SEMMELWEIS EGYETEM

DOKTORI ISKOLA

Ph.D. értekezések

3072.

ARADI PETRA

Celluláris és molekuláris élettan
című program

Programvezető: Dr. Hunyady László, egyetemi tanár

Témavezető: Dr. Jakus Zoltán, egyetemi docens

LYMPHATIC- AND NEUTROPHIL-DEPENDENT TISSUE DAMAGE IN CONTACT HYPERSENSITIVITY AND RHEUMATOID ARTHRITIS

PhD thesis

Petra Aradi

Molecular Medicine Doctoral School

Semmelweis University

Supervisor: Zoltán Jakus, MD, Ph.D., Habil.

Official reviewers: Győző Szolnoky, MD, Ph.D., Habil.
Norbert Kiss, MD, Ph.D.

Head of the Complex Examination Committee:
Edit Buzás, MD, D.Sc.

Members of the Complex Examination Committee:
Anna Erdei, D.Sc.
Miklós Sárdy, MD, D.Sc.

Budapest
2024

Table of Contents

List of Abbreviations

ACD – Allergic Contact Dermatitis

APC – Antigen-Presenting Cell

BglII – Type II Restriction Endonuclease of *Bacillus globigii*

CCBE1 – Collagen and Calcium Binding EGF Domains 1

CCL21 – Chemokine Ligand 21

CCR7 – Chemokine Receptor 7

CHS – Contact Hypersensitivity

CLEC-2 – C-type Lectin-like Receptor 2

DAMP – Damage-associated Molecular Pattern

DAPI – 4’,6-Diamidino-2-phenylidole

DC – Dendritic Cell

DNFB – 2,4-dinitro-1-fluorobenzene

DT – Diphtheria Toxin

DTR – Diphtheria Toxin Receptor

E10.5 – Embryonic Day 10.5

ER – Estrogen Receptor

FOXC2 – Forkhead Box Protein C2

HE – Hematoxylin and Eosin

HEV – High Endothelial Venule

GFP – Green Fluorescent Protein

IC – Immune Complex

ICAM-1 – Intercellular Adhesion Molecule 1

IFN-γ – Interferon-γ

IL-1β – Interleukin-1β

ITAM – Immunoreceptor Tyrosine-based Activation Motif

KO – Knock Out

LC – Langerhans Cell

LEC – Lymphatic Endothelial Cell

LN – Lymph Node

LYVE-1 – Lymphatic Vessel Hyaluronan Receptor-1

MHC – Major Histocompatibility Complex

PAMP – Pathogen-associated Molecular Pattern

PBS – Phosphate Buffered Saline

PDPN – Podoplanin

PFA – Paraformaldehyde

PGE2 – Prostaglandin E2

PLCγ2 – Phospholipase Cγ2

PROX1 – Prospero Homeobox Protein 1

RA – Rheumatoid Arthritis

ROS – Reactive Oxygen Species

RT – Room Temperature

S1P – Spingosine-1-Phosphate

SYK – Spleen Associated Tyrosine Kinase

SPL76 – Lymphocyte Cytosolic Protein 2

SOX18 – Sex Determining Region of Chromosome Y-box transcription factor 18

Th Cell – T helper Cell

Tg – Transgenic

TLR – Toll-like Receptor

TNCB – 2,4,6-trinitrochlorobenzene

TNF-α – Tumor Necrosis Factor-α

Treg Cells – Regulatory T Cells

VCAM-1 – Vascular Cell Adhesion Molecule 1

VEGF-C/D – Vascular Endothelial Growth Factor C/D

VEGFR3 – Vascular Endothelial Growth Factor Receptor 3

1. Introduction

In recent decades, the understanding of the lymphatic system's role has expanded beyond maintaining interstitial fluid homeostasis to include significant involvement in various conditions. This thesis provides an overview of the lymphatic system and its connection to inflammatory diseases. The research focuses on the interaction between the lymphatic structure and neutrophils in different inflammatory contexts, specifically examining allergic contact dermatitis and rheumatoid arthritis using mouse models. By investigating these interactions, this study aims to enhance understanding of the lymphatic system's impact on inflammatory processes, potentially offering new therapeutic approaches for these conditions.

1.1. Lymphatic system: Structure, function and disorders

1.1.1. Historical background

The human vascular system consists of two connected, but functionally separated networks: the blood and the lymphatic vasculature. While the blood vascular system has been intensively investigated for a long time, the lymphatic system has received less medical and scientific attention until recent years (1, 2).

Even though the lymphatic system has been known for a long time, we still have a partial knowledge about it. Hippocrates was one of the first people in the 5th century BC, who mentioned the lymphatic system. He has already described the lymph nodes as spongy structures which contained oily fluid. Later on, the lymphatic system was reported several times by ancient anatomists without knowledge of its function (3). In the 17th century, there was a feud about describing the lymphatic vessels as first between the Swedish medical student, Olaus Rudbeck and the Danish Professor, Thomas Bartholin which caused the immunology's first priority dispute. The Swede discovered transparent vessels with valves containing clear fluid in the liver and established they emptied into the thoracic duct. Similar findings were published by Bartholin, who described these vessels everywhere in the body, not only in the liver. He named these vessels "lymphatic vessels". Rudbeck charged Bartholin with plagiarism and antedating his discoveries (4).

In the last few decades, due to the modern genetic, molecular, and cellular approaches, our understanding of the lymphatic system's function and clinical importance in both

physiological and pathophysiological conditions have progressed. The lymphatic vasculature, which is a characteristic feature of higher vertebrates, plays a really important role not only in the maintenance of fluid balance, but in the pathogenesis of several diseases, such as lymphedema, cancer or inflammatory conditions (5).

1.1.2. Development of the mammalian lymphatic vascular tree

The lymphatic endothelial cells (LECs) have been already differentiated in the embryonic age distinct from blood vascular endothelial cells. The development of the lymphatic vasculature starts in the early embryonic age: in case of humans at about weeks 6-7 during pregnancy, while in mice at embryonic day 9.5-10.5 (E9.5-10.5) (6). At the beginning of the lymphatic vessel development, the lateral subpopulation of cardinal vein endothelial cells expresses lymphatic vessel hyaluronan receptor-1 (LYVE-1) at E9.0 in mice, which is one of the most specific lymphatic endothelial markers. These LYVE-1-positive LECs induce the expression of the homeobox transcription factor Sex Determining Region of Chromosome Y-box transcription factor 18 (SOX18), which can bind to the SOX18-binding site of Prospero homeobox protein 1 (PROX1) promoter, indicating that SOX18 is required for the initiation of the LEC differentiation program. The transcription factor PROX1 expression at E9.5 in the presence of vascular endothelial growth factor C (VEGF-C) initiates the determination, proliferation, and migration of LECs to form a primordial lymphatic vascular structure – so-called primary lymph sacs (Figure 1) (7-9). It affects the upregulation of specific lymphatic endothelial markers, while the blood vessel-specific endothelial markers are downregulated (8, 10-12). In PROX1-deficient mice the development of blood vessels is not affected, while the lymphatic vasculature does not form (7, 8).

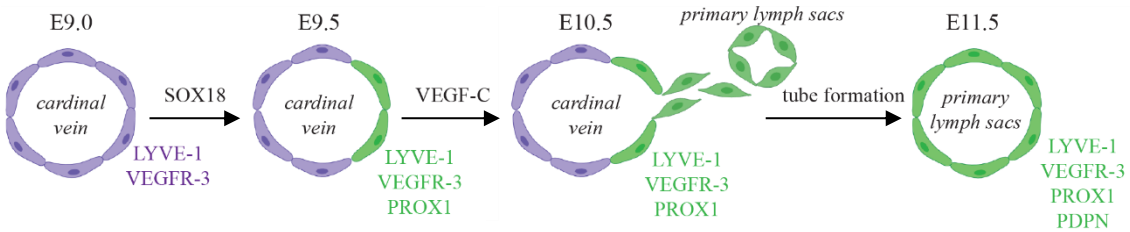

Figure 1. Development of the murine lymphatic vasculature in embryos

Lymphatic precursors begin to express SOX18 transcription factor, which induces PROX1 expression helping to maintain the LEC phenotype. The mesenchymal source of VEGF-C facilitates the sprouting of LEC and the formation of primary lymph sacs.

VEGF-C signals, arising from the lateral mesenchyme, are required for the sprouting of the LECs and can activate the receptor tyrosine kinase vascular endothelial growth factor receptor 3 (VEGFR3). During the early stages of development, VEGFR3 represented in all endothelia helps to develop the cardiovascular system independently from the VEGF-C (13, 14). Later, VEGFR3 expression remains high only in the LEC precursors but is downregulated in the blood vessels at the E10.5 time point. (15). This signaling pathway is crucial for the survival, maintenance, and migration of LECs, furthermore, it is necessary to regulate PROX1 by establishing a feedback loop to maintain the identity of LEC progenitors (16). Besides VEGF-C, VEGF-D can also bind to the VEGFR3 (17). However, the lack of the VEGF-D only resulted in the partial absence of lymphatic vessels suggesting that VEGF-D does not play a crucial role in the lymphatic development (18).

