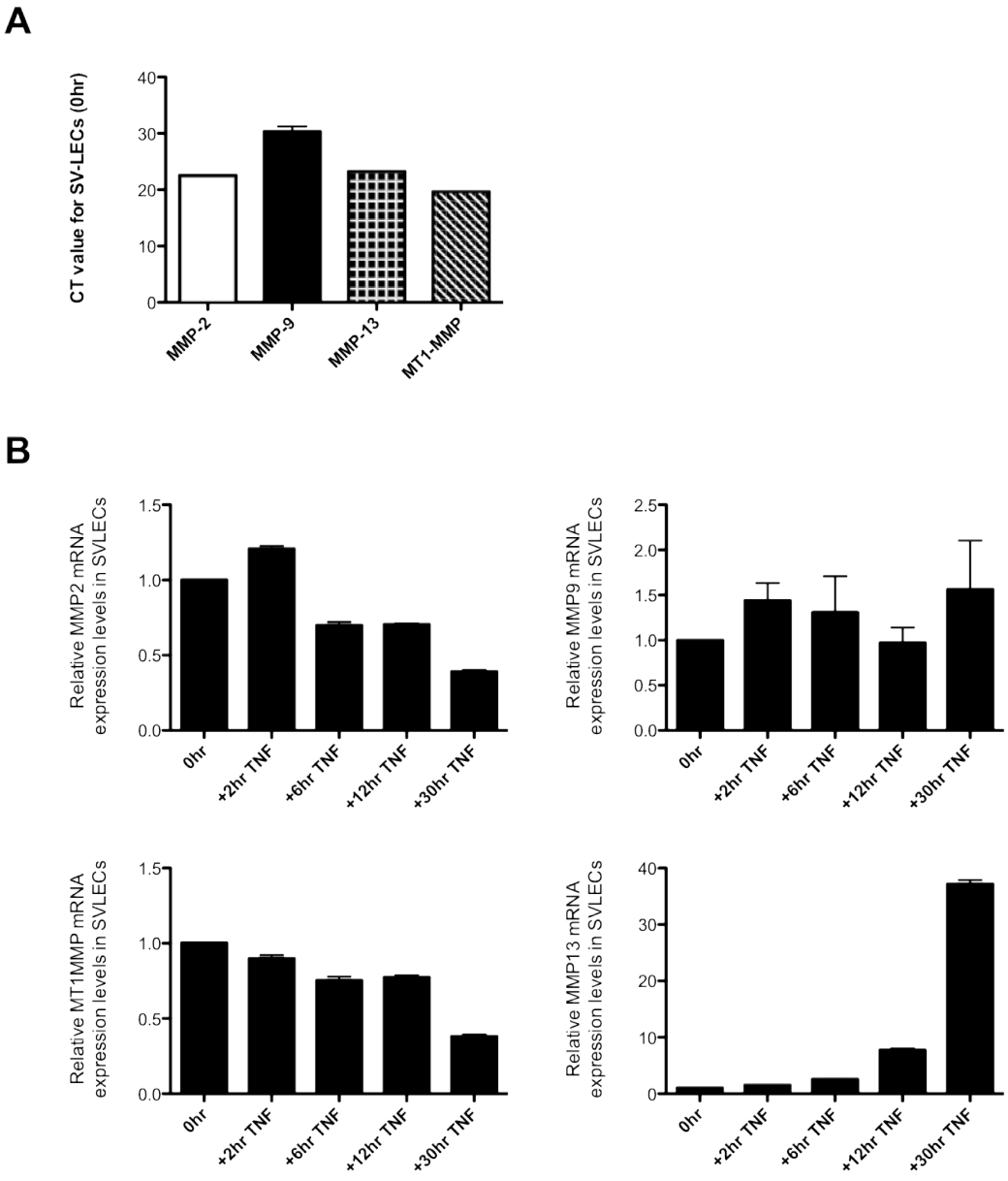


Figure 6.1: Expression of MMP-2, -9, -13 and MT1-MMP in SV-LECs after stimulation by TNFα. (A) Starting CT value (absolute mRNA level) of each MMP in SV-LECs. (B) SV-LECs treated with recombinant TNFα for various time points up to 30 hr were collected and MMPs mRNA levels were quantified by qPCR and normalized to GAPDH mRNA levels. Data shown are representative of more than 5 independent experiments.

examined so far, the mRNA expression of MMP-13 increased steadily with time after TNFα stimulation, finally reaching a 35-fold increase in mRNA expression compared to baseline level (Figure 6.1). These results indicated that stimulation of SV-LECs by TNFα resulted in a drastic upregulation in MMP-13 mRNA expression, while there were no increase in mRNA expression of MMP-2, MMP-9 and MT1-MMP. More significantly, these findings highlighted the ability of LECs to upregulate the expression of MMP-13 upon stimulation, suggesting that LECs may be the cellular source of MMP-13 in the LNs during inflammation.

While we have shown that LECs are capable of producing and upregulating the expression of MMP-13, there exist several cell types in the LNs that have been known to produce MMPs. Of these cells, macrophages have been reported to produce several MMPs including the gelatinases and the collagenases (Shapiro et al., 1990; 1993). To determine the contribution of MMP-13 by macrophages, we made use of a mouse macrophage cell line, RAW 264.7, to stimulate with TNF for the same time points as the previous experiment.

Similar to SV-LECs, MMP-2, MMP-9, MMP-13 and MT1-MMP were found to be expressed in RAW cell. However, their expression pattern after TNFα stimulation were different compared to SVLECs. The mRNA expression of MMP-2 increased to 2-fold after 2 hours of TNFα, and dropped to baseline levels after 6 hours before increasing to 2-fold of the mRNA expression level of the

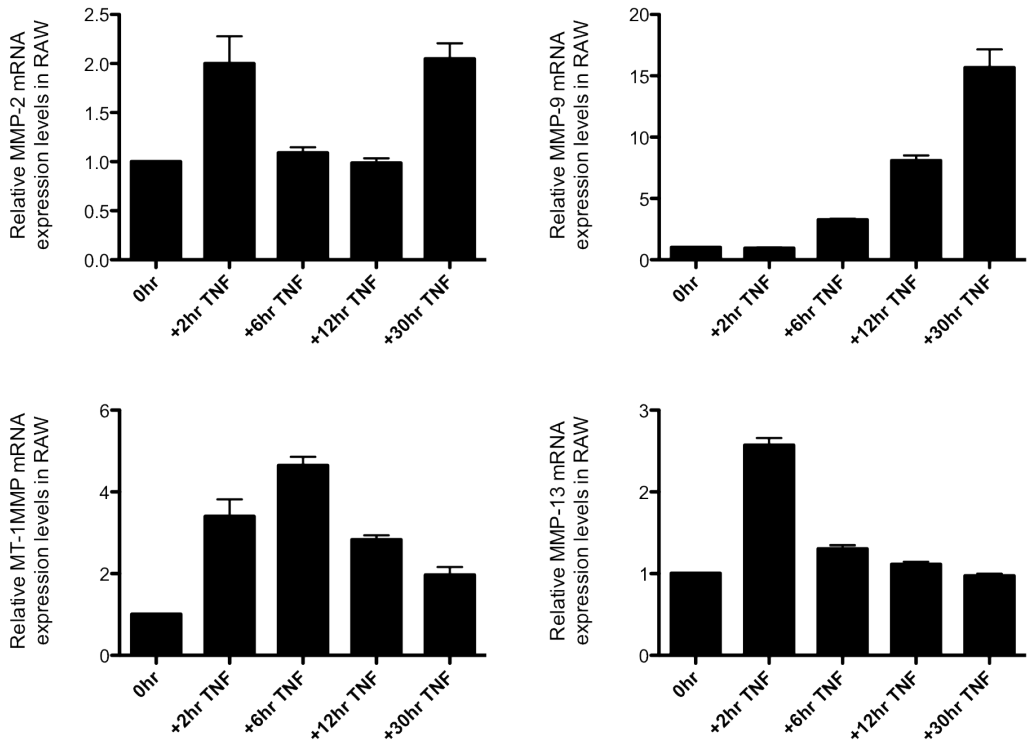


Figure 6.2: Expression of MMP-2, -9, -13 and MT1-MMP in RAW cells after stimulation by TNFα. RAW cells treated with recombinant TNFα for various time points up to 30 hr were collected and MMPs mRNA levels were quantified by qPCR and normalized to GAPDH mRNA levels. Data shown are representative of 4 independent experiments.

resting RAW cells at 30 hours of TNFα stimulation (Figure 6.2). On the other hand, there was a continual increase in the mRNA expression of MMP-9 starting at 6 hours after TNFα stimulation and peaking at 30 hours after stimulation with a 16-fold increase in the expression of MMP-9 (Figure 6.2). For MT1-MMP, upregulation of mRNA expression could be detected at 2 hours after stimulation and continued to 6 hours of TNFα treatment with an expression of almost 5-fold the initial level before dropping off at subsequent time points (Figure 6.2). There was also an early 2.5-fold increase in the expression of MMP-13 mRNA after 2 hours of TNFα treatment, though the expression dropped to resting RAW cells level after 6 hours and remained low for the length of the experiment (Figure 6.2). These data disclosed that while macrophages were capable of producing MMP-2, MMP-13 and MT1-MMP and even increasing their expression to a minor extent in response to TNFα stimulation, the chief protease whose expression was largely upregulated after prolonged TNFα stimulation was MMP-9. Thus, macrophages are unlikely to be the major source of MMP-13 in the early stages of LN lymphangiogenesis since we do not detect a significant increase in MMP-13 expression.

6.2.2 Blocking MMP-13 proteolytic activity prevents tube formation by SV-LECs

Having shown that LECs are likely the main producers of MMP-13 in the LNs upon immunization, we proceed on to investigate the capacity of SV-LECs to undergo *in vitro* lymphangiogenesis by using the tube formation assay. The tube

formation assay was chosen as our *in vitro* lymphangiogenesis model because it comprises several steps in lymphangigogenesis such as the adhesion of endothelial cells to the substrate, migration, alignment, protease activity as well as tubule formation (Arnaoutova et al., 2009; Arnaoutova and Kleinman, 2010). SV-LECs were seeded onto matrigel on a 96-well plate and incubated overtime to allow for tube formation to occur. We found that SV-LECs were able to spontaneously form tubes on the matrigel in the absence of any lymphangiogeneic factors (**Figure 6.3A**). As early as an hour of incubation, the cells could be seen aligning themselves on the matrigel substrate. At 6 hours after seeding, a network formed by the lumens of the tubes could be observed (**Figure 6.3A**). To explore the role of MMP-13 in the tube formation of SV-LECs, we measured the amount of MMP-13 secreted by the cells when they were undergoing the tube formation process. After allowing SV-LECs to grow on matrigel for 3 hours, we took the conditioned media for analysis of MMP-13 protein expression by western blot. For our control, the cells were grown on an empty well without the addition of any substrate. Interestingly, the blot showed that there was a marked increase in the secretion of MMP-13 by SV-LECs when they were incubated on matrigel for 3 hours (**Figure 6.3B**). No band could be detected for SV-LECs when they were grown on the culture plate alone without any substrates (**Figure 6.3B**). We also looked at the secretion of MMP-9 by SV-LECs but could not detect any bands through western blot between cells that were grown on matrigel and on empty well (data not shown). Together, these data suggested that the ability of SV-LECs to spontaneously undergo tube formation when grown on matrigel is associated with an increased secretion of MMP-13.