At E11.5, the small transmembrane glycoprotein podoplanin (PDPN) is expressed on LECs, which triggers platelet aggregation via C-type lectin-like receptor 2 (CLEC-2) to block the entry of blood into the lymphatic vessels causing the separation of the lymphatic vessels from the blood circulation (19). This so-called lymphovenous clot occurs between the *vena cardinalis* and the primordial lymph sacs at E12.5-13.5 (20-22). The inhibition of platelet aggregation was described in podoplanin knockout embryos leading to blood-filled, diluted lymphatic vessels (20). The same phenotype was observed in CLEC-2-, spleen associated tyrosine kinase (SYK)-, lymphocyte cytosolic protein 2 (SLP76)-, and phospholipase Cγ2 (PLCγ2)-deficient mice as well (21-24), which suggest the activation of platelets is triggered via the CLEC-2 – SYK – SLP76 – PLCγ2 signaling pathway (25, 26). Furthermore, it has been revealed in *Slp76*-null mice that LECs exposed to elevated shear stress rapidly lost expression of PROX1, resulting in lymphatic endothelial cells reprogramming, which means that blood flow can reprogram lymphatic vessels to blood vessels (27). Periodically in normal conditions, the lymphovenous clots are resolved and rebuilt enabling lymph flow to the venous system and blocking the retrograde blood flow (28). The peripheral lymphatic vasculature is generated by centrifugal sprouting of PDPN-, LYVE-1-, and VEGFR3-positive lymphatic vessels from the lymph sacs, driven by the VEGF-C/VEGFR3 signaling pathway (19, 29).

In the final steps of lymphatic vessel development around E14.5, the differentiation to lymphatic capillaries and collecting lymphatic vessels occurs. Forkhead box protein

C2 (FOXC2) transcription factor plays an essential role in the establishment of collecting lymphatic vessels and the morphogenesis of lymphatic valves cooperating with VEGFR3. FOXC2-deficient mice show agenesis of lymphatic valves in the collecting vessels and abnormal lymphatic vascular patterning causing lymphatic dysfunction (30, 31).

1.1.3. Development of lymphatic vessels in different organs

The most accepted theory about the development of lymphatics is that the lymph sacs originate from the embryonic veins and then the vessels grow by proliferation and centrifugal sprouting towards the surrounding tissue (32). An alternative model of lymphatic development suggests that lymph sacs arise in the mesenchyme via distinct progenitor cells, independently of veins (33). The organ-specific lymphatic structure shows heterogeneity not only in its origin but also in its development and maturation program. The developmental and maturation processes typically occur during the embryonic age; however, several studies support that organ-specific lymphatic development and maturation can be formed partially or fully even after birth.

A dual origin of lymphatic progenitors has been revealed in the developing heart of the embryonic mouse model, furthermore, the fully developed lymphatic structure in the heart is observable two weeks after birth (33). A large part of the superficial dermal lymphatic vessels does not form via transdifferentiation of venous endothelial cells, instead, it develops from non-venous progenitors through a lymphovasculogenesis process involving the recruitment of LECs into clusters around embryonic age 12.5 (34). The first $PROX1^+$ lymphatic capillaries are observed in the intestinal tube around E17.5 originating from the mesenteric lymphatic vessels through an active branching process (35).

Interestingly, it is important to mention that aging can alter the lymphatic structure. Under physiological conditions, dermal lymphatics contribute to fluid filtration and the immune response against the harmful agents. However, the function of the lymphatic collector is impaired with age due to the loss in the cell-to-cell gap junctions and reduced crosslinking between hyaluronan and extracellular matrix causing hyperpermeable vessels. Furthermore, decreased contractile pressure and reduced drainage are related to the smaller and fewer lymphatic vessels. Therefore, it seems that dermal lymphatic vessel vasculature is essential to maintain the skin homeostasis (36, 37). Moreover, it is also

shown nowadays that collagen and calcium binding EGF domains 1 (CCBE1)-dependent mechanisms proved to be crucial in preventing the age-related regression of meningeal lymphatics, possibly through its involvement with VEGF-C (38).

1.1.4. Anatomy of the lymphatic vascular system

The lymphatic system is composed of blind-ended capillaries' network being responsible for collecting the extravasated tissue fluid, macromolecules, and cells from the surrounding tissues, which are collectively called lymph. Lymphatic capillaries are thin-walled vessels composed of partially overlapping, single-layer, oak-leaf-shaped LECs, not supported by smooth muscle cells or pericytes and covered by interrupted basement membrane (6, 39). The diameter of the lymphatic capillaries is more variable (10-60 μm) compared to the blood capillaries, and often appear collapsed (40). Lymphatic capillaries have discontinuous junctions (button-like) making them highly permeable. Therefore, they work as leukocyte entry sites and help with the uptake of lymph components (41). Furthermore, the LECs are linked to the surrounding extracellular matrix by anchoring filaments attaching to the collagen fibers via emilin-1 and fibrillin. When the interstitial fluid pressure increases, taut collagen fibers help to open the gaps between the LECs and increase the uptake of tissue fluid (42, 43).

The lymph moves to a pre-collector lymphatic vessel, which is characterized by the presence of smooth muscle cells, discontinuous basement membrane and containing intraluminal valves preventing the backflow of lymph. In contrast to the lymphatic capillaries, LECs of pre-collector vessels are connected to each other in a more continuous, 'zipper-like' junction inhibiting the lymph uptake (41). The one-way lymph flow is secured towards the venous system by the intrinsic contractility of smooth muscle cells as well as the contraction of surrounding skeletal muscles and arterial pulsations (39, 44). Afferent collecting vessels go through lymph nodes (LNs), bringing foreign antigens presented by antigen-presenting cells (APCs) (6). Following that, these precollector vessels link to a larger collecting vessel and finally it returns to the blood circulation through the *ductus thoracicus* and *ductus lymphaticus dexter* via connection with the subclavian veins (Figure 2) (45, 46).

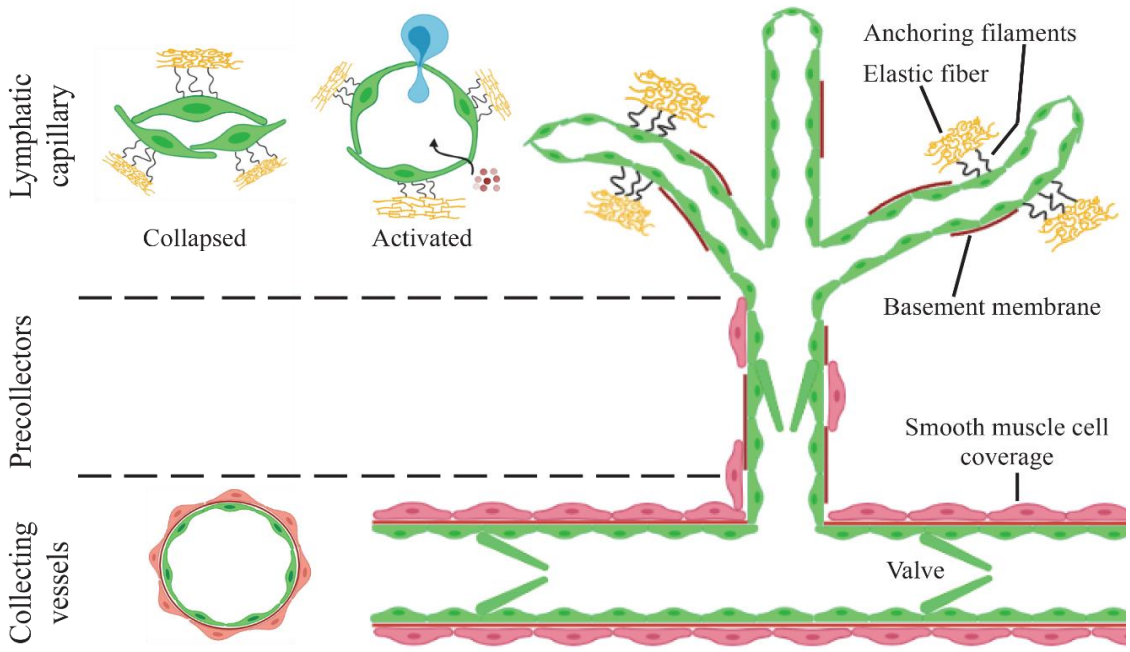

Figure 2. Schematic figure of the lymphatic vascular tree

Lymphatic capillaries are blind-ended vessels partially covered by basement membrane. LECs are linked to the extracellular matrix by anchoring filaments. The interstitial fluid with molecules or immune cells enters the capillary through paracellular and transcellular routes due to the button-like junctions. Precollectors have partial smooth muscle cell coverage, but still incomplete basement membrane, which converge into collecting lymphatics having complete smooth muscle cell layer and basement membrane. Lymphatic valves are distributed to prevent backflow.