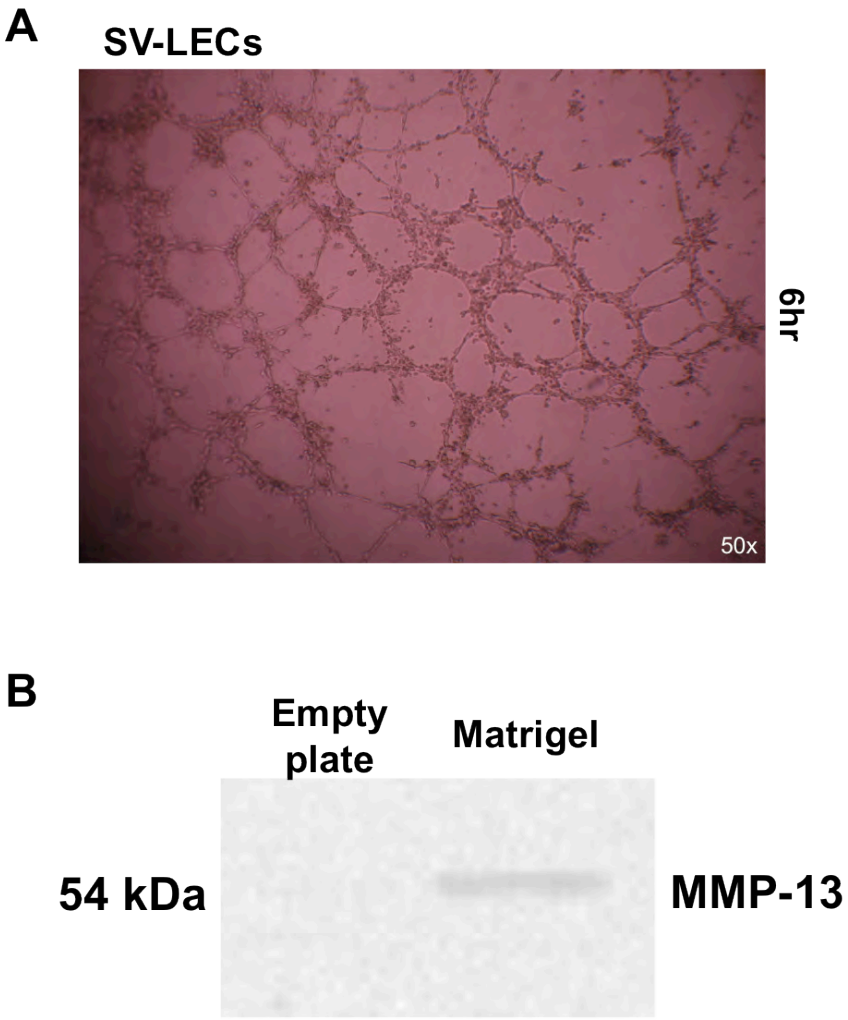


Figure 6.3: Spontaneous tube formation of SV-LECs on matrigel. (A) Formation of tubular structures by SV-LECs grown on matrigel after 6 hr. (B) Western blot analysis of the MMP-13 protein levels in the conditioned media of SV-LECs grown on culture plate without substrate versus matrigel after 3 hr. Results shown are representative of more than 10 independent experiments.

As we have demonstrated earlier, SV-LECs appeared to only upregulate the expression of MMP-13 upon stimulation although they also expressed other MMPs including MMP-2, MMP-9 and MT1-MMP. Therefore, we could not rule out the possibility that other MMPs, other than MMP-13 might be involved in the tube formation process. To address this issue, we made use of three separate MMPs inhibitors: GM6001, MMP-9/MMP-13 inhibitor and a MMP-13 inhibitor, to block the activity of different MMPs and evaluate their effects on tube formation by SV-LECs. First, GM6001 is a broad-spectrum MMP inhibitor, targeting MMP-1, MMP-2, MMP-3, MMP-8 and MMP-9. MMP-9/MMP-13 inhibitor primarily targets MMP-9, and has MMP-13 as its secondary target. And lastly, MMP-13 inhibitor as its name suggests, predominantly targets MMP-13. After 8 hours of incubation, SV-LECs treated with DMSO as control formed a distinctive network of tubule lumens on matrigel (Figure 6.4). For SV-LECs treated with the pan-MMP inhibitor GM6001, tubes could still be formed, although the formation process is diminished judging by the number of branch points observed (Figure 6.4). When the MMP-9/MMP-13 inhibitor blocked the activities of MMP-9 and, to a lesser degree, MMP-13, no tube formation was observed (Figure 6.4). SV-LECs were only able to attach to the matrigel and briefly aligned themselves (Figure 6.4). Some cells could be seen stretching and trying to establish contact with other cells, but the majority of the cells remained in clusters (Figure 6.4). Intriguingly, the blocking of MMP-13 alone brought about the most severe inhibition of tube formation (Figure 6.4). There were no tubular structures and no rearrangement of the SV-LECs positions and only clusters of cells that appeared slightly rounded could be detected on the matrigel

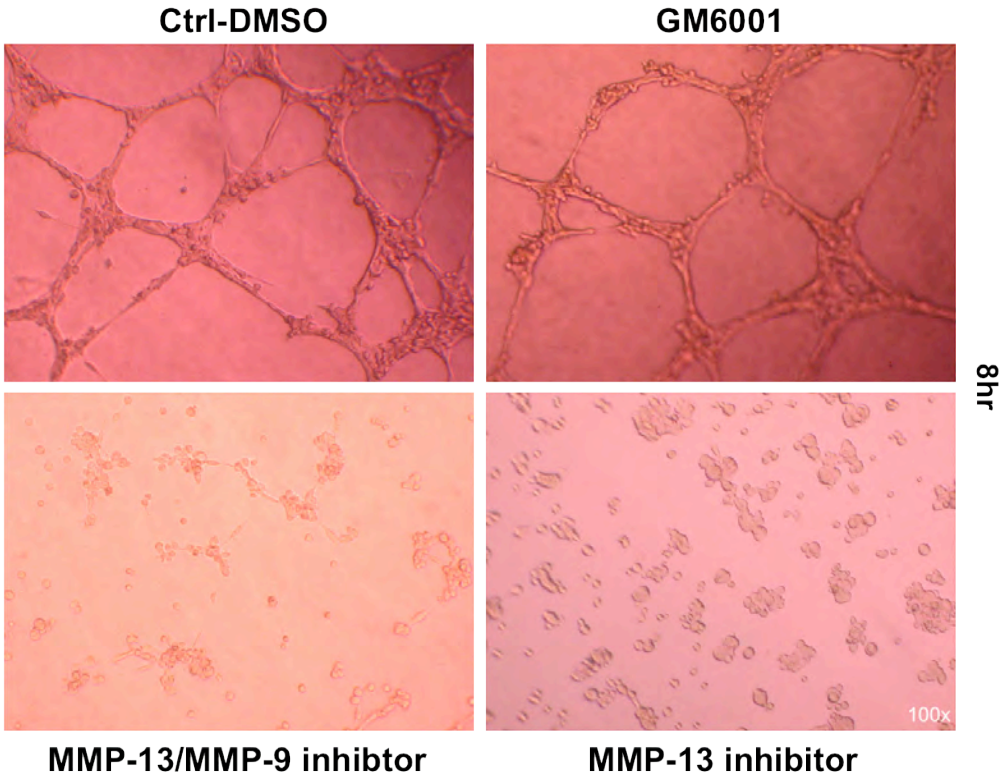


Figure 6.4: Effects of different MMP inhibitors on tube formation of SV-LECs. Appearance of SV-LEC tubes on matrigel in media with DMSO (control), GM6001, MMP-9/MMP-13 inhibitor or MMP-13 inhibitor after 8 hr. Data shown are representative of more than 5 independent experiments.

(Figure 6.4). Overall, these results revealed that MMP-13 was the principal protease involved in the tube formation by SV-LECs (Figure 6.4).

The tube formation assay involves several steps implicated in the lymphangiogenesis process including endothelial cells adhesion, migration, matrix degradation and tubule formation as described previously. Therefore, while we have demonstrated that blocking MMP-13 was able to prevent tube formation, it was not clear to us which of the earlier step of the process was being inhibited. To answer this question, the tube formation assay was repeated with live-cell imaging to obtain a better understanding of how inhibiting MMP-13 was able to stop SV-LECs from forming tubes. Similarly, SV-LECs were treated with DMSO as control or MMP-13 inhibitor and seeded on matrigel for 3 hours. Due to the brief time that we incubated our SV-LECs, we did not observe the formation of the lumens of tubes by the SV-LECs being treated with the control DMSO after 3 hours (Figure 6.5). However, the cells had already attached to the matrigel, and re-positioned themselves in order to form tubes (Figure 6.5). When MMP-13 inhibitor was added to the SV-LECs, the cells only managed to cluster together in rounded clumps (Figure 6.5).

From the tube formation time-lapse video of the SV-LECs under control treatment of DMSO, we could see that the SV-LECs first attached to the matrigel as rounded solitary cells (Video 6.1). As time passed, the cells grouped together in small clusters before they slowly become elongated and reached out to other cell

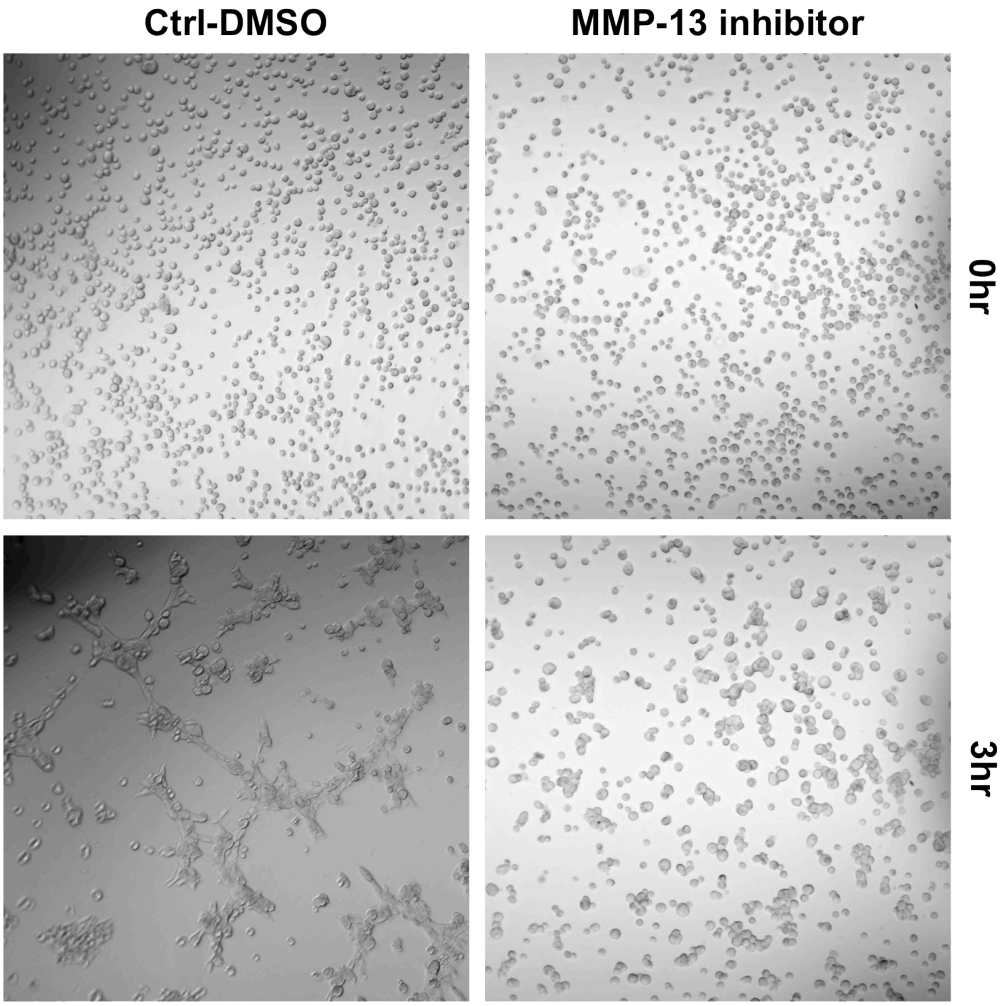


Figure 6.5: Live-cell imaging of the effects of blocking MMP-13 on the tube formation process by SV-LECs. Before and after images of SV-LECs on matrigel in media with DMSO or MMP-13 inhibitor. Data shown are representative of 3 independent experiments.

clumps, forming what appeared to be branches of the tubes (Video 6.1). These activities illustrated that SV-LECs underwent several processes necessary for lymphangiogenesis to take place, namely attachment to a surface matrix, protease activity required for the migration of the cells, and lastly, tubule formation. When MMP-13 inhibitor was added to the culture media, SV-LECs also existing as rounded isolated cells were inadequately attached to the matrigel substrate (Video 6.2). Following that, SV-LECs gradually grouped together with neighbouring cells to form small clusters of rounded cells and remained at that stage till the end of the experiment without any further migration or changes in the morphology of the cells necessary for tube formation (Video 6.2). These observations implied that inhibiting MMP-13 likely reduced the protease activity necessary for the proper attachment of SV-LECs on the matrigel as well as degradation of the matrigel that is obligatory for cell migration.

Next, we assessed the effects of blocking the proteolytic activities of MMP-13 on LEC migration with the scratch wound assay. SV-LECs were grown on a 6-well plate until they formed a confluent monolayer before we induced a wound by scratching across the SV-LEC monolayer. We then determined the migration of SV-LECs by looking at the closure of the wound after 12 hours. Images captured, both at the start and at the end of the 12 hours incubation period, demonstrated significant migration of SV-LECs toward the scratch (Figure 6.6A). However, no obvious difference in the rate of SV-LEC migration could be observed between the group treated with MMP-13 inhibitor and the control group treated with

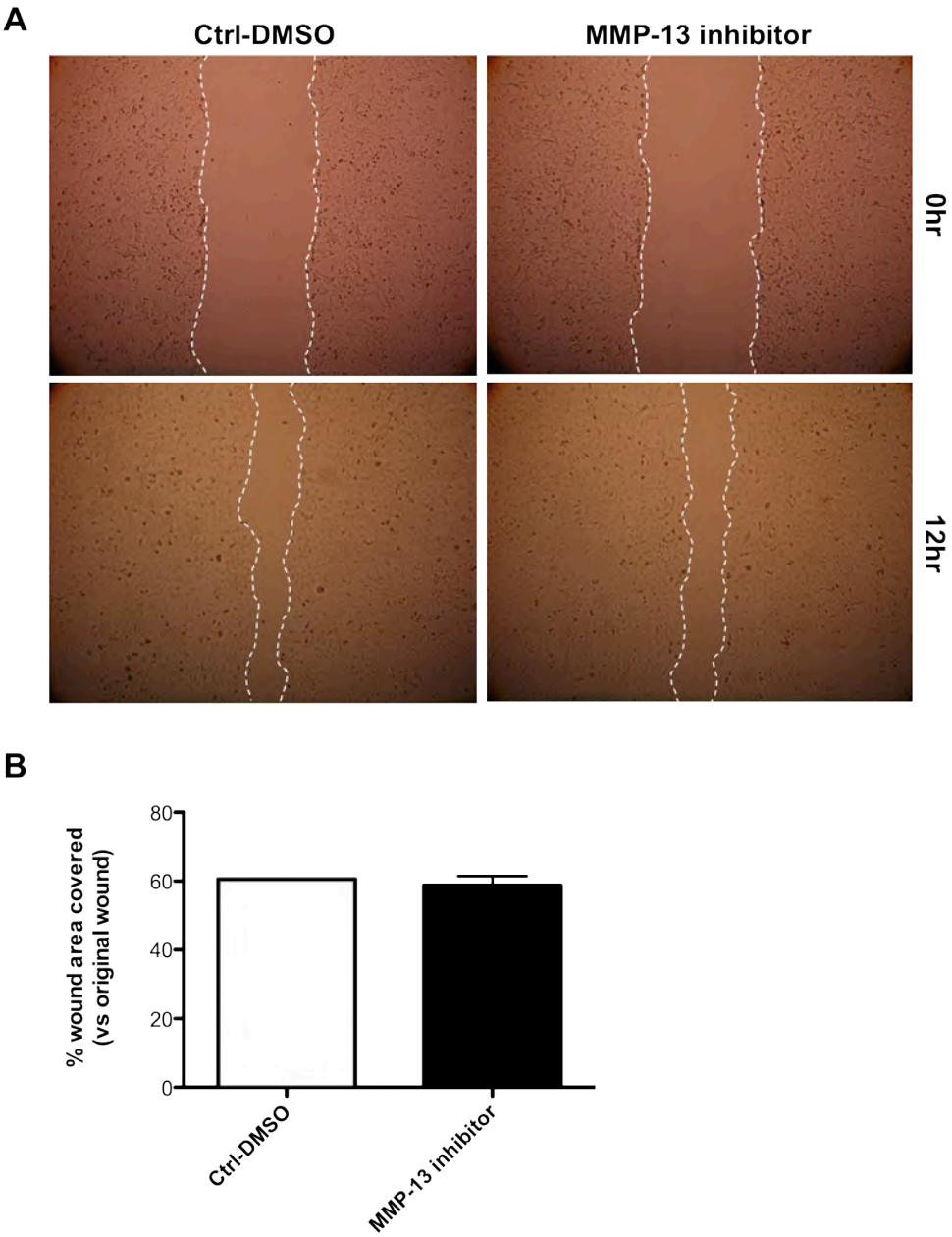


Figure 6.6: Effects of blocking MMP-13 on wound closure by SV-LECs in the scratch wound assay. (A) Confluent monolayers of SV-LECs scratched with a pipette tip (0 hr) were incubated with MMP-13 incubator or DMSO (control) for 12 hr to allow the closure of the wound. (B) Percentage of wounded area that was closed by the SV-LECs after 12 hr. Results shown are representative of 3 independent experiments.

DMSO (Figure 6.6A). Quantifying the percentage of wound area that was closed after 12 hours revealed that both SV-LECs treated with DMSO as control and MMP-13 inhibitor had about 60% wound area recovered and there was no significant difference between the two groups (Figure 6.6B). Overall these results indicated that MMP-13 was not required for the migration of SV-LECs, and confirmed that blocking MMP-13 proteolytic activities prevented the degradation of matrigel that is necessary for tube formation.

Having outlined the prohibiting effects of inhibiting MMP-13 on tube formation with a selective MMP-13 inhibitor, we then tried to use siRNA to knock down the expression of MMP-13. The decision to make use of siRNA to silence MMP-13 is based on its specificity along with the added physiological relevance compared to using a synthetic chemical inhibitor. First we tested for the specificity of the MMP-13-targeted siRNA, before carrying out tube formation with the siRNA-transfected SV-LECs *in vitro*, with the hope of ultimately using the MMP-13-targeted siRNA in our *in vivo* model. Following the transfection of SV-LECs with the MMP-13 siRNA for 48 hours, we measured the expression of MMP-13 mRNA in the cells as well as secreted MMP-13 in the media. MMP-13 mRNA expression analysis revealed that the MMP-13 siRNA achieved a greater than 80% knock down in the gene expression compared to the untransfected control, the mock transfection control (only transfection reagent added to the cells) and the negative scrambled siRNA control (Figure 6.7A). Similarly, secreted MMP-13 protein analysis by western blot of the conditioned media showed that the SV-

LECs transfected with the MMP-13 siRNA secreted a considerably reduced amount of the protease as illustrated by the lack of a band as compared to the other three controls (Figure 6.7A).

After verifying the specificity and efficacy of the MMP-13 siRNA, we proceeded on to carry out the tube formation assay with the siRNA-transfected SV-LECs. Non-specific scrambled siRNA was used as a control for transfecting SV-LECs. From the images taken of the cells after 6 hours, we could clearly spot formation of tubes from the control SV-LECs as well as from the SV-LECs transfected with MMP-13-targeted siRNA, although the extent of tube formation appeared to be diminished in the latter SV-LECs (Figure 6.7B). To ascertain the effects of silencing MMP-13 by siRNA on tube formation by the transfected SV-LECs, we carried out quantification of the tube formation by measuring three parameters of the process: total tubes present, total tube length and total branch points. Each parameter was measured in both groups of cells and normalized against the control group. Analysis of the total tubes present indicated that while knocking down MMP-13 in the SV-LECs led to less tubes formed, the difference was not significant (Figure 6.7C). Similarly, studies of the total tube length and total branch points presented minor reductions in tube formation by the MMP-13 siRNA transfected SV-LECs (Figure 6.7C). These findings stated that while SV-LECs transfected with the MMP-13-targeted siRNA led to significant knockdown of the gene in the cells, this silencing effect was not sufficient to result in a dramatic decrease in tube formation.

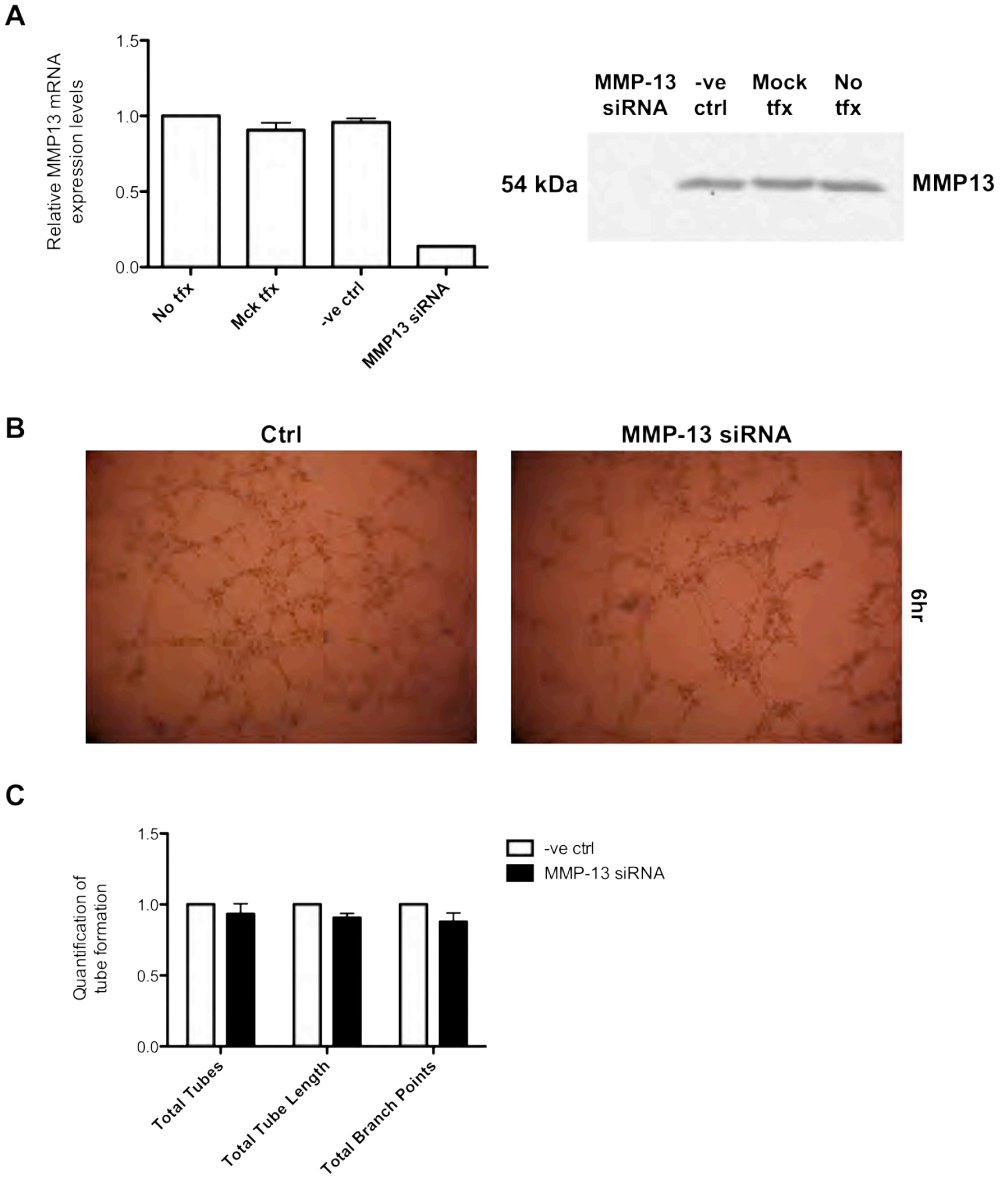


Figure 6.7: Effects of silencing MMP-13 with siRNA on tube formation. (A) Efficiency of the MMP-13 siRNA as assessed by mRNA levels via qPCR and protein levels via western blot. (B) Formation of tubular structures on matrigel by SV-LECs transfected with control scrambled siRNA or MMP-13 siRNA. (C) Tube formation by SV-LECs transfected with MMP-13 siRNA was quantified by the number of total tubes, total tube length and total branch points in a blinded fashion and presented as fold increase relative to control SV-LECs transfected with scrambled siRNA. Data shown are representative of 3 independent experiments.