1.1.5. Lymph nodes

The hundreds of lymph nodes are located sporadically in the body – firstly in the mucosa – filtering the interstitial fluid. These encapsulated bean-shaped lymphoid organs play a highly important role in immune cell trafficking, in which the naive lymphocytes can encounter antigens presented by APCs, such as dendritic cells (DCs), leading to tolerance or activation. Immune cells can enter lymph nodes via afferent lymphatic vessels connected to subcapsular sinus or high endothelial venules (HEVs) and egress through efferent lymphatic vessels and medullary or cortical sinuses. HEVs are specialized postcapillary venules found only in the secondary lymphoid organs, where the naive lymphocytes enter these organs from the blood (47). Histologically, LNs can be divided into two main regions: the cortex and the medulla. Furthermore, the cortex also has two components: the germinal center, otherwise known as the B cell area consisting of primary follicles (in the resting state) and the paracortex, predominantly populated by T cells. Circulating lymphocytes enter the LNs and APCs present the antigens to T cells in the paracortex, while the germinal center is the main site of the humoral responses.

Follicular dendritic cells also reside within the B cell follicles, presenting intact antigens to B cells. After an immune response, the effector cells produce cytokines and express homing molecules, which help them to migrate toward the source of their antigen in the peripheral tissues. Lymph flows through the cortex via trabecular sinuses reaching the medullary sinuses. The medullary region is separated by medullary cords, which contain memory T cells, plasma cells, macrophages; however, their function is less known (48).

Taken together, the lymph nodes have several crucial functions to maintain the normal immune function: to collect antigens from the peripheral tissues presented by APCs; to recruit naive lymphocytes from the blood; to provide the environment for antigen-specific tolerance or create effector responses and to modulate the homing process of effector or memory T cells. These show the essential role of the innate and adaptive cellular components of immune cells.

1.1.6. Function of the lymphatic vascular system

The lymphatic system is a highly structured vascular network, and one of its most important roles is to maintain the interstitial fluid homeostasis. The hydrostatic pressure is the driving force for fluid filtration from plasma to the surrounding tissues, and the osmotic gradient is facilitated by macromolecules. Although part of the capillary filtrate can be absorbed by venules, the lymphatic system is mainly required to uptake and return the lymph to the bloodstream. Due to the unique LEC button-like junction structure, fluid, proteins, macromolecules, and certain immune cells enter blind-ended lymphatic capillaries via passive paracellular absorption. The lymphatic system returns approximately 1-2 liters of lymph with 20-30 g protein per liter to the venous circulation daily in a healthy adult person (49, 50).

Another crucial role of the lymphatic vasculature is to traffic the leukocytes and the soluble antigens from the peripheral tissues through the lymph nodes, as described above. Depending on the features of the antigens, it can lead to the activation of the adaptive immune response or tolerance (51).

It is well known that the lymphatic vessels inside the intestinal villi absorb and transport the lipids as chylomicrons. In the last decades, it has been also revealed that the lymph contains a lot of cholesterol and high density lipoprotein (52), demonstrating that lymphatic drainage is also required for reverse cholesterol transport from the peripheral

tissues to the liver, which maintains cellular homeostasis and protects against atherosclerosis (53, 54).

Furthermore, it was also investigated in rodent models that skin microenvironment is hypertonic compared to the plasma, in which mononuclear phagocytes can regulate the blood pressure by local organization of interstitial electrolyte clearance (55, 56).

Lymphatic vessels play a highly important role in the embryonic lung development as well (57). Before birth, lymphatic function is required to increase lung compliance in an independent way from surfactant and change lung mechanics in preparation for infiltration at birth. In animal experiments, the lack of lymphatics causes respiratory failure in pups after birth leading to death (58).

The above data shows the numerous new functions of the lymphatic vasculature that have been discovered in the last few years, which contributed to the understanding of their physiological and pathophysiological role.

1.1.7. Lymphatic insufficiency

In the last few years, the lymphatic system has been associated with several pathological conditions, such as metabolic diseases (59), tumor metastasis (60), cardiovascular diseases (54) and chronic inflammation (61). Impairment of lymphatic transport due to abnormal vessel development or injury of lymphatics leads to the accumulation of interstitial fluid, resulting in swelling and dysfunction of limbs. If the protein-rich lymph stays in the peripheral tissue for a longer period, it can also activate inflammatory responses, causing fibrosis and accumulation of subcutaneous fat. Based on the etiology, we can distinguish primary and secondary lymphedemas (62, 63).

Primary lymphedema is a result of a heritable failure of lymphatic vessels development. Several pathogenic variants of genes, especially in the VEGF-C – VEGFR3 signaling pathway, have been shown to cause primary lymphedema (64). The *flt4* gene was first described, encodes VEGFR3, its pathogenic variants were identified to cause hereditary lymphedema. A heterozygous single nucleotide polymorphism in the tyrosine kinase domain of the receptor leads to the inhibition of VEGFR3 signaling, causing the so-called Milroy disease. It is characterized by the painless but chronic leg edema already present at birth (65). In 2001, a new mouse model with an inactivating *Vegfr3* mutation

in their germ line was introduced, having similar symptoms as the human lymphedema patients, caused by the lack of subcutaneous lymphatic vessels (66).

Secondary lymphedema is a slow but progressive condition, which develops due to infections, surgery, or inflammation. Globally, the most common form of secondary lymphedema is nematode infection (filariasis) with over 140 million affected people. The adult *Wuchereria bancrofti* gets into the lymphatic system of humans, transmitted through mosquitoes, obstructing lymphatic vessels and blocking lymph transport (51). Another main cause of secondary lymphedema can be the surgical removal and irradiation of breast-associated axillary LNs dissection in 6-30% of patients. Furthermore, lymphatic dysfunction has been well described after the treatment of a variety of cancers, such as melanoma, urological, head or neck tumor. These therapies can damage the lymphatic vessels causing dermal fibrosis, inflammation and dysregulated adipogenesis (64, 67).

1.2. Overview of the inflammatory response

Traditionally, homeostasis and inflammation are depicted as contrasting states within biological systems, often linked with health and disease, respectively. Inflammation serves as a protective reaction that engages immune cells, blood vessels, and molecular messengers. Its primary role is to eliminate the initial source of cellular damage, remove impaired cells and tissues, and kickstart the process of tissue restoration. Inflammation plays a pivotal role in numerous physiological and pathological processes. While the pathological aspects of various types of inflammation are well understood, their physiological functions remain largely obscure (68, 69).

Unlike typical inflammatory triggers such as infection and injury, systemic chronic inflammation seems to be linked to tissue malfunction rather than direct host defense or tissue repair. The initial response of the inflammatory process is the local release of inflammatory exudate, containing plasma proteins and mainly neutrophils, from blood vessels to the affected tissues. This migration is facilitated by interactions between endothelial cells and leukocytes, driven by selectins, integrins, and chemokine receptors (70).

Once at the site of infection or injury, neutrophils become activated, aiming to eliminate pathogens by releasing granule contents, including reactive oxygen and nitrogen species. However, this process can inadvertently harm host tissues. A successful

acute inflammatory response culminates in pathogen elimination, followed by a resolution and repair phase, mediated mainly by tissue-resident and recruited macrophages. Transitioning from inflammation to resolution relies on a shift in lipid mediators from pro-inflammatory prostaglandins to anti-inflammatory lipoxins, which aid in monocyte recruitment for tissue repair (71).

Failure to eliminate pathogens leads to a persistent inflammatory state, characterized by a shift from neutrophil to macrophage dominance. Prolonged inflammation, known as chronic inflammation, may result in granuloma formation and tertiary lymphoid tissues, influenced by the effector class of T cells present. Besides persistent pathogens, chronic inflammation can arise from autoimmune responses or undegradable foreign bodies, leading to granuloma formation as a protective mechanism (72, 73).

1.2.1. Immune response in inflamed skin

The skin is the largest organ, that separates our body from the outer environment and is continuously exposed to external pathogens. It serves not only as a physical barrier to protect the host from physical and chemical attacks but also as an immunological barrier composed of various immune cells maintaining the skin homeostasis upon inflammatory challenges (74).

One of the most crucial functions of the lymphatic vessels is facilitating immune cell migration and antigen transport from the peripheral tissue to the lymph nodes. This migration plays an important role in immune surveillance, and activates the adaptive immune responses or initiation of tolerance (75). The lymphatic network often changes in inflammatory conditions; for example, the lymphatic vessels are enlarged, extremely leaky and tortuous, indicating reduced drainage capacity (76, 77). Besides the increase of lymphatic vessel permeability, the number of immune cells - mostly T cells or DCs - in the afferent lymph is elevated as well (78).

1.2.1.1. Lymphatic migration of dendritic cells

In the skin, the dendritic cells (DCs) are one of the most important professional antigen-presenting cells besides the keratinocytes and macrophages, and they are key players in bridging the innate and adaptive immune systems. Cutaneous DCs are distinguished into two major subtypes: Langerhans cells (LCs) in the epidermis and

dermal DCs. During a tissue steady state, DCs constantly migrate to the lymph nodes carrying self-antigens to maintain peripheral tolerance by facilitating self-reactive T cell anergy or clonal depletion. Upon sensing inflammatory stimuli, the foreign antigens are captured by pattern recognition receptors of DCs, which initiates an early immune response in the skin. Then, antigen-carrying DCs enter lymphatic vessels and migrate to skin-draining lymph nodes, where they present antigens to $CD4^+$ T cells, thereby inducing the adaptive immunity (79, 80).

The chemokine receptor 7 (CCR7) is the most important regulator of DCs, which is required for directed migration toward lymphatic vessels from the skin (78, 81). Interestingly, in CCR7-deficient mice, the lymphatic permeability is also reduced, indicating bidirectional communication between immune cells and the lymphatic network (82). The ligand of CCR7, chemokine ligand 21 (CCL21), is expressed by lymphatic endothelial cells both under homeostatic and inflammatory conditions binding to glycoproteins like podoplanin. DCs enter the lymphatic vessels at the site of the highest chemokine gradient, indicating that CCL21 directly regulates the entry into the lymphatic networks (83, 84). A recent study demonstrated that DCs dock to the basolateral surface of lymphatic vessels and transmigrate across LECs through hyaluronan-mediated interactions with LYVE-1 (85). Lymphatic vessels are crucial for DC migration to the lymph nodes, because a lack of lymphatic drainage leads to a deficiency in immune response or tolerance induction (86). Upon inflammation, keratinocytes produce various cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), which promotes DC migration into LNs as well. These pro-inflammatory cytokines are also responsible for neutrophil recruitment from the circulation to sites of tissue damage or pathogen entry (87).

1.2.1.2. Antigen presentation in the lymph node

After the entry into the lymphatic network, DCs with the captured antigens migrate to the lymph node subcapsular sinus via afferent collecting lymphatic vessels. First, the DCs cross the LEC layer to reach the T cell zone, which depends on CCR7-CCL21 chemotaxis as well (88). DCs can directly interact with the naive $CD4^+$ T helper (Th) cells, presenting the antigens on major histocompatibility complex (MHC) in the primer follicle, which then activates, and changes them to effector T cells (89). Furthermore, due

to the proper cytokine milieu and encounter with antigen-loaded DCs, selected B cells undergo clonal expansion, proliferation and differentiation to long-lived memory B cells, leading to the development of the second follicle. After that, the antigen-specific effector T cells and the memory cells egress from the lymph node via efferent lymphatics regulated by spingosine-1-phosphate (S1P), returning to the blood circulation and they migrate back to the inflammatory site of tissue (80). Immunization causes increased lymph node lymphangiogenesis, leading to improved DC mobilization from the periphery (90).

1.2.1.3. Role of the lymphatic endothelial cells in immune regulation

Nowadays, it is getting clear, that lymphatic endothelial cells play a truly important role in evolving peripheral tolerance, suggesting that they directly participate in immune regulation (91-93). LECs can express immunomodulatory cytokines and MHC class I and class II molecules, which confirm the ability to potentially function as APCs, activating adaptive immune responses through their interaction with key immune cells, such as DCs, macrophages, and lymphocytes. By presenting peripheral self-antigens released from tissue homeostatic turnover, it can induce $CD4^+$ T cell anergy and clonal depletion of $CD8^+$ T cells (94). Bidirectional crosstalk between LEC and T cells is confirmed by a regulatory function of $CD4^+$ T cells on LEC growth. During inflammatory conditions, interferon-γ (IFN-γ) secreted by T lymphocytes can negatively regulate lymph node lymphangiogenesis (95, 96). Interestingly, it has also been revealed that antigen-bearing DCs and antigen-specific T cells can already interact within the lymphatic capillaries, where adaptive immune interaction and modulation occur (97).

Recently, novel data have been reported on the role of macrophages in lymphangiogenesis. Besides providing VEGF-C, they can also transdifferentiate into lymphatic endothelial cells under inflammatory conditions, forming cell aggregates that are integrated into existing lymphatic vessels. (98, 99).

1.2.1.4. Inflammatory mediators

Inflammation is associated with increased lymphangiogenesis, induced by the activation of macrophages and granulocytes, and as a response to proinflammatory cytokines such as TNF-α (5, 100). TNF-α can induce VEGF-C expression, produced by

leukocytes and LECs, promoting the proliferation and migration of lymphatic endothelial cells (101). Macrophages are a well-known source of VEGF-C inducing local sprouting of pre-existing LECs and their role is crucial in inflamed tissue (102). In addition, IL-1β can upregulate the VEGF-C transcription through NF-κB-mediated promoter activation as well, thereby indirectly stimulate lymphatic vessel growth and function (103). The effective drainage is influenced by pumping activity and vascular permeability of lymphatic vessels in the inflamed tissue. IL-1β, TNF-α, VEGF-C – VEGFR3 axis can trigger an increased lymphatic vessel permeability (104-106). Interestingly, while VEGF-C can enhance lymphatic contractions, nitric oxide, histamine and prostaglandin are responsible for negative regulation of the pumping function (107-109). Inflammatory mediators, like TNF-α, VEGF-A, IL-1β, and IL-6, also increase the expression of adhesion molecules in endothelial cells, such as E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1), promoting the interaction with immune cells (110). Lymphangiogenesis in inflammatory conditions contributes to the absorption of accumulated interstitial fluid and increases the immune responses by promoting DC and macrophage mobilization (111, 112).

1.2.2. Neutrophils at the crossroads of innate and adaptive immunity

Circulating neutrophils form an essential part of innate immunity. Normally, they are found in the bloodstream but upon an inflammatory stimulus, they are one of the first responders, who can be rapidly recruited to the site of inflammatory tissue (113). Toll-like receptors (TLRs) are expressed on the cell surface of the granulocytes recognizing the pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) (114). Another important receptor is the Fc receptor γ-chain (FcRγ), mediating the internalization of IgG-opsonized pathogens and antigen-antibody immune complexes (IC), which can lead to the autoimmunity (115).

Over the past decade, it has become increasingly clear that neutrophils are highly complex cells, possessing not only effector functions in the innate immune response but also the ability to modulate the adaptive immune response. This modulation occurs through direct interaction with other immune cells or by secreting cytokines that impact dendritic cells and lymphocytes (116). Furthermore, although lymphocyte recirculation is a well-known process for exploring pathogens, neutrophils seem to circulate as well,

entering and egressing lymph nodes via HEVs (117). This movement is mediated by CCR7 and its ligand CCL21, just like in DCs. Studies on CCR7-deficient mice revealed reduced migration of neutrophils into tissue-associated lymphatic vessels and draining lymph nodes compared to wild-type mice (118). Moreover, TNF-α was identified as crucial for facilitating neutrophil migration into lymphatic vessels during inflammatory conditions (119). Furthermore, it has been shown that neutrophils can present antigens to $CD4^+$ and $CD8^+$ T cells as well (120, 121). After IC stimulation, neutrophils showed high MCH-II expression and upregulation of costimulatory molecules, helping to activate antigen specific $CD4^+$ T cells which stimulate the antigen-specific proliferation of both naive and memory T cells (122). Therefore, investigating the detailed molecular mechanisms of neutrophils can be essential to better understand their involvement in inflammatory conditions.

1.3. Allergic contact dermatitis

Besides infection or injury, many chronic diseases result from imbalances in the immune system's interaction with environmental factors. Inducers of inflammation can be exogenous or endogenous. Non-microbial exogenous inducers of inflammation can include allergens, irritants, foreign substances, and toxic compounds (69). In the last few years, several studies have been published investigating the connection between lymphatic vasculature and inflammatory diseases. It is known that lymphatic vessels are often aberrant in inflamed skin, but how the lymphatics exactly influence the pathomechanism of inflammatory skin diseases, like contact dermatitis, has remained unclear.

Contact dermatitis is a widespread, inflammatory eczematous skin disease. Two main types of contact dermatitis can be distinguished according to the pathological mechanisms: irritant contact dermatitis is a non-specific response of the skin to direct chemical damage, while allergic contact dermatitis activates the antigen-specific immunity. Allergic contact dermatitis (ACD) is a common inflammatory skin disorder, a T cell-mediated immune reaction caused by repeated skin exposure to contact allergens. It is classified as a delayed-type hypersensitivity response, which is characterized by redness, the presence of papules and vesicles, followed by itchy and dry skin (123, 124).

Due to one of the most frequently used animal models for this condition, the murine contact hypersensitivity (CHS) model, our knowledge about the immunological mechanisms of ACD has increased significantly in the past decade.

1.3.1. Epidemiology

ACD is one of the most common inflammatory skin diseases, demonstrating prevalence rates of 15-20% of the general population worldwide (125). Genetic predisposition and environmental exposures contribute to higher risk of developing ACD, for example, it is more common in women due to jewels or hair dye use (126, 127). Furthermore, another endangered group is the building or metal workers, healthcare workers, cleaners who have close and repeated contact with common allergens, such as nickel or triphenyl phosphite in polyvinyl chloride gloves (128-130).

1.3.2. Pathophysiology

Two immunologically distinct steps are required to develop the clinical features of ACD. In the sensitization phase, naive antigen-specific T lymphocytes mature and differentiate into memory or effector T cells upon the first antigen application. In the elicitation phase, re-exposure of the same antigen leads to the recruitment of allergen-specific T lymphocytes in the inflammatory site of the tissue, triggering clinically visible tissue inflammation characterized by edema, erythema and blistering (131).