We have proved the importance of MMP-13 in tube formation by SV-LECs through inhibition experiments. Next, we decided to look at the effects of upregulating MMP-13 on tube formation. In order to increase the expression of MMP-13 on SV-LECs, we seeded the cells on matrigel with TNFα added into the media. We also added a receptor agonist, LTβRag, to investigate the effects of stimulating the LTβR signaling on tube formation. At 6 hours after incubation of the SV-LECs, the expected formation of tubes was detected in the control group as well as in both the treatment groups (Figure 6.8A). Microscope images of the results seemed to suggest that the degree of tube formation was intensified in SV-LECs treated with rLTαβ and TNF (Figure 6.8A). Again we quantified the extent of tube formation by the total tubes present, the total length of the tubes and the total branch point to substantiate our observations. Our analysis revealed that, compared to the control group, there were minimal differences seen in the treatment groups although the treatment groups showed increases in all three parameters measured (Figure 6.8B). Contrasting the differences in tube formation between the two treatment groups showed that stimulation of SV-LECs with rLTαβ did not give rise to any significant differences to treating the SV-LECs with TNF (Figure 6.8B). These results suggested that MMP-13 expressed by SV-LECs at baseline levels was already sufficient for tube formation to occur spontaneously, thus, any upregulation of MMP-13 or other forms of stimulation of the SV-LECs did not result in any significant increase in the extent of tube formation.

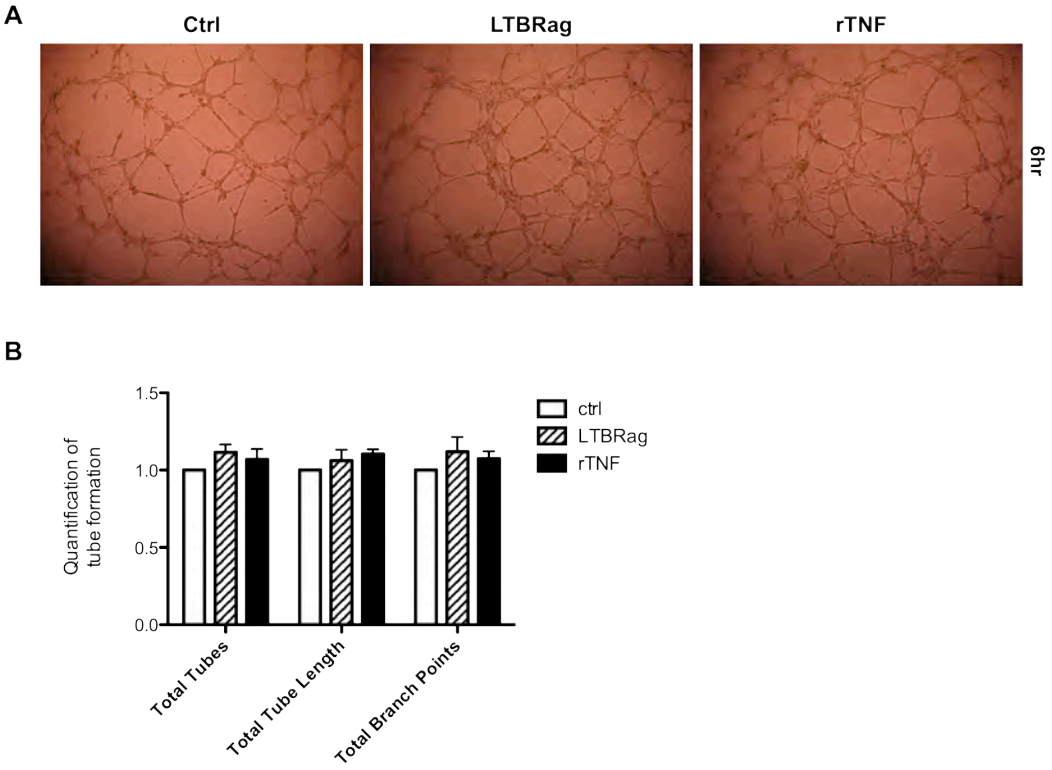


Figure 6.8: Effects of stimulation of LTβR and TNFR signaling on tube formation. (A) Appearance of SV-LEC tubes on matrigel in media with PBS (control), LTβRag or recombinant TNFα after 6 hr. (B) Tube formation by SV-LECs stimulated with LTβRag or recombinant TNFα was quantified by the number of total tubes, total tube length and total branch points in a blinded fashion and presented as fold increase relative to control SV-LECs. Results shown are representative of 3 independent experiments.

6.2.3 MMP-13 is not involved in the regulation of LECs proliferation

Proliferation of LECs is a key step in the expansion of the lymphatic vessel network (Adams and Alitalo, 2007). Furthermore, blocking LTβR signaling has been shown to reduce endothelial cells proliferation in the LNs (Chyou et al., 2008). Therefore, we next wondered if MMP-13 has any regulatory role in LECs proliferation as well as if the reduced lymphangiogenesis we observed in LNs at 4 days post-immunization is a direct consequence of the reduced MMP-13 expression brought about by LTβRIg treatment. To address this, we injected a MMP-13 inhibitor subcutaneously into the footpads of the mice before injection of CFA/KLH for immunization. The mice were then subjected to *in vivo*-labeling of cells with BrdU for cell proliferation analysis via flow cytometry.

We first assessed LECs proliferation in the LNs of mice after 2 and 3 days of immunization without any treatment (Figure 6.9A). LN cellularity and LEC numbers increased over time following immunization (Figure 6.9A). After 2 days of immunization, we could already detect a 2-fold increase in the LN cellularity and the amount of LECs present in the enlarged LNs was twice that of the non-immunized LNs, while this increase in both the LN cellulary and LEC numbers were greater at day 3 of immunization (Figure 6.9A). The rate of BrdU uptake by LECs also increased from about 5% at day 2 to about 12% at day 3 post immunization, associating the increased LEC numbers with cell proliferation (Figure 6.9A). Blocking the activity of MMP-13 by the MMP-13 inhibitor reduced the LN cellularity of LNs after 2 and 3 days of immunization, although

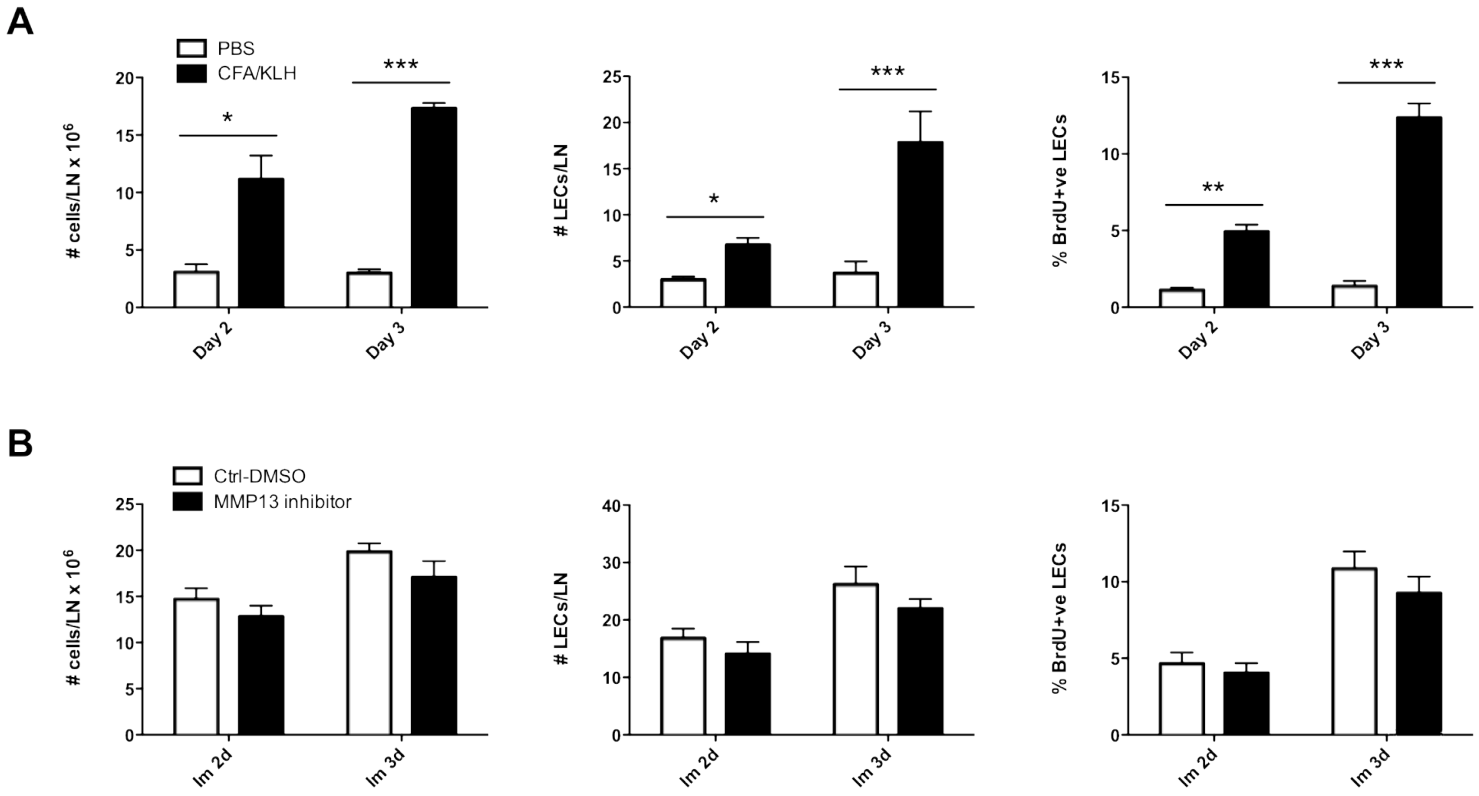


Figure 6.9: Effects of blocking MMP-13 on LECs proliferation. (A) LN cellularity, number of LECs and LEC proliferation as assessed by BrdU uptake were evaluated in LNs after 2 and 3 days of CFA/KLH immunization. (B) LN cellularity, number of LECs and LEC proliferation were evaluated in LNs after 2 and 3 days of immunization with and without inhibiting MMP-13. Data shown are representative of 3 independent experiments with 3 mice per group in each experiment and significant differences are designated by *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

the difference was not significant (Figure 6.9B). Similarly, LEC numbers of the immunized LNs treated with MMP-13 inhibitor were decreased slightly at both time points, but the difference was again not significant (Figure 6.9B). The minor reduction in LEC numbers of these treated LNs was reflected in the rate of cell proliferation where the differences in the percentage of $BrdU^+$ LECs were small and not statistically significant (Figure 6.9B). These results indicated that blocking MMP-13 did not have any influence on LECs proliferation.

6.2.4 Inhibition of MMP-13 activity prevents tube formation in a primary human LEC line

While the tube formation assays that we have conducted with murine SV-LECs thus far have illustrated clearly the importance of MMP-13 in the process, we decided to test the role of MMP-13 in *in vitro* lymphangiogenesis in another system, the primary human dermal adult lymphatic microvascular endothelial cells, HMVEC-dLy, for further validation. After verifying the expression of MMP-13 by the HMVEC-dLy (data not shown), we repeated the tube formation assay on the human LECs with the MMP-13 inhibitor.

Unlike the stable SV-LEC line, the primary HMVEC-dLy took a much longer time for tube formation to occur. After 18 hours of incubation, control HMVEC-dLy treated with DMSO spontaneously developed into a characteristic network of tubule lumens on the matrigel (Figure 6.10). As expected with blocking MMP-13

in the tube formation assay, HMVEC-dLy treated with MMP-13 inhibition did not exhibit any tubular structures (Figure 6.10). Instead, clusters of cells were observed with a few clusters having extensions to other clusters of cells (Figure 6.10). These results indicated that MMP-13 was also an essential player in the tube formation by HMVEC-dLy, thus, further confirming its significance in the lymphangiogenesis process.

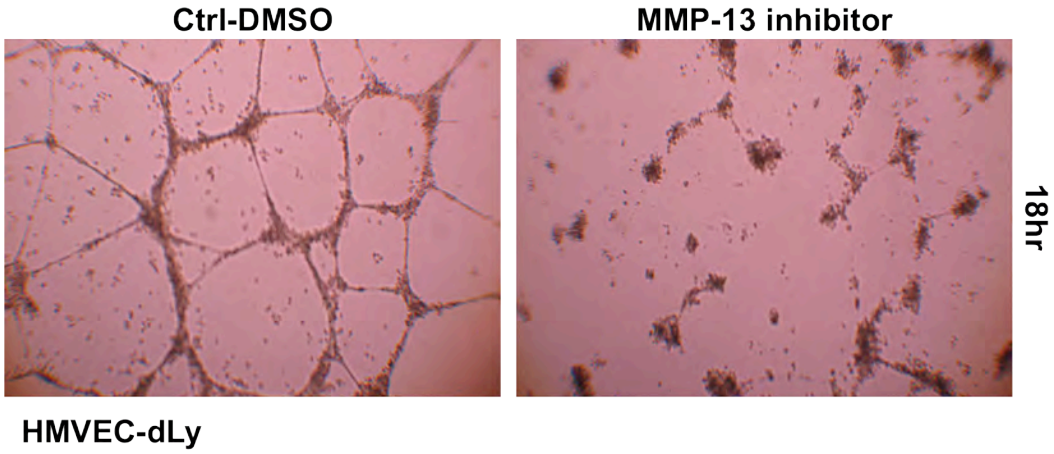


Figure 6.10: Blocking MMP-13 inhibits tube formation in a primary human LEC line. Appearance of HMVEC-dLy on matrigel in media with DMSO or MMP-13 inhibitor after 18 hr. Results shown are representative of 3 independent experiments.