1.3.2.1. The sensitization phase

The skin's barrier function plays a crucial role in preventing the entry of allergens into the body. The stratum corneum, the uppermost layer of the epidermis and its tight junctions can block antigen penetration into the skin (132). Most chemicals and drugs are defined as low-molecular weight antigens, which typically have a molecular mass of <500 Da (haptens). They are not immunogenic by themselves, and need to be bound to larger protein carriers (like albumin) in the skin tissue, eliciting adaptive immune responses (133). Most haptens have lipophilic features, which facilitate crossing through the skin's corneal barrier (123). In experimental conditions, strong contact sensitizers, such as 2,4-dinitro-1-fluorobenzene (DNFB), 2-chloro-1,3,5-trinitrobenzene (TNCB) or oxazolone are used to obtain CHS, which are not present in our daily environment.

Upon hapten engulfing, keratinocytes are activated through pattern recognition receptors, like membrane-associated TLRs or NOD-like receptors and produce various proinflammatory cytokines, such as TNF-α, IL-1β and prostaglandin E2 (PGE2) via the activation of the NF-κB cascade, thereby facilitating the migration and maturation of DCs. Certain allergens can indirectly activate TLRs via the degradation of hyaluronic acid, and the resulting DAMPs (134, 135). The activation of TLRs induces the production of reactive oxygen species (ROS), contributing to further skin inflammation (136). MCH class I and II molecules are expressed at the dendritic cell surface to present the antigens (137, 138). Allergen-bearing DCs migrate to regional lymph nodes of the skin, where they prime allergen-specific $CD4^+$ and $CD8^+$ T cells in the para-cortical area, which then proliferate and circulate back to the blood (Figure 3).

Other essential immune cells required to initiate the immune response are the mast cells and neutrophils. Mast cells are colonized in a large number in the dermis and are crucial for stimulating DCs via ICAM-1 and the recruitment of neutrophils (132, 139). Allergen can also enhance vascular permeability via mast cell-derived histamine pathway, thereby increasing neutrophil infiltration to the inflammatory site (140). Upon activation of neutrophils, metalloproteinase and granzyme B are secreted contributing to the degradation of the extra cellular matrix and attraction of macrophages to the inflammatory tissue (141, 142).

The sensitization phase takes 10-15 days in humans and 5-7 days in mice. This first step has no clinical consequences in the majority of cases, but being recognized by the local innate immunity is essential for further development of the disease (123).

1.3.2.2. The elicitation phase

Upon the second antigen exposure, allergen-specific effector and memory T cells are activated rapidly, evolving within 24 to 48 hours, which often leads to immune-mediated tissue injury, like redness, rash and skin lesions (143). CHS is primarily a $CD8^+$ T cell-driven response demonstrated by monoclonal antibody-dependent depletion of $CD4^+$ or $CD8^+$ T cells *in vivo*. The depletion of $CD8^+$ cells showed a considerable decrease in inflammation, while the elimination of $CD4^+$ cells resulted in a strong enhancement of immune reaction, suggesting that $CD8^+$ T lymphocytes have effector functions, whereas $CD4^+$ T cells have regulatory roles (144). The inflammatory effects of $CD4^+$ and $CD8^+$ T

cells are largely dependent on different cytokines, such as IL-1α, IL-6, IL-17, TNF-α, TGF-β, and IFN-γ secreted by keratinocytes, APCs, and lymphocytes. Cytokines produced by activated T cells can stimulate skin-resident cells, which help the further recruitment of T cells, amplifying the immune response in the inflamed tissue (Figure 3) (145).

Besides the antigen-specific inflammation, keratinocytes, mast cells and neutrophils play a really important role in the initiation of the elicitation phase as well creating antigen-nonspecific inflammation (Figure 3) (132, 142).

After the re-exposure of the antigen, keratinocytes produce IL-1α, which activates the M2 macrophages. The CXCL2 production from M2 macrophages is crucial to develop the perivascular formation of DC clusters which can help to activate the memory T cells. The antigen is recognized in the DC clusters by antigen-specific memory T cells, leading to the development of the clinical symptoms of the disease (146).

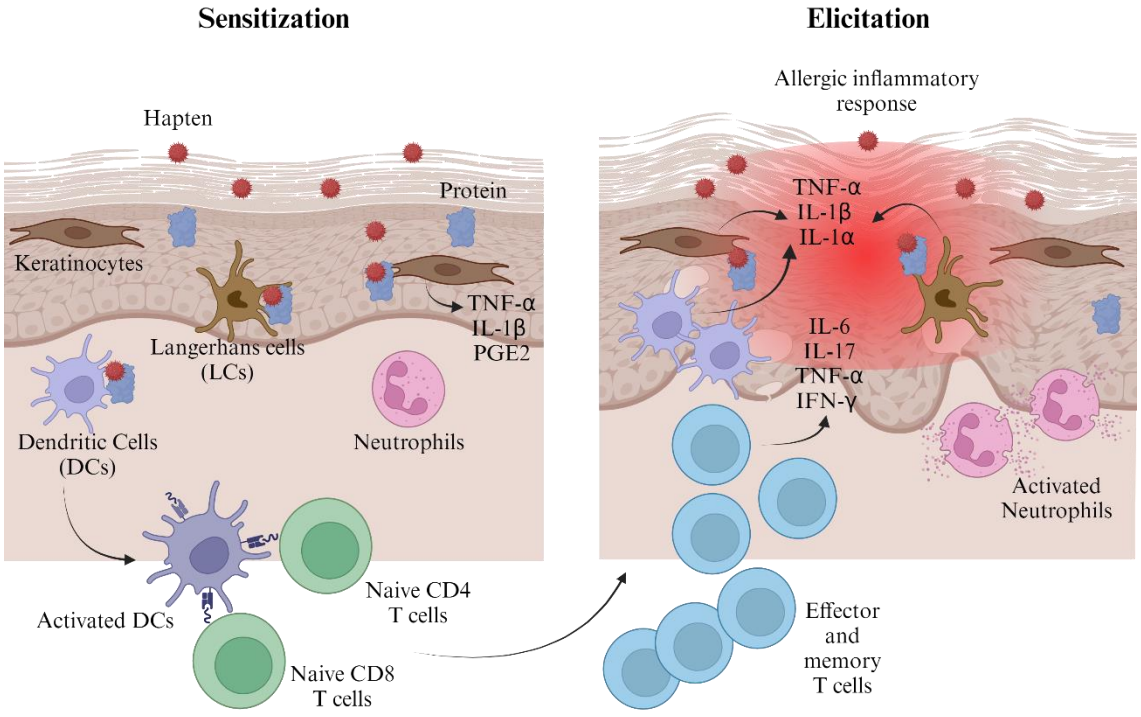

Figure 3. Pathophysiology of allergic contact dermatitis

In the sensitization phase, the dendritic cells become activated followed by encountering a haptenized peptide. Exiting the skin, they mature fully, enabling them to effectively present antigens to naive T cells. During the elicitation phase, re-exposure of the skin to the same contact allergen prompts hapten-specific effector T lymphocytes to release inflammatory cytokines, leading to disease-specific local skin injuries.

Despite extensive knowledge on the pathomechanism of contact dermatitis, the exact role of the lymphatic vasculature in this process is still unknown. As mentioned above, some other studies have already been investigating the role of the lymphatic vessels in inflammatory conditions, but none of them studied in a comparable way to characterize the importance of lymphatics separately in the two phases of CHS. By answering these questions, we are getting closer to understanding the pathophysiological mechanism of ACD, providing new therapeutic approaches.

1.3.3. Modulation of the lymphatic vessels in inflammatory skin conditions

Inflammation is part of a complex biological response of the body against pathogens or irritants, characterized by five classical symptoms: rubor (redness), calor (increased heat), tumor (swelling), dolor (pain), and functio laesa (impaired function). These are partially mediated by the activation and expansion of the blood and the lymphatic vessels. As detailed above, the role of the lymphatic vessels is essential in the process of inflammation. First, they maintain tissue fluid homeostasis by draining the increased extravasated fluid from leaky blood vessels. Furthermore, they transport immune cells and inflammatory mediators to the lymph nodes from the inflamed tissue (147). In chronic inflammatory diseases, the lymphatic vasculature remains enlarged, activated, and hyperpermeable, which can trigger the accumulation of immune cells and fluid. Due to the increased interstitial fluid pressure, the overlapping LECs open, and cell- and macromolecule-rich fluid can enter the lymphatic network (110). Additionally, strong proliferation and morphologic changes of the lymphatic vessels can occur under inflammatory conditions, however, the nature of the lymphangiogenesis is highly stimulus- and tissue-specific (148).