6.3 Discussion

While MMP-2, MMP-9 and MT1-MMP have been shown to be produced by LECs (Nakamura et al., 2004), this is the first report on the expression of MMP-13 by murine LECs. With the SV-LECs, we could detect the expression at baseline of all four MMPs tested, MMP-2, MMP-9, MMP-13 and MT1-MMP. Surprisingly, when SV-LECs were stimulated with TNFα, a known activator of MMPs transcription (Galis et al., 1995; Siwik et al., 2000), only MMP-13 displayed a dramatic increase in expression levels. However, we did not detect similar increases in MMP-13 after stimulation of macrophages with TNFα, suggesting that LECs may be the predominant cellular source of MMP-13 in the LNs in response to immunization. This is particularly interesting, as we have shown earlier that LECs express LTβR. Together, these findings suggest that LTβR signaling may act directly on LECs leading to production of MMP-13 following immunization.

After demonstrating that SV-LECs are capable of spontaneous tube formation on matrigel, we showed that SV-LECs increased the secretion of MMP-13 when they are grown on matrigel. Blocking MMP-13 activity with a synthetic inhibitor prevents the formation of tubular structures by SV-LECs on matrigel. Our time-lapse study of the tube formation process together with the results from the scratch wound assay suggest that MMP-13 is important in the matrix degradation necessary for the proper attachment of SV-LECs on the matrigel as well as for the cell migration process. However, inhibiting a wide range of MMPs including

MMP-1, MMP-2, MMP-3, MMP-8 and MMP-9 with GM6001 and MMP-9 with the MMP-9/MMP-13 inhibitor did not result in the complete blockage of tube formation by SV-LECs. This is unexpected due to the reports of MMP-2 involvement in lymphangiogenesis (Nakamura et al., 2004; Matsuo et al., 2007; Bruyère et al., 2008; Detry et al., 2012). In our model, only blocking of MMP-13 is necessary to prevent tube formation.

Silencing the expression of MMP-13 by SV-LECs with siRNA did not lead to significant inhibition of SV-LECs tube formation, suggesting that the >80% knockdown of MMP-13 expression is still not enough to impede the process. Similarly, increasing the expression of MMP-13 by stimulation of the SV-LECs with LTβRag or TNFα did not result in any significant changes in the generation of tubular structures by SVLECs suggest that the levels of MMP-13 is not a limiting factor in the tube formation process.

While LTβR signaling inhibition has been reported to reduce endothelial cells proliferation (Chyou et al., 2008), we show here that blocking MMP-13 did not have any effect on LECs proliferation. Our results suggest that LTβR signaling may have other roles in regulating the growth of lymphatic vessels in addition to regulating the expression of MMP-13. Furthermore, these results reveal that the role of MMP-13 in lymphangiogensis may be solely as a collagenase on the degradation of the ECM and BM.

We observed similar inhibition of the tube formation in primary human LECs by blocking MMP-13, thus further confirming MMP-13 significance in the lymphangiogenesis process. Collectively, our findings suggest that MMP-13 promotes lymphangiogensis through the degradation of the ECM necessary for sprouting and cell migration.

Chapter 7: Discussion

The discovery of molecular mechanisms underlying the remodeling of lymphatic vessels in the LNs during inflammation is still in its infancy. Similarly, the role of LTβR signaling in the control of the LN architecture, especially in the context of inflammation remains uncertain. Through the work presented in this study, we have shown that the B cell mediated lymphangiogenesis in the LNs is regulated by LTβR signaling. Furthermore, we have demonstrated that this regulation of lymphatic vessel growth by LTβR is only at the early stage of the process, via the expression of MMP-13. The proteolytic activity of MMP-13 is vital for the degradation of the ECM and BM to enable the sprouting of new lymphatic vessels. In this chapter, results of all the research work done so far are summarized and discussed, and suggestions for future directions are put across as well.

7.1 LTβR signaling in B cells-mediated LN lymphangiogenesis

7.1.1 Importance of B cells in LN lymphangiogenesis

Expansion of the LNs during inflammatory conditions is a result of the increased recruitment of lymphocytes and monocytes into the LNs via the HEVs in order to optimize antigen-presenting between the antigen-presenting cells and the lymphocytes for an effective immune response (Gretz et al., 2000; Palframan et al., 2001; Soderberg et al., 2005). The increase in LN size is accompanied by remodeling of the lymphatic vessel network of the LN, which is vital for improving the mobilization of DCs to the draining LNs (Angeli et al., 2006). LN lymphangiogenesis can be regulated by locally produced VEGF-A (Angeli et al.,

2006), or remotely mediated by VEGF-A released at the inflamed peripheral tissues (Halin et al., 2007). B cells in inflamed LNs have been shown to contribute to the expansion of the lymphatic vessel network through the expression of VEGF-A (Angeli et al., 2006; Shrestha et al., 2010). Here, we provide evidence that B cells mediate LN lymphangiogenesis through the expression of LTα. The expansion of the lymphatic vessel network after immunization was not observed in μMT mice that lack B cells. While reconstitution of μMT mice with B cells restored LN lymphangiogenesis after immunization, the growth of lymphatic vessels was not detected in μMT mice reconstituted with B cells that were $LT\alpha^{-/-}$. LTα is expressed on both B and T cells, although it appears that the expression of LTα on T cells is not able to compensate the loss of expression of LTα on B cells in mediating LN lymphangiogenesis. LTα exists in two forms: a secreted homotrimer $LT\alpha_3$ that binds to TNFRs, as well as a membrane tethered heterotrimer $LT\alpha_1\beta_2$ with LTβ that binds to LTβR. Besides playing known critical roles in both inflammation and lymphoid organ development (De Togni et al., 1994; Hjelmström et al., 2000), $LT\alpha_3$ has been recently described to be important in lymphatic vessel function and lymphangiogenesis (Mounzer et al., 2010). While we have demonstrated the importance of LTα expression on B cells in the expansion of the lymphatic vessel network, further work is required to substantiate the role of $LT\alpha_3$ in our model of lymphangiogenesis.

There was also a lack of FDCs in the LNs of chimeric mice that do not express LTα on B cells. This is not surprising, due to the well-recognized role of $LT\alpha_1\beta_2$

on B cells in the development and maintainance of the FDCs (Mackay and Browning, 1998; Ansel et al., 2000). However, what is intriguing is that the lack of FDCs in the LNs coincides with the absence of LN lymphangiogenesis, suggesting that FDCs may have a role in regulating the growth of lymphatic vessels. Further indications of the role of FDCs in lymphangiogenesis comes from the same chimeric mice experiments. Even though LTα was also expressed by T cells, this expression was not able to make up for the loss of LTα on B cells. This implies that the role of LTα on B cells in mediating lymphangiogenesis may not simply be in the secreted $LT\alpha_3$ form, but also in the membrane bound $LT\alpha_1\beta_2$ form, where signaling is restricted by both the location of the ligand-expressing cells as well as the receptor-expressing cells. Stromal cells, including the FDCs and FRCs, have been recently recognized to participate in maintaining the homeostasis of the immune system as well as launching efficient immune responses (Mueller and Germain, 2009). Moreover, FRCs have been shown to regulate LN remodeling and vascularity through VEGF-A expression (Chyou et al., 2008). Given that the growth of new lymphatic vessels in the B cell follicles are of close proximity to the FDCs, it is possible that FDCs may promote lymphangiogenesis directly by releasing lymphangiogenic factors such as VEGF-A that promotes the growth and proliferation of LECs (Konstantinou et al., 2009). On the other hand, FDCs may also regulate the remodeling of lymphatic vessels indirectly by organizing the B cell follicles. FDCs are known to secrete CXCL13 that attracts B cells to migrate into B cell follicles (Ansel et al., 2000), as well as producing the B cell activating factor (BAFF) that is important for B cell homeostasis (Woodland et al., 2006). The reverse scenario may also be plausible,

where B cells modulate lymphangiogenesis indirectly by maintaining the FDC network through the expression of $LT\alpha_1\beta_2$ (Ansel et al., 2000)

Interestingly, both the production of CXCL13 and BAFF by FDCs are regulated by LTβR signaling (Ansel et al., 2000; Nishikawa et al., 2006). Due to the close interactions and interdependency between B cells and FDCs, development and maintenance of functional FDCs by LTβR signaling is indispensable for normal B cell functions, and vice versa. However, additional research is needed to clarify the role of FDCs in LN lymphangiogenesis.

7.1.2 Regulation of LN lymphatic vessel growth and function by LTβR signaling

LTβR signaling has been shown to be important in regulating the lymphatic vessels and HEVs in the remodeling of the LNs (Browning et al., 2005; Liao and Ruddle, 2006; Chyou et al., 2008). Our study on the short-term inhibition of the LTβR signaling in the LNs through the use of the soluble decoy LTβRIg shows that the expansion of the LN and lymphangiogenesis following immunization were reduced. The process of lymphocytes entry into the LNs begins with the adherence of the L-selectin expressed on lymphocytes to the peripheral lymph node addressins (PNAd) found on the luminal surfaces of HEV (Miyasaka and Tanaka, 2004). The diminished increase in LN cellularity is likely an effect of impaired lymphocyte migration into the LNs. PNAd and mucosal vascular

addressin cell-adhesion molecule (MAdCAM) on HEVs, molecules important for lymphocyte trafficking, have been demonstrated to be downregulated when LTβR signaling is blocked (Drayton et al., 2003; 2004; Browning et al., 2005). The reduced LN expansion may also be a result of reduced growth of the HEVs as LTβR blockage has been shown to result in decreased VEGF-A expression and endothelial cell proliferation (Chyou et al., 2008). Consistently, we also observed a reduction in lymphangiogenesis after LTβRIg treatment. That LECs express LTβR indicates that LTβR signaling may control the growth of lymphatic vessels directly through LECs. Furthermore, inhibition of the LTβR signaling pathway was revealed to reduce DC migration from the periphery to the LNs following immunization. Increased buildup of DCs in enlarged inflamed LNs was previously shown to be a result of the expanded lymphatic vessel network in these LNs that leads to enhance migration of DCs into the LNs (Angeli et al., 2006). While LTβR signaling is vital in mediating the expression of CCL19 and 21 in the spleen (Ngo et al., 1999), blocking LTβR did not appear to reduce the expression of these chemokines that are necessary to direct DC trafficking in the LNs (Browning et al., 2005). Therefore, the decline in DC migration is most probably a direct consequence of the decrease in lymphangiogenesis by the blocking of LTβR, indicating the regulation of lymphatic vessel function by LTβR signaling.

We first used the TNFαKO mice model in an attempt to study LN lymphangiogenesis in an environment with B cells in the absence of FDCs. However, the abnormal growth of lymphatic vessels in the LNs of these mice

makes it apparent that they are not the most appropriate model available. A previous study on airway inflammation has also demonstrated the exaggerated response to infection, including abnormal lymphangiogenesis, by the TNFαKO mice, indicating that genetic deletion of the gene does not give the same result as blocking with a synthetic inhibitor (Baluk et al., 2009). Given the central role that TNFα plays as a proinflammatory cytokine involved in instigating the inflammatory cascade through the NF-κB signaling pathway (Karin and Greten, 2005; Hayden and Ghosh, 2008), it is not suprisingly that compensatory pathways arise from the deletion of the gene in these mice (Marino et al., 1997; Baluk et al., 2009). However, which signaling pathways are upregulated remain to be determined. Our experiment with LTβRIg in the TNFαKO mice suggests that while the entry of lymphocytes is still regulated by LTβR signaling, the compensatory pathways controlling lymphangiogenesis is independent of this signaling pathway.

7.1.3 Role of B cells in LTβR signaling

Corresponding to our findings that the expression of LTα on B cells is central to LN lymphangiogenesis, analysis of LTβR ligands expression by B and T cells highlights the role of B cells in modulating LTβR signaling. Upon immunization, the expression of LTα is upregulated in B cells but not T cells, indicating that B cells may be critical as a cellular source of ligand of the regulation of LTβR signaling in lymphangiogenesis. Lending support to our proposition, a previous study has reported the involvement of B cells and LTβR signaling in the cross-talk

between lymphatic vessels and HEVs in the LNs (Liao and Ruddle, 2006). The remodeling of LNs induced by virus infection was also orchestrated by $LT\alpha_1\beta_2$-expressing B cells (Kumar et al., 2010). However, B cells were demonstrated to be dispensable in the *de novo* lymphangiogenesis within tertiary lymphoid structures (Furtado et al., 2007). Although T cells was shown to be required for this *de novo* lymphangiogenesis, they did not seem to play similar vital role in our model of lymphatic growth in immunized LNs. We also provided evidence for alternative splicing of the LTα gene in mice. Alternative splicing is the process where a single gene can give rise to different mRNAs resulting in the generation of various protein isoforms with differing and even opposing functions. Because of its contributions to proteome diversity in humans, alternative splicing is decidedly applicable to disease and therapy (Garcia-Blanco et al., 2004). Although the amount of isoforms of the LTα gene has not been determined, recent work has illustrated the differential expression of up to seven LTα isoforms in human following lymphocyte activation (Smirnova et al., 2008). Future work on the functional studies of LTα ought to consider the possible influence of the various LTα isoforms and characterization of the splice variants may be valuable in refining our knowledge of the role of LTα in mediating inflammation and immune response.

In contrast to LTα and LTβ, LIGHT do not appear to have a critical influence in the development of secondary lymphoid organs (Scheu et al., 2002; Tumanov et al., 2003). One of the most prominent role of LIGHT is in the activation of T cells

in the regulation of inflammatory responses (Ware, 2008). While the expression and role of LIGHT in T cells are well-recognized , the understanding of the function of LIGHT in B cells is still inadequate. Consistent with a previous study (Kang et al., 2007), we detected a weak expression of LIGHT by B cells that increases after immunization. Additional studies will be required to comprehend the implications of this upregulation of LIGHT expression by B cells in inflammatory conditions.

While blocking LTβR signaling is able to inhibit the expansion of the lymphatic vessel network in inflamed LNs, activating the LTβR via a receptor agonist in the absence of immunization is insufficient to initate lymphangiogenesis. This is perhaps not surprising as a complex multifactorial network of chemical mediators, including cytokines and chemokines, are known to be secreted in response to inflammation that may be necessary for the growth of lymphatic vessels (Nathan, 2002). However, activation of LTβR together with CFA/KLH immunization is still not able to drive lymphangiogenesis in μMT mice. These observations suggest that the role of B cells in lymphangiogenesis is not simply that of supplying the ligand $LT\alpha_1\beta_2$ for LTβR signaling. It is possible that B cells may regulate lymphangiogenesis through the production of lymphangiogenic factors. Indeed, B cells have been shown to express a substantial amount of VEGF-A when activated *ex vivo* (Angeli et al., 2006). In addition to the absence of B cells, μMT mice also do not have FDCs in the LNs. As discussed previously, the

presence of FDC network in the LNs may also be essential for the growth of lymphatic vessels.

7.1.4 Temporal control of LTβR signaling in LN lymphangiogenesis

Studies have generally focused on the effect of blocking LTβR signaling on lymphatic vessels in resting LNs (Browning et al., 2005; Chyou et al., 2008); here, we explored the therapeutic potential of inhibiting LTβR on lymphangiogenesis in inflamed LNs. While LN cellularity was reduced after LTβRIg treatment, blocking LTβR signaling did not reduce the extent of lymphangiogenesis in the inflamed LNs. The absence of effect on lymphatic vessel growth with blocking LTβR signaling after immunization was also supported in a previous study using a different immunization protocol (Liao and Ruddle, 2006). It appears that once lymphangiogenesis has been initiated, the process is no longer subjected to LTβR regulation. These observations are significant because they hint at the possible temporal control of LTβR signaling in lymphangiogenesis. Indeed, our subsequent results revealed the importance of LTβR signaling in the early stages of the expansion of the lymphatic vessel LNs in the LNs through the expression of MMP-13.

7.2 Role of MMP-13 in lymphangiogenesis

7.2.1 Regulation of MMP-13 expression by LTβR signaling

The regulation of lymphangiogenesis by MMPs is an area of research that has been relatively overlooked. While the role of MMPs in angiogenesis is widely recognized, data on the evidence of MMPs in lymphangiogenesis have not been as substantial. Early studies looking at similar links between MMPs and lymphangiogenesis often resulted in dismissive outcomes. One such report showed that the Ets 1 transcription factor, which is involved in angiogenesis through the induction of several matrix-degrading proteases, including MMP-2, MMP-9 and MT1-MMP, is not expressed by LECs (Wernert et al., 2003). However, later studies have verified the expression of these three MMPs in LECs as well as the involvement of MMP-2 in lymphangiogenesis (Nakamura et al., 2004; Matsuo et al., 2007; Bruyère et al., 2008; Detry et al., 2012). Studies on MMPs in lymphangiogenesis have mainly looked at MMP-2, MMP-9 and MT1-MMP perhaps due to their undisputed role in regulating angiogenesis. However, as mentioned earlier, differences btween the exposure of the lymphatic vessels and blood vessels to the ECM as well as the lack of a continuous BM for lymphatic vessels suggest that MMPs that are implicated in lymphangiogenesis may be different from those that are vital for angiogenesis. In our study, we have established the novel role of MMP-13 in mediating lymphangiogenesis.

MMP-13 belongs to the group of MMPs known as collagenase. As the name suggests, the main substrates for this group of MMPs are the interstitial collagens

I, II and III. In addition, having similarities in the active site sequence with gelatinases imply that MMP-13 can also cleave collagen IV and gelatin (Knäuper et al., 1996a). MMP-13 was originally cloned from human breast cancer tissue in 1994 (Freije et al., 1994). Human MMP-13 is homologous to mouse MMP-13, which is also known as its main interstitial collagenase (Henriet et al., 1992; Freije et al., 1994). Interestingly, similar expression pattern of mouse MMP-13 to human MMP-1, and the failure to isolate MMP-1 in mouse, imply a functional homology between mouse MMP-13 and both the human MMP-1 and MMP-13 (Henriet et al., 1992; Balbín et al., 2001). The ability of MMP-13 to cleave a varity of ECM components, including type IV collagen which is one of the main constituent of the BM, strongly suggests that it may be vital for lymphangiogenesis. Furthermore, as MMP-13 is highly entangled in the MMP activation cascade(Leeman et al., 2002), it may also link up other MMPs in the process of driving lymphangiogenesis. Our data show that the regulatory role of LTβR signaling in the initiation of lymphangiogenesis is through the expression of MMP-13 in the LNs. Among the MMPs that we studied including MMP-2, MMP-9 and MT1-MMP, only the mRNA expression of MMP-13 in the LNs increased dramatically upon immunization and this increase in mRNA level is impeded by blocking LTβR signaling. However, we could also detect an increase in the proteolytic activity of MMP-9 after immunization in the LNs through the zymography assay along with MMP-13 activity. Correspondingly, increase in the proteolytic activities of both MMPs was reduced with LTβRIg treatment.

7.2.2 Compartmentalization of MMP-13 in the LNs

Analysis of the compartmentalization of MMP-13 and MMP-9 in the LNs revealed the close association of these MMPs with the lymphatic vessels, blood vessels, FRCs as well as type I and IV collagen. MMP-13 and MMP-9 were found to be surrounding the lymphatic vessels, which were also enclosed by collagen I (representing the ECM) and also a considerable amount of collagen IV (the main component of the BM). Interestingly, both collagens are recognized substrates of MMP-13. Reports have suggested that both MMP-13 and MMP-9 are capable of binding to cell surface and ECM molecules (Murphy and Nagase, 2011). The binding of MMPs to a specific location in the pericellular environment, otherwise known as compartmentalization, is an important aspect of MMP activity regulation. Attaching MMPs to a fixed location is able to build up a pool of enzymes and focus their activities to specific targets in the extracellular space. Our observations suggest that MMP-13 may be involved in the sprouting of the lymphatic vessels through the degradation of the surrounding ECM and BM. Among the various functions of MMP-13, MMP-13 is known to activate proMMP-9 (Knäuper et al., 1997). Furthermore, activated MMP-9 can in turn activate proMMP-13, resulting in a positive feedback loop (Han et al., 2007). Hence as both MMPs are bound at similar locations of the pericellular environment of the LNs, even though we did not detect any increase in the mRNA expression of MMP-9 in the LNs, it is possible that MMP-13 may also act indirectly in driving lymphangiogenesis through the activation of MMP-9. Interestingly, in addition to MMP-13, the expression of MMP-9 has been shown to increase in the regeneration region of the skin in a mouse model of

lymphangiogenesis (Rutkowski et al., 2006). Through observations in MMP-9-deficient mice, they determined that MMP-9 is not required for adult dermal lymphangiogenesis (Rutkowski et al., 2006). We have also examined LN lymphangiogenesis induced by CFA/KLH immunization in MMP-9-deficient mice and found that there were no significant differences in the lymphatic vessel network in the LNs after immunization between WT and MMP-9-deficient mice (data not shown). However, even though various MMPs have been recognized to be vital for angiogenesis, studies have demonstrated that mice deficient in most of the MMPs do not have any significant differences in the blood vasculature due to overlapping in their proteolytic activities (Rundhaug, 2005). Further studies are required to evaluate the exact involvement of MMP-9 in the remodeling of lymphatic vessels.

In our study, we also noticed the discontinuous expression of LYVE-1 on the lymphatic vessels in the LNs. This is a phenomenon that has also been recently observed in lymphatic vessels in various organs and tissues including the skeletal muscle and cornea (Gehlert et al., 2010; Wardrop and Dominov, 2011; Nakao et al., 2012), and the sinusoidal endothelium of the liver (Arimoto et al., 2010). The proinflammatory cytokine TNFα has been reported to downregulate the expression of LYVE-1 in some inflammatory conditions (Johnson et al., 2007). While the reason behind the local loss of LYVE-1 on the lymphatic vessels is unclear, this occurance coincides with lymphatic vessels that are undergoing remodeling. That we detect a brighter staining of MMP-13 and MMP-9 around

regions of the lymphatic vessels with decreased LYVE-1 expression suggests that a higher concentration of the enzymes are assembled at these locations. These observations lend additional support to our hypothesis in the role of MMP-13 in mediating lymphatic vessel remodeling.

7.2.3 *In vitro* modulation of lymphangiogenesis by MMP-13

While MMPs are well-recognized to be produced by BECs (Newby, 2012), the expression of MMPs by LECs have not been thoroughly investigated. In agreement with previous reports, we demonstrated the expression of MMP-2, MMP-9 and MT1-MMP by LECs (Nakamura et al., 2004; Bruyère et al., 2008). On top of that, we showed for the first time that LECs express MMP-13. Among the cell types present in the LNs, macrophages are also a well-established producer of MMP-13 in addition to endothelial cells (Fallowfield et al., 2007). When comparing the expression of MMPs between LECs and macrophages, we found that only LECs display a dramatic increase in MMP-13 upon inflammatory stimulation, thus suggesting that LECs may be the main supplier of MMP-13 in our lymphangiogenesis model. Because we have confirmed the expression of LTβR by LECs, these findings together imply that LTβR signaling may act directly on LECs in modulating the release of MMP-13.

In vitro study of lymphangiogenesis allowed us to dissect the specific step in the process that is regulated by MMP-13. Through the tube formation assay and

scratch wound assay, we verified the proteolytic activity of MMP-13 in regulating the growth of lymphatic vessels. When grown on matrigel, LECs simultaneously upregulate the expression of MMP-13 and form tubular structures resembling the lumen of a vessel. This tube formation by LECs was prevented when we blocked the activites of MMP-13 with a synthetic inhibitor. Because tube formation involves several steps of the lymphangiogenesis process, our time-lapse video on the formation of tubes indicates that the proteolytic activities of MMP-13 is required for the adhesion of LECs on the matrigel. The inability of LECs to adhere properly to the matrigel likely affects subsequent steps in the process like the migration and alignment of the LECs to form tubular structures. We confirmed the above observations with the scratch wound assay, which measures the ability of LECs to migrate on a surface without the need for any proteolytic degradation. That inhibiting MMP-13 did not affect LEC migration in the scratch wound assay indicates that the primary role of MMP-13 in lymphangiogenesis may be in the degradation of the matrix.