In recent years, several studies demonstrated the lymphatic vasculature's dominant role in inflammation. Modulation of the lymphatics contributes to the change in the extent of inflammation in the skin. For example, lymphatic vessel stimulation can alleviate inflammation severity due to increased drainage and reduced immune cell infiltration, indicating a valid therapeutic approach. Promoting the lymphatic vasculature by VEGF-C/VEGFR3 signaling might account for an anti-inflammatory effect, as it is shown to reduce the production of proinflammatory cytokines and edema (147, 149). Previous studies showed that adenovirus-induced VEGF-C expression could decrease the skin

inflammation (150); furthermore, administering recombinant VEGF-C exhibited significantly limited chronic skin inflammation (151). On the other hand, inhibition of the lymphatic vessels leads to exacerbated inflammation. Tissue swelling, epidermal thickening and leukocyte numbers are significantly increased by blocking VEGFR3 in the CHS model, indicating a more severe inflamed skin phenotype (150).

These studies suggest that the VEGF-C – VEGFR3 signaling axis is crucial in reducing local skin inflammation. However, we must mention that utilizing K14 promoter-dependent transgenic overexpression of VEGF-C in all keratinocytes, adenovirus-mediated overexpression of VEGF-C or VEGFR3-Ig, and administration of VEGFR3 blocking antibodies have important limitations. These limitations include the uncontrollable expression of the lymphangiogenic factor VEGF-C or VEGFR3 ligand used for blocking its function, as well as the potential nonspecific effects of the applied blocking antibody. Developing a reliable method to study the lymphatic system is essential as it facilitates the way towards novel, targeted therapeutic approaches.

1.4. Rheumatoid arthritis

In contrast to exogenous inducers of inflammation, the endogenous inducers originate from internal sources, generated by stressed, damaged, or dysfunctional tissues, triggering inflammatory responses, but the nature and features of these signals are not clearly defined. However, they likely belong to different functional categories depending on the type and severity of tissue abnormalities they indicate (69).

One of the most common inflammatory diseases is rheumatoid arthritis (RA), affecting 0.5-1% of the population worldwide (152). This chronic autoimmune joint disease is characterized by leukocyte invasion of the synovial lining and hyperplasia of the resident synoviocytes causing swelling limbs, pain, and irreversible connective tissue damage (153). RA is caused by the dysregulation of the innate and adaptive immune systems. In inflammatory conditions, neutrophils are often disregarded as short-lived, terminally differentiated bystander cells with little relevance. However, neutrophils play an essential role in innate immune defense, although an overactivation and the release of proteases can lead to tissue damage in autoimmune RA (154, 155). As it was mentioned above, Fcγ receptors are essentially involved in neutrophil activation at the site of

inflammation, for example in IC-triggered cellular activation, clearance of immune complexes, or phagocytosis of opsonized particles (156, 157).

Resting human neutrophils express single-chain transmembrane FcγRIIA containing immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tail of the molecule. At the same time, FcγRI appears only upon stimulation associating with ITAM-bearing transmembrane adapter protein called Fc receptor γ-chain (FcRγ). In murine neutrophils, two low affinity (FcγRIII and FcγRVI) and one high affinity (FcγRI) Fcγ receptors are expressed, all of them are associated with ITAM-containing FcRγ (Figure 4) (158, 159).

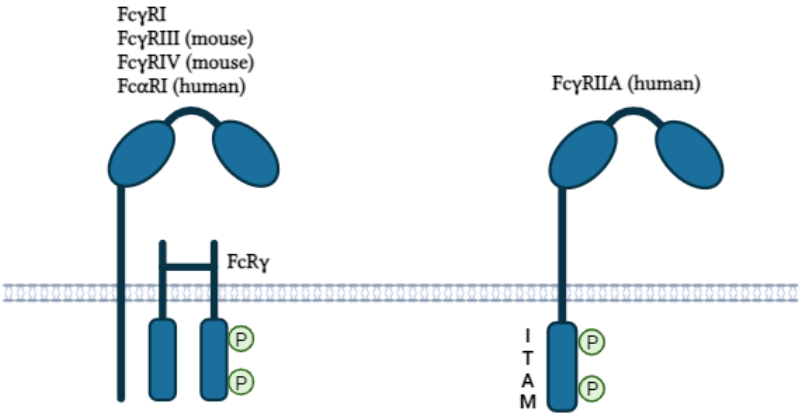

Figure 4. Neutrophil Fc-receptors

Low-affinity activating Fcγ-receptors signal through cytoplasmic ITAM motifs which recruit the Syk tyrosine kinase and activate further signaling. Most ITAM-coupled Fc-receptors (except FcγRIIA) are noncovalently linked to the FcRγ adapter (based on (114)).

It has already been published, that the absence of FcRγ causes inhibition of FcR-dependent neutrophil effector responses and leads to protection from autoimmune arthritis (160, 161). However, it was never investigated whether the obligate function of FcRγ enables the receptor expression, or it is also involved in the signaling process via ITAM tyrosines.

2. Objectives

Inflammation is a crucial part of the body's defense process, by which the immune system recognizes and eliminates the harmful stimuli followed by the healing process. Inflammation involves a coordinated interaction between immune cells, blood and lymphatic vessels, and molecular mediators to restore tissue homeostasis. Based on the previously presented literature, we have broad knowledge about the crucial role of the lymphatic network and neutrophils in the regulation of different inflammatory processes, but their detailed task in tissue injury remains unclear.

Allergic contact dermatitis affects many people worldwide; however, the exact mechanism of the lymphatic vasculature's function is still unknown in the two phases of the disease. During our study, we investigated the acute role of lymphatic vessels separately in the sensitization and elicitation phases of the contact hypersensitivity mouse model. Another very common inflammatory disease with significant tissue damage is rheumatoid arthritis. The importance of FcRγ ITAM tyrosines in neutrophil activation has not been directly tested. In our experiment, we used an autoantibody-induced experimental arthritis mouse model to define the *in vivo* role of FcRγ ITAM tyrosines and to investigate the inflammation in rodent ankles. Understanding the lymphatics- and neutrophil-dependent mechanisms could contribute to new therapeutic targets in the treatment of inflammatory diseases in the future.

We aimed to address the following questions:

1. Finding and characterizing mouse models to investigate the role of the lymphatics separately in the two phases of CHS.
2. Investigating the effect of presence or absence of lymphatic vessels in the sensitization and elicitation phase of CHS model.
3. Specifying the immune cells which contribute to the development of CHS.
4. Identifying the exact role of FcRγ ITAM tyrosines in an inflammatory arthritis mouse model *in vivo*.

3. Material and Methods

3.1. Experimental animals

8–16-week-old wild type and Flt4 kinase-dead point mutant mice on NMRI genetic background were used for the experiments (*Flt4*$^{kd/+}$, also named as *Vegfr3*$^{kd/+}$ (MRC Harwell, UK) (Table 1) (66).

To delete lymphatics locally in the skin, a transgenic inducible lymphatic vessel elimination model, the Flt4-CreER^{T2}, *iDTR*$^{fl/fl}$ strain (on C57Bl/6 background) was used (Table 1) (162-164). The strain was maintained in homozygous form.

To visualize the lymphatic vessels in *Flt4*$^{kd/+}$and the Flt4-CreER^{T2}; *iDTR*$^{fl/+}$ mice, these strains were crossed with the *Prox1*^{GFP} lymphatic reporter mice (165) (maintained on C57Bl/6 background).

FcRγ-deficient (Fcer1g$^{tm1Rav/tm1Rav}$, referred to as γ$^{-/-}$) mice were purchased from Taconic Farms (Hudson, NY, USA) (160). Animals expressing the wild type (WT γ) and the ITAM tyrosine mutant FcRγ (YF γ; where tyrosines at positions 65 and 76 were replaced by phenylalanines) were described previously and were crossed with FcRγ KO mice (referred to as γ$^{-/-}$ WT γ Tg and γ$^{-/-}$ YF γ Tg animals, respectively) (166). To augment the expression of the transgenic wild type and mutant FcRγ chain, the mice were crossed to obtain homozygous, double transgenic animals (referred to as FcRγ KO + 2x WT FcRγ Tg and FcRγ KO + 2x YF FcRγ Tg mice, respectively). Single and double transgenic animals were differentiated by quantitative PCR. Mice carrying the KRN T-cell-receptor transgene were maintained in heterozygous form by mating with C57BL/6 mice (167). All transgenic mice were backcrossed to the C57BL/6 genetic background. Genotyping was performed by allele-specific PCR.

Wild type control C57BL/6 mice were purchased from Charles River (Wilmington, MA) or the Hungarian National Institute of Oncology (Budapest, Hungary). NOD mice, as well as a congenic strain carrying the CD45.1 allele on the C57BL/6 genetic background (B6.SJL-Ptprca) were purchased from the Jackson Laboratory (Bar Harbor, ME).

All animal experiments were approved by the Animal Experimentation Review Board of the Semmelweis University and the Government Office for Pest County (License numbers of ethic votes: PE/EA/1654-7/2018, PE/EA/00658-6/2023 and PE/EA/00659-

6/2023). Experimental animals were housed in either specific pathogen-free or conventional animal facilities between 18−22 °C, 45% humidity, and 12/12 hours dark–light cycles. Food and water were supplied as *ad libitum*.