The importance of MMP-13 in lymphangiogenesis was further emphasized through our efforts to hinder tube formation by silencing MMP-13 gene expession in LECs. When the expression of MMP-13 was knocked down using siRNA, we did not detect any major reduction in the tube formation by LECs. The difference between blocking MMP-13 activity with a synthetic inhibitor and silencing the gene expression is that the inhibitor exerts its effects after the enzyme is secreted into the media, whereas gene silencing greatly reduces the amount of MMP-13

that is being secreted. If the inhibitor is not limiting, it will be able to bind to and inhibit the increased release of MMP-13 by LECs on matrigel. Although verification of the gene silencing, via both mRNA and protein expression, showed more than 80% knockdown of MMP-13 in LECs at basal conditions, the extent of the upregulation of MMP-13 secretion by LECs when growing on matrigel is unclear. Our observations seem to indicate that only a miminal amount of MMP-13 is required to induce *in vitro* lymphangiogenesis. Further prove of this deduction is provided when we demonstrated that no significant changes in the extent of lymphangiogenesis was observed when we attempt to increase the secretion of MMP-13 by activating LECs with rTNFα.

Although the proliferation of LECs is naturally essential in the expansion of the lymphatic vessel network, our results showed that blocking the activites of MMP-13 in the LNs did not result in any significant decrease in LECs proliferation. This suggests that inhibiting the proteolytic activityies of MMP-13 may have an effect on the morphology of the lymphatic vessel network, as the remodeling of lymphatic vessels has been thought to be directed by the physical contraints due to the components of the ECM, leading to either a stationary phenotype or branching in the presence of dense matrix (Detry et al., 2012). While the total LEC count did not reveal any discrepancies in the lymphangiogenesis process, a detailed analysis on the morphology of the lymphatic vessels and collagen deposition in the LNs may reveal more about the effects of blocking MMP-13 on lymphatic vessel remodeling.

7.3 Proposed mechanims driving lymphangiogenesis in the inflamed LNs

Based on our findings, we propose a model where B cells mediate the initiation of lymphangiogenesis through the expression of MMP-13 via LTβR signaling (Figure 7.1). As the LTβR ligand, $LT\alpha_1\beta_2$ is tethered to the membrane, binding to the receptor have to be a cell-cell interaction. This indicates that the LTβR-expressing cells participating in the signaling ought to be in the vicinity of B cells. Because we have validated the expression of LTβR on LECs and illustrated the expansion of the lymphatic vessel network within the B cell follicles, as well as the capacity of LECs to secrete MMP-13, the LTβR signaling pathway could be a direct interaction between B cells and LECs. Upon LTβR activation, LECs then increase the expression of MMP-13 to initiate the sprouting of new lymphatic vessels through the degradation of ECM and BM. Interactions of B cells with LECs via LTβR signaling may lead to the production of lymphangiogenic factors by B cells to further aid in the lymphangiogenesis process. This pathway is also probable given that B cells are capable of producing an elevated amount of VEGF-A that can induce lymphangiogenesis and LN expansion (Angeli et al., 2006; Shrestha et al., 2010). A second possible pathway is through B cells interactions with FDCs via LTβR signaling, or BECs, macrophages and other stromal cells present in the LNs that express LTβR. This interaction then leads to the activation of B cells and/or FDCs (other cells) in producing lymphangiogenic factors to drive lymphangiogenesis.

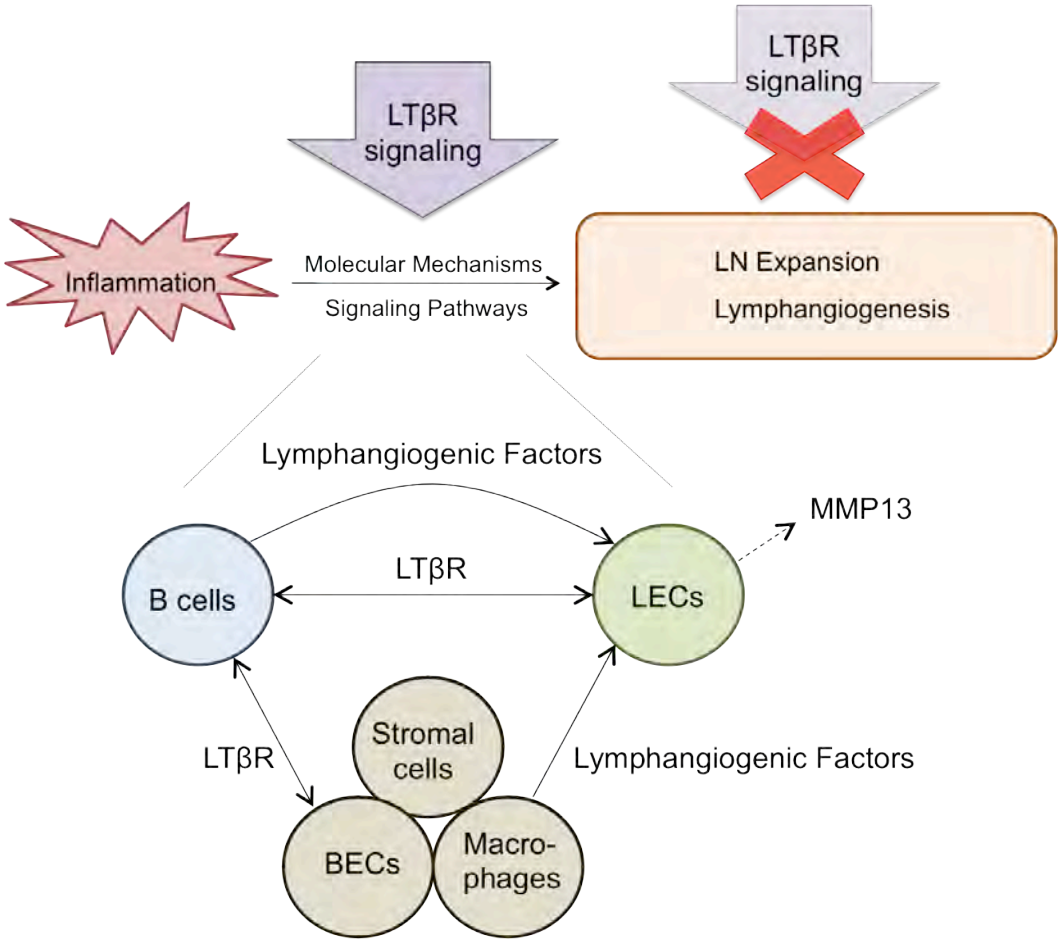


Figure 7.1: Diagram depicting the possible pathways of lymphangiogenesis mediated by LTβR signaling. B cells may drive lymphangiogenesis directly through activating LTβR signaling on LECs, leading to production of lymphangiogenic factors such as VEGF-A and the secretion of MMP-13. B cells may also induce lymphangiogenesis indirectly through LTβR signaling on stromal cells such as FDCs, BECs or macrophages.

7.3.1 Regulation of lymphangiogenesis through the role of MMP-13 in the activation cascade of MMPs

Current literature on the role of MMPs in lymphngiogenesis have all agreed on the involvement of MMP-2 to some degree (Nakamura et al., 2004; Rutkowski et al., 2006; Matsuo et al., 2007; Bruyère et al., 2008; Detry et al., 2012). However, when we blocked the activities of a wide range of MMPs including MMP-2 and MMP-9 with a broad spectrum inhibitor, we did not detect any significant effects on LECs in forming tube-like structures on matrigel. These differing observations are surprising due to the above mentioned studies describing the contributions of MMP-2. Part of the explanation may be attributable to the source of LECs as well as the experimental models used. Even though blocking MMP-2 along with other MMPs did not have an effect on tube formation by LECs in our study and we did not detect any significant increase in mRNA levels of the other three MMPs we evaluated in the LNs, we cannot rule out their role in our model of lymphangiogenesis. Recent studies are beginning to uncover the complexity of the role of MMPs in regulating tissue homeostasis, providing evidence that these proteases do not normally function alone, but rather they regulate the activity of other proteases forming cascades and regulatory networks (Overall and Kleifeld, 2006; Morrison et al., 2009). As proMMP-13 can be activated by MMP-2 as well as MT1-MMP (Knäuper et al., 1996b), and in turn, proMMP-2 is activated by MT1-MMP, these MMPs may also influence the lymphangiogenesis process indirectly through the activation of MMP-13. Studies on MMP-13 in tumors have shown the coexpression of MMP-2 and MT1-MMP with MMP-13, suggesting that these expression patterns may be necessary to provide an environment for the

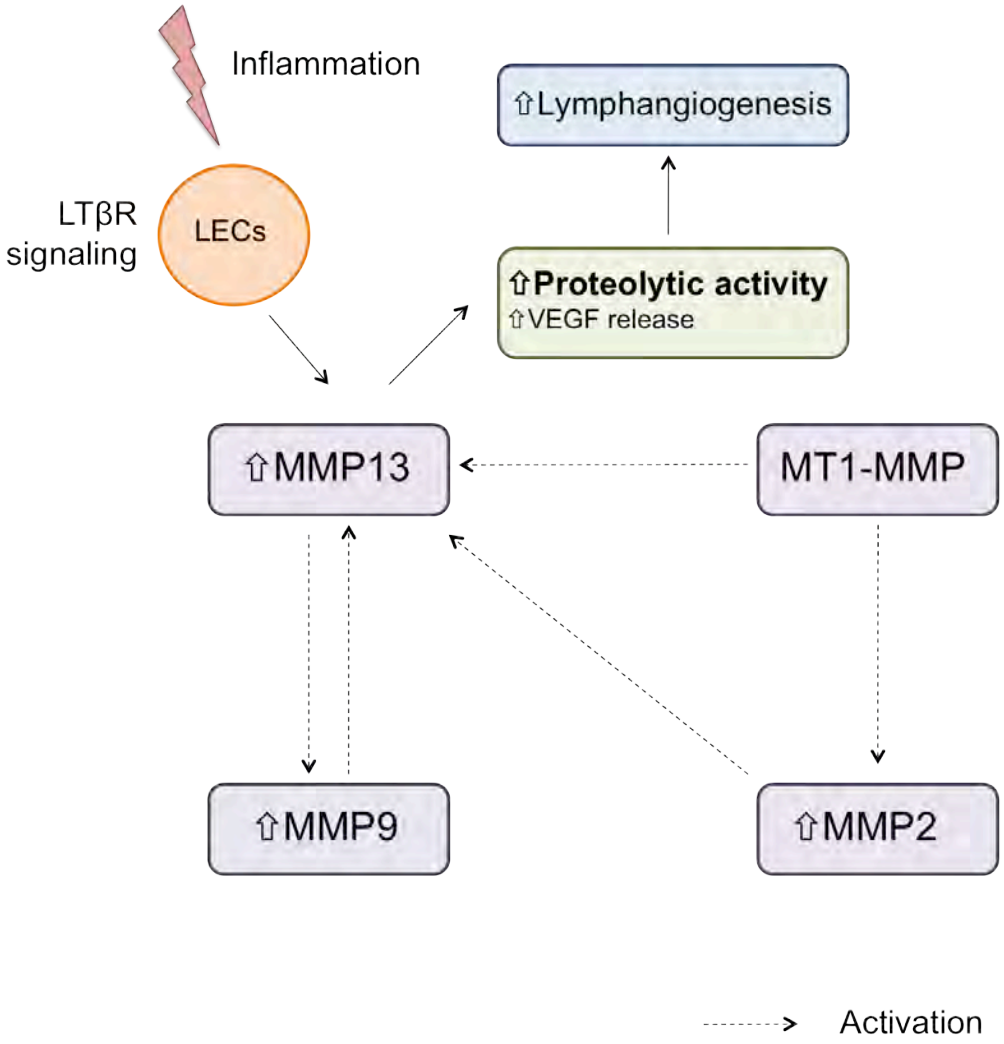


Figure 7.2: Proposed model of how the various MMPs may work to stimulate lymphangiogenesis upon LTβR stimulation in a cascade. LECs (possible cellular source of MMP-13) is mediated by LTβR signaling upon inflammation to increase the expression of MMP-13. Increase expression of MMP13 leads to activation of proMMP-9. In turn, MMP-9 can also activate proMMP-13, resulting in a positive feedback loop. MMP-2 and MT1-MMP can also activate MMP-13. The presence of these other MMPs may be in providing an optimal working environment for MMP-13 leading to to increase proteolytic activity. Degradation of the ECM and BM then faciliates sprouting lymphangiogenesis. The degradation of the ECM may also lead to the increase of VEGF-A bioavailability, further promoting lymphangiogenesis.

activation of MMP-13 (Cazorla et al., 1998; Johansson et al., 1999). It is worth noting that MMP-2 can also activate proMMP-9 (Fridman et al., 1995). Interestingly, MMP-2, MMP-9, MMP-13 and MT1-MMP together have been implicated in a MMP activation cascade in a human chondrosarcoma cell line (Cowell et al., 1998). Therefore, we propose a model on the initiation of lymphangiogenesis by MMP-13 and how the various MMPs may regulate the process (Figure 7.2).

7.4 Relevance of this study

Lymphangiogenesis has been associated with a number of inflammatory disorders, including the chronic inflammatory disease psoriasis (Kunstfeld et al., 2004), rheumatoid arthritis (Xu et al., 2003; Zhang et al., 2007), inflammatory bowel disease (Alexander et al., 2010), Kawasaki disease (Ebata et al., 2011; Hirono and Ichida, 2011) and chronic airway inflammation (Baluk et al., 2005; 2009). Typically, it is assumed that lymphangiogenesis aids in the resolution of chronic inflammation by increasing lymph flow in draining away accumulated fluids and inflammatory cells and cytokines. Indeed, studies targeting the stimulation of lymphangiogenesis and improving lymphatic drainage have shown to be effective in alleviating the severity of inflammation and edema in chronic inflammatory diseases (Polzer et al., 2008; Guo et al., 2009; Kajiya et al., 2009; Huggenberger et al., 2010; 2011b). However, increased lymphangiogenesis can also leads to the inception of an inflammatory response through the enhanced drainage of APCs to the draining LNs. Undesirable inflammation due to

lymphangiogenesis observed in organ transplantation are found to be associated with transplant rejections (Cursiefen et al., 2003; Kerjaschki et al., 2004; Cursiefen et al., 2004a; Kerjaschki, 2006). Considering the evidence presented in these studies, while the influence of lymphangiogenesis in these inflammatory disorders still require further study, targeting the lymphatic vasculature may represent an effective mode of therapy.

The structuring of the LN and the remodeling of the lymphatic vessel network are the principal determinants of an efficient immune response during inflammation. Lymphangiogenesis in the LNs can serve to increase migration of inflammatory cells from the inflamed peripheral tissues for antigen presentation and inflammatory resolution (Kataru et al., 2009). Increased drainage to the LNs by the growth of the lymphatic vessel network may also be advantageous in clinical settings, such as improving the delivery of DC vaccines to LNs. *Ex vivo* manipulation of DCs has been experimented as therapeutic vaccines for infectious disease and cancer for more than a decade (Steinman and Banchereau, 2007; Palucka and Banchereau, 2012). Despite the benefits of DC vaccines to conventional methods, one of the major deficiencies is the inability of the injected DCs to migrate to the draining lymphoid organs, with only about 1% success rate (de Vries et al., 2003). Our study on the regulation of lymphangiogenesis in the LNs which boosts DC migration may present new approaches in optimizing DC vaccination protocols.

On the other hand, LN lymphangiogenesis has also been associated with pathological conditions. While the spread of malignant cells from tumor through the lymphatics or blood vessels to distant organs has been established as a hallmark of cancer (Hanahan and Weinberg, 2000), recent studies have shown that tumors can induce lymphangiogenesis within sentinel LNs before metastasis to facilitate their dissemination (Hirakawa et al., 2005; Qian et al., 2006; Harrell et al., 2007; Hirakawa et al., 2007). As the concept behind the expansion of the lymphatic vessel network within the LNs before tumor metastasis is comparable to the growth of the lymphatic vessels within the draining LNs during inflammation, our proposed model of the LTβR-mediated lymphangiogenesis may be relevant to tumor LN lymphangiogenesis. Particularly of interest is the potential link between MMP-13 and tumor lymphangiogenesis. MMP-13 has been implicated in a variety of malignant tumors such as breast cancer, chondrosarcoma, head and neck carcinoma, and melanoma, where high expression of MMP-13 may be necessary for metastasis of specific tumors through the proteolytic degradation of the extracellular environment (Pendás et al., 2000; Ala-aho and Kähäri, 2005; Zigrino et al., 2009). Recent studies have also demonstrated the newfound role of MMP-13 in tumor angiogenesis, in part through the regulation of VEGF-A bioavailability (Lederle et al., 2010; Kudo et al., 2012; Wang et al., 2012). A thorough understanding of the role of MMP-13 in cancer processes may assist in the development of therapeutic approaches to contain the progression and metastatic capacity of tumor cells.

7.5 Future directions

The degradation of the ECM and BM is the most apparent role of MMPs in the regulation of angiogenesis, and lymphangiogenesis, as it allows the sprouting of new vessels from pre-existing ones by directly removing the imposing physical constraints. However, studies have shown that proteolytic cleavage of the ECM and BM can also release factors that may promote or inhibit angiogenesis (Rundhaug, 2005; van Hinsbergh and Koolwijk, 2008; Ribatti, 2009). The capacity of MMP-13 to free ECM-bound VEGF-A was put forward through an *in vitro* study which investigated the amount of VEGF-A that was released from collagen type I gel containing the growth factor via MMP-13 (Lederle et al., 2010). The releasing of sequestered VEGF-A by MMPs was first reported in a study of MMP-9 in triggering the angiogenic switch in pancreatic islet tumors (Bergers et al., 2000). In addition, MMP-13 can also indirectly stimulate angiogenesis through promoting VEGF-A secretion by fibroblasts and endothelial cells (Kudo et al., 2012). In line with previous studies in the role of VEGF-A in LN lymphangiogenesis (Angeli et al., 2006; Halin et al., 2007; Shrestha et al., 2010), the ability of MMP-13 to increase the bioavailibity of VEGF-A is particularly intriguing. As LTβR signaling has been shown to regulate the expression of VEGF-A in the LNs (Chyou et al., 2008), releasing sequestered VEGF-A may be an additional way in which the signaling pathway increases the bioavailability of the lymphangiogenic factor. It would be interesting to explore if the ability of MMP-13 in modulating the increase of VEGF-A in the LNs during inflammation may represent additional control of lymphangiogenesis.

In our current study, we studied the involvement of MMP-13 in lymphangiogenesis through its mRNA expression and proteolytic activities, as well as compartmentalization in the LNs. Another important determinant in the regulation of MMP activity is through enzyme inactivation, of which TIMPs play a major role (Baker et al., 2002; Brew and Nagase, 2010). Characterization of MMP-13 has shown that the enzyme can be inhibited by TIMP-1, TIMP-2 and TIMP-3 (Knäuper et al., 1996a). Interestingly, the expression of TIMPs has also been shown to be regulated by NF-κB signaling (Bommarito et al., 2011). Therefore, to further examine the role of MMP-13 in the LTβR signaling-mediated lymphangiogenesis, it may be interesting to examine the contributions of TIMPs in LN lymphangiogenesis, as well as its regulation by LTβR.

7.6 Conclusion

In summary, we have identifed the LTβR signaling pathway as a key molecular mediator in the B cell-mediated lymphangiogenesis. We have also shown that LTβR signaling regulates the expression of MMP-13 that is important for the sprouting of lymphatic vessels through the degration of the matrix components. While much progress have been made in the elucidation of the various regulatory pathways involved in lymphangiogenesis, a clear understanding of the interactions between lymphatic vesels and the surrounding matrix environment could provide new insights into the lymphangiogenesis process and the role of main players such as those that govern the degradation of the ECM.

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Appendix 1: List of antibodies used for flow cytometry

Antibody (conjugation)	Company	Clone
Goat anti-mouse LTβR (biotin)	R & D systems	Polyclonal
Hamster anti-mouse CD3e (APC)	eBioscience	145-2C11
Hamster anti-mouse CD11c (PE)	eBioscience	N418
Hamster anti-mouse podoplanin	DKSH	8.1.1
Mouse anti-FITC (biotin)	Jackson	1F8-1E4
Mouse anti-mouse CD45.2 (FITC)	eBioscience	104
Mouse anti-mouse CD45.2 (PerCP-Cy5.5)	eBioscience	104
Rat anti-mouse B220 (FITC)	eBioscience	RA3-6B3
Rat anti-mouse B220 (PerCP-Cy5.5)	eBioscience	RA3-6B4
Rat anti-mouse CD11b (PerCP-Cy5.5)	BD pharmingen	M1/70
Rat anti-mouse CD31	Serotec	ER-MP12
Rat anti-mouse CD31 (FITC)	BD Pharmingen	390
Anti-rat IgG (APC)	Invitrogen	N.A.
Anti-syrian hamster IgG (PE)	Calbiochem	N.A.
Streptavidin Cy2	Jackson	N.A.
Streptavidin Per-Cy5.5	eBioscience	N.A.

Appendix 2: List of antibodies used for immunofluorescence analysis

Antibody (conjugation)	Company	Clone
Armenian hamster anti-mouse CD31	Millipore	MAB1398Z
Armenian hamster anti-mouse TCRβ	BD Pharmingen	H57-597
Goat anti-mouse LTβR (biotin)	R & D systems	Polyclonal
Rabbit anti-type IV collagen	Cosmo Bio	Polyclonal
Rabbit anti-mouse collagen type I	Millipore	Polyclonal
Rabbit anti-mouse MMP-9	Abcam	Polyclonal
Rabbit anti-mouse MMP-13	Santa Cruz	Polyclonal
Rat anti-mouse B220	eBioscience	RA3-6B2
Rat anti-mouse B220 (biotin)	eBioscience	RA3-6B3
Rat anti-mouse B220 (biotin)	eBioscience	RA3-6B3
Rat anti-mouse CD11b	BD Pharmingen	M1/70
Rat anti-mouse CD31 (FITC)	BD Pharmingen	390
Rat anti-mouse ERTR7	Acris	ER-TR7
Rat anti-mouse FDC	ImmunoKontact	FDC-M2
Anti-armenian hamster IgG (DyLight549, DyLight647)	Jackson	N.A.
Anti-rabbit IgG (Cy2, Cy3 or Cy5.5)	Jackson	N.A.
Anti-rat IgG (Cy2, Cy3 or Cy5.5)	Jackson	N.A.
Streptavidin Cy3 or DyLight549	Jackson	N.A.

Appendix 3: List of primers used for semi-quantitative PCR

Target gene		Primer sequence (from 5' to 3')
β-actin	forward	TGGCACCACACCTTTCTACAATGAGC
	reverse	GCACAGCTTCTCCTTAAGCAGAAAGAGG
LTα (1)	forward	TGCCAGGACAGCCCATCCAC
	reverse	TGAGCAGGAACACAGCCCC
LTα (2)	forward	CCAGGACAGCCCATCCACT
	reverse	GTGGACAGCTGGTCTCCCTT
LTβ	forward	TGGATGACAGCAAACCGTCG
	reverse	AACGCTTCTTCTTGGCTCGC
LIGHT	forward	GGCTGGAACAGAACCACCG
	reverse	CCAAGTCGTGTCTCCCATAAC

Appendix 4: List of primers used for qPCR

Target gene		Primer sequence (from 5' to 3')
GAPDH	forward	AGGCCGGTGCTGAGTATGTCG
	reverse	GCAGAAGGGGCGGAGATGAT
MMP-2	forward	TAACCTGGATGCCGTCGT
	reverse	TTCAGGTAATAAGCAAAATTGAA
MMP-9	forward	CGGCACGCCTTGGTGTAGCA
	reverse	AGGTGAGGGGGCGCCTGTAG
MMP-13	forward	GCCAGAACTTCCCAACCAT
	reverse	TCAGAGCCCAGAATTTTCTCC
MT1-MMP	forward	AACTTCGTGTTGCCTGATGA
	reverse	TTTGTGGGTGACCCTGACTT

Appendix 5: List of antibodies used for immunoblotting

Antibody (conjugation)	Company	Clone
Rabbit anti-MMP-9	Abcam	Polyclonal
Rabbit anti-MMP-13	Santa Cruz	Polyclonal
Mouse anti-GAPDH (HRP)	Sigma	GAPDH-71.1
Anti-rabbit IgG (HRP)	Jackson	N.A.