3.2. Inducible lymphatic vessel elimination mouse model

The Flt4-CreER^{T2}; *iDTR$^{fl/fl}$* transgenic mouse model was used to eliminate the lymphatic vessels locally in a tamoxifen-dependent manner as described before (168). Tamoxifen (Sigma-Aldrich, St. Louis, MO) was dissolved at a concentration of 20 mg/ml in corn oil (Sigma), and 4.5 mg/40 g body weight dose of tamoxifen was injected intraperitoneally to adult Flt4-CreER^{T2}, *iDTR$^{fl/fl}$* and *Prox1^{GFP}*; Flt4-CreER^{T2}; *iDTR$^{fl/+}$* mice once daily for 5 consecutive days. One week after the last tamoxifen treatment 60 ng diphtheria toxin (DT; Sigma-Aldrich, D0564) dissolved in 30µl phosphate buffered saline (PBS) was injected once daily for three days either into the hind limb (to ablate the lymphatics specifically during sensitization phase) or into the right ear (to ablate the lymphatics specifically during elicitation phase). As a control, PBS was administered into the contralateral ear of the same animal or into the hind paw of a littermate (Table 1).

Table 1. Mouse strains to investigate the lymphatic vessels

Genotype	Phenotype	Inducible	Alternative name
Vegfr3$^{+/+}$	Intact lymphatics	No	Mice with intact lymphatics
Vegfr3$^{kd/+}$	Lack of lymphatics in skin	No	Lymphatic-deficient in both phases
Flt4-CreER^{T2}, *iDTR$^{fl/fl}$*	Intact lymphatics	Yes	Mice with intact lymphatics
Flt4-CreER^{T2}, *iDTR$^{fl/fl}$*	Lack of lymphatics in hind paw skin	Yes	Lymphatic-deficient in sensitization phase
Flt4-CreER^{T2}, *iDTR$^{fl/fl}$*	Lack of lymphatics in ear skin	Yes	Lymphatic-deficient in elicitation phase

3.3. Contact hypersensitivity mouse model

Isoflurane (Baxter) was used to anesthetize the mice during the treatment. Mouse CHS model induction was based on the protocol described before (142, 169) .

Single antigen exposure: In the sensitization phase, mice were treated with 100 µl acetone as a vehicle control on the shaved abdominal skin of the *Vegfr3*$^{+/+}$ and *Vegfr3*$^{kd/+}$ mice (lacking lymphatics both in the sensitization and the elicitation phase) and on the shaved abdominal skin of Flt4-CreER^{T2}, *iDTR*$^{fl/fl}$ mice (after the administration of PBS and DT into the ears of the mouse – lacking lymphatics in the elicitation phase) and on the hind paw skin of Flt4-CreER^{T2}, *iDTR*$^{fl/fl}$ mice (after the administration of PBS or DT into the hind paws of the mouse - lacking lymphatics in the sensitization phase). After 5 days, the initial ear thickness of mice was measured using a caliper (Käfer Messuhrenfabrik GmbH & Co). For elicitation, mice were treated by epicutaneous application of 20 µl 1% 2-chloro-1,3,5-trinitrobenzene (TNCB; Sigma-Aldrich, 79874) diluted in acetone on both ears. 20 µl acetone on ear skin was also applied as an absolute control group. 24 hours after the challenge the ear swelling was measured again. The increase in ear thickness was assessed as the difference between the values prior and 24 hours after the challenge, indicated by blue coloring in the graphs.

Repeated antigen exposure: In the sensitization phase, the abdominal skin/paw skin of mice was treated with 100 µl 3% TNCB diluted in acetone as an antigen, as detailed above. Five days after sensitization, the initial ear thickness was measured using a caliper. For elicitation, mice were treated by the epicutaneous application of 20 µl 1% TNCB diluted in acetone on both ears as a second exposure of antigen. The ear swelling was determined as described above, indicated by orange coloring in the graphs.

Neutrophil depletion: Flt4-CreER^{T2}, *iDTR*$^{fl/fl}$ mice were treated with tamoxifen followed by DT or PBS injection in the ear skin as described. The animals were then sensitized with 100 µl 3% TNCB diluted in acetone as an antigen or 100 µl of acetone as a control, on abdominal skin as detailed above. Three days after the sensitization, mice were intraperitoneally injected with 100 µg of murinized anti-Ly6G antibody (Absolute Antibody, Ab00295-2.0) to deplete neutrophil granulocytes, or with 100 µg of functional grade IgG2a kappa isotype antibody (Invitrogen, 16-4724-85) serving as control. Elicitation on ear skin was performed 2 days after the injection as described above. The success of the depletion of neutrophils (labeled as Ly6G^{+}, CD11b^{+}, CD45^{+} cells) was

confirmed with flow cytometry from peripheral blood samples using anti-Ly6G PerCP-Cy5.5 (BD Biosciences, 560602), anti-CD11b-allophycocyanin (BD Biosciences, 553312) and anti-CD45-FITC (Biolegend, 103108) staining, also considering the characteristic population in the forward scatter-side scatter plot.

Due to the circadian rhythm of the immune cells, we treated the animals around noon, because the recruitment of the mature immune cells to the tissues is the highest in this time point (170).

3.4. K/BxN Serum-Transfer Arthitis

K/BxN mouse model of RA are commonly used to study the onset of joint inflammation based on crossbreeding the mouse strain transgenic for T cell receptor specific for bovine ribonuclease with NOD strain (167, 171).

We mated the mice carrying KRN T-cell receptor transgene on the C57BL/6 genetic background with NOD mice and obtained transgene-positive (arthritic) K/BxN and transgene-negative (control) BxN mice (167, 172). We collected blood from the arthritic and control mice via retroorbital bleeding, and we used 400 µl sera intraperitoneally to induce inflammatory arthritis in the $\gamma^{-/-}$ WT γ Tg, $\gamma^{-/-}$ YF γ Tg and control animals. We followed the procession of the arthritis severity for 2 weeks as described before (157). The histological analysis of the inflamed ankles after 8 days has been detailed below.

3.5. Histological procedures and immunostaining

Isolated tissues (ear, back skin, small intestine, lung) were fixed in 4% paraformaldehyde (Sigma-Aldrich) overnight at 4°C, dehydrated in a series of ethanol solutions (50%, 70%, 95%, 100% concentration), and embedded into paraffin (Leica) using a Leica EG1150H embedding station. 7-8 µm thick sections were produced using a Thermo Scientific microtome (HM340E) and were processed for hematoxylin and eosin (HE) (Leica) staining and different types of fluorescent immunostainings. The immunostainings were performed using the following antibodies in 1:50 dilution incubating at 4°C, overnight: anti-LYVE1 (R&D systems, AF2125), anti-CD31 (R&D systems, mab3628), anti-Ly6G/Ly6C (anti-Gr1; BD Biosciences, 550291, clone: RB6-8C5), anti-CD45 (clone: IBL-5/25), anti-PDPN (BioLegend, 127402) and anti-GFP (Life Technologies, A11122). The secondary antibodies conjugated with fluorophores (Life

Technologies) were diluted in 1:250, incubating at room temperature for one hour: Alexa Fluor 488 goat anti-rabbit IgG (A11034); Alexa Fluor 488 donkey anti-goat IgG (A11055); Alexa Fluor 568 donkey anti-goat IgG (A11057); Alexa Fluor 488 donkey anti-rat IgG (A21208); Alexa Fluor 594 donkey anti-rat IgG (A21209); Alexa Fluor 594 donkey anti-hamster IgG (A21113). As a nuclear staining, 4',6-Diamidino-2-phenylidole (DAPI) containing mounting medium (Vector Laboratories, H-1200) was used. Microscopic images were taken by a Nikon ECLIPSE Ni-U microscope connected to a Nikon DS-Ri2 camera.

The ankles were isolated from mice after injection with PBS or DT and after arthritic or control serum injection. They were fixed in 4% paraformaldehyde at 4°C for 4 days. After two days long PBS washing steps, samples were decalcified in Osteomoll (Merck) for 3 weeks. The samples were dehydrated, embedded, and sectioned as described above.

Lymphatic vessel number and area (mean of all visible lymphatic vessels in one mouse ear section) were quantified in NIS-Elements Imaging Software (Nikon) from anti-LYVE1 fluorescent immunohistochemical images taken with a 20X objective. Blood vessel numbers (mean of 3 field of view per tissue section) were quantified in NIS-Elements Imaging Software (Nikon) from anti-CD31 fluorescent immunohistochemical images taken with a 20X objective. The number of $CD45^+$ and $Gr1^+$ immune cells were counted in FIJI Software (version: 1.53q) (173) using anti-CD45 and anti-Gr1 fluorescent immunohistology images (average of 2 fields of view per tissue section).

3.6. Whole mount immunostaining of the ears

The 4% paraformaldehyde-fixed ears were blocked and then incubated with anti-LYVE1 primary antibody in 1:150 dilution at 4°C overnight followed by anti-goat secondary antibody conjugated to Alexa Fluor 488 in 1:150 dilution. Fluorescent stereo microscopic images were taken using a Nikon SMZ25 microscope connected to a Nikon DS-Ri2 camera.

3.7. Digestion of skin samples

Ear skin was collected and cut into small pieces. The samples were digested with Liberase II kit (Roche, 492430) in a microtube Thermo-Shaker (BioSan TS-100) for 1 hour at 37°C and at 1400 rpm. Alternatively, ear skin samples were digested in a solution

of 2.5 mg/ml Collagenase D (Roche, 1108888201) and 10mg/ml DNase I (Roche, 11284932001) dissolved in RPMI (contains glutamine, 10% FBS, Penicillin, Streptavidin) in a microtube Thermo-Shaker (BioSan TS-100) for 30 minutes at 37°C and at 250 rpm. Single-cell suspension was prepared by passing through a 70 μm cell strainer (Falcon). The supernatant was collected for mouse cytokine array.

3.8. Flow cytometry

Single cell suspension prepared as described above was stained with anti-CD45-PE (BD Biosciences, 553081) and anti-Ly6G-PerCP-Cy5.5 (BD Biosciences, 560602) or anti-CD45R/B220-PE (BD Biosciences, 553090), and anti-CD3-Alexa Fluor 647 (BD Biosciences, 557869) in a 1:200 dilution of PBS based buffer containing 2% heparin and 5% heat inactivated fetal bovine serum (FBS). The conjugated primary antibody incubation was carried out for one hour at 4 °C. After staining, the samples were analyzed with BD Biosciences FACSCalibur cytometer.

Alternatively, the single cell suspension was stained with anti-CD45-FITC (BioLegend, 103108), anti-CD45R/B220-PE (BD Biosciences, 553090), anti-Ly6G-PerCP-Cy5.5 (BD Biosciences, 560602), anti-CD25-APC (Invitrogen, 17-0251-82), anti-CD279 (PD-1)-APC/Fire ™ 750 (BioLegend, 135240), anti-CD3-PE/Cyanine 7 (BioLegend, 100220), anti-CD8a-Pacific Blue ™ (Invitrogen, MCD0828) and anti-CD4-Pacific Orange ™ (Invitrogen, MCD0430) in a 1:200 dilution of PBS based buffer containing 2% heparin and 5% heat inactivated FBS. The conjugated primary antibody incubation was carried out for one hour at 4 °C. Following washing, pellets were dissolved in PBS and were analyzed with Beckman Coulter CytoFLEX cytometer.

3.9. Mouse Cytokine Array Panel A

The tissue lysates from the mouse ear samples were prepared as described in the "Digestion of skin samples" section. 500 μl of the supernatant was run on the array as described in the kit protocol (R&D Systems, ARY006). Representative images are shown from a 5-10-minute exposure to the X-ray film. The pixel intensity of dots on X-ray film was evaluated by FIJI Software (version 1.51n) (173).

3.10. Restimulation of lymph node cells (passive CHS model)

The hind paw of Flt4-CreER^{T2}, *iDTR$^{fl/fl}$* mice were injected with PBS or DT once daily for three days after tamoxifen treatment. Eight days after the first diphtheria toxin injection the hind paw skin was treated with 100 µl acetone or 3% TNCB in acetone for the sensitization. Five days after sensitization, the regional lymph nodes were collected, and single-cell suspension was prepared by homogenizing the lymph nodes through a 70 µm cell strainer using the hard end of a syringe plunger. For activation, the cells were incubated with PBS as a control or 3 mM solution of TNBS (2,4,6-trinitrobenzenesulfonic acid - water soluble form of TNCB; Sigma, P2297) for 7 min at 37°C in the dark. After washing, 10^6 cells were incubated in RPMI medium (contains glutamine, 10% FBS, Penicillin, Streptavidin) in a 96-well plate for 48 hours. After that, the supernatant was collected and IFN-γ production was measured by ELISA (Thermo Fisher Scientific, BMS606TWO) according to the manufacturer's protocol.

3.11. Presentation of data and statistical analysis

Microscopic image processing and analysis were performed using Nikon NIS-Elements Imaging Software, Adobe Illustrator and Adobe Photoshop. Flow cytometry data was evaluated using FCS express or CytExpert software. Experiments were performed the number of times indicated in the figure legends. Bar graphs show the mean and SEM of all mice or samples from indicated number of independent experiments. The effect of the second antigen exposure in ear thickness/immune cell infiltration/cytokine expression is calculated by subtracting the mean of the corresponding control group after single antigen exposure from the individual data points after repeated exposure, indicated by purple coloring in the graphs. Statistical analysis was performed in GraphPad Prism 7.0 and Excel 2018. Normal distribution of all datasets was assessed using the Shapiro-Wilk test. The significance of difference between groups was assessed by paired or unpaired Student's t-test, Mann-Whitney U test, Wilcoxon signed-rank test or two-way ANOVA. An $\alpha<0.05$ was considered statistically significant, showing *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

All figures in the introduction are created with BioRender.com.

4. Results

4.1. Characterization of the genetic mouse models used for studying the role of lymphatics in the two distinct phases of CHS

We utilized multiple mouse strains to explore the function of lymphatics in the two distinct phases of CHS. The *Vegfr3*$^{kd/+}$ mouse line carries a heterozygous mutation in the tyrosine kinase domain of VEGFR3, crucial for lymphatic vessel development and maintenance. VEGFR3 is predominantly expressed by lymphatic endothelial cells in adults (66, 174, 175). This mutation disrupts normal lymphangiogenesis, resulting in the absence of skin lymphatics in these mice. Crossbreeding *Vegfr3*$^{kd/+}$ animals with the *Prox1*^{GFP} lymphatic reporter mouse strain facilitated the visualization of lymphatic structures (165). Histological analysis confirmed the lack of lymphatics in the ear and back skin of *Vegfr3*$^{kd/+}$ mice (Figure 5a), while other tissues such as the lungs and small intestine remained unaffected. Notably, blood vessels in all tissues of *Vegfr3*$^{kd/+}$ mice were unchanged. Moreover, no significant increase in immune cell infiltration was observed in the ears of *Vegfr3*$^{kd/+}$ mice compared to wild type controls (Figure 5b). These observations establish the *Vegfr3*$^{kd/+}$ mouse strain as a reliable model for studying lymphatic involvement in both phases of CHS. Subsequently, we will refer to *Vegfr3*$^{kd/+}$ mice as "lymphatic-deficient in both phases."

Subsequently, we assessed the inflammation induced by single versus repeated antigen treatments in the NMRI genetic background mouse model with intact lymphatics. The findings showed a notable elevation in ear thickness following repeated antigen exposure compared to single antigen treatment (Figure 5c). Histological analysis using HE staining illustrated significant cell infiltration into the ear tissue after repeated antigen exposure (Figure 5d). Additionally, flow cytometry characterization confirmed a substantial increase in leukocyte infiltration, particularly neutrophils and T cells (Figure 5e).

To examine the distinct roles of lymphatics in each phase of CHS, we employed an alternative method to selectively eliminate lymphatic vessels with spatial and temporal precision. For this purpose, we utilized Flt4-CreER^{T2}; *iDTR*$^{fl/+}$ mice, crossed with the *Prox1*^{GFP} lymphatic reporter strain for visualizing lymphatics, wherein the diphtheria toxin receptor expression in lymphatic endothelial cells can be induced by tamoxifen

administration, and local diphtheria toxin (DT) injection prompts the deletion of lymphatics at specific sites (162, 163, 165). To validate the model, DT was injected into the ears of tamoxifen-treated Flt4-CreER^{T2}; *iDTR*$^{fl/+}$ mice. Eight days post-injection, lymphatic vessels were assessed using the *Prox1*^{GFP} signal and anti-LYVE1 immunostaining, confirming successful deletion in the ears without affecting the contralateral side injected with PBS as a control or other organs including back skin, lungs, and the small intestine (Figure 5f). DT injection did not affect local blood vessels or induce proliferation. Additionally, moderate immune cell infiltration, primarily CD3-positive cells, was observed in the ears, accompanied by edema development (Figure 5g). These results demonstrate the applicability of the Flt4-CreER^{T2}; *iDTR*$^{fl/+}$ mouse strain for locally deleting lymphatics in specific tissues, thereby mimicking their absence in either the sensitization or elicitation phase of CHS. The diphtheria toxin-injected mice will be referred to as "lymphatic-deficient in sensitization phase" or "lymphatic-deficient in elicitation phase," respectively.

To evaluate the extent of inflammation relative to another mouse strain, we investigated the CHS model on the C57Bl/6 genetic background in ears with intact lymphatics, injected with PBS. Following repeated antigen treatment, ear thickness showed a notable increase compared to single antigen exposure (Figure 5h). Histological examination confirmed heightened immune cell infiltration (Figure 5i), a finding further corroborated by flow cytometry analysis, revealing a significant rise in leukocyte infiltration, particularly neutrophils and T cells, akin to the other mouse strain (Figure 5j).

These results highlight that, in contrast to single antigen exposure, repeated antigen treatments lead to robust inflammation in the CHS model. Moreover, both mouse strains showed specific and dramatic inflammatory phenotypes upon repeated antigen exposure and responded similarly to antigen treatment. Therefore, these data support that the results of the subsequent experiments are comparable.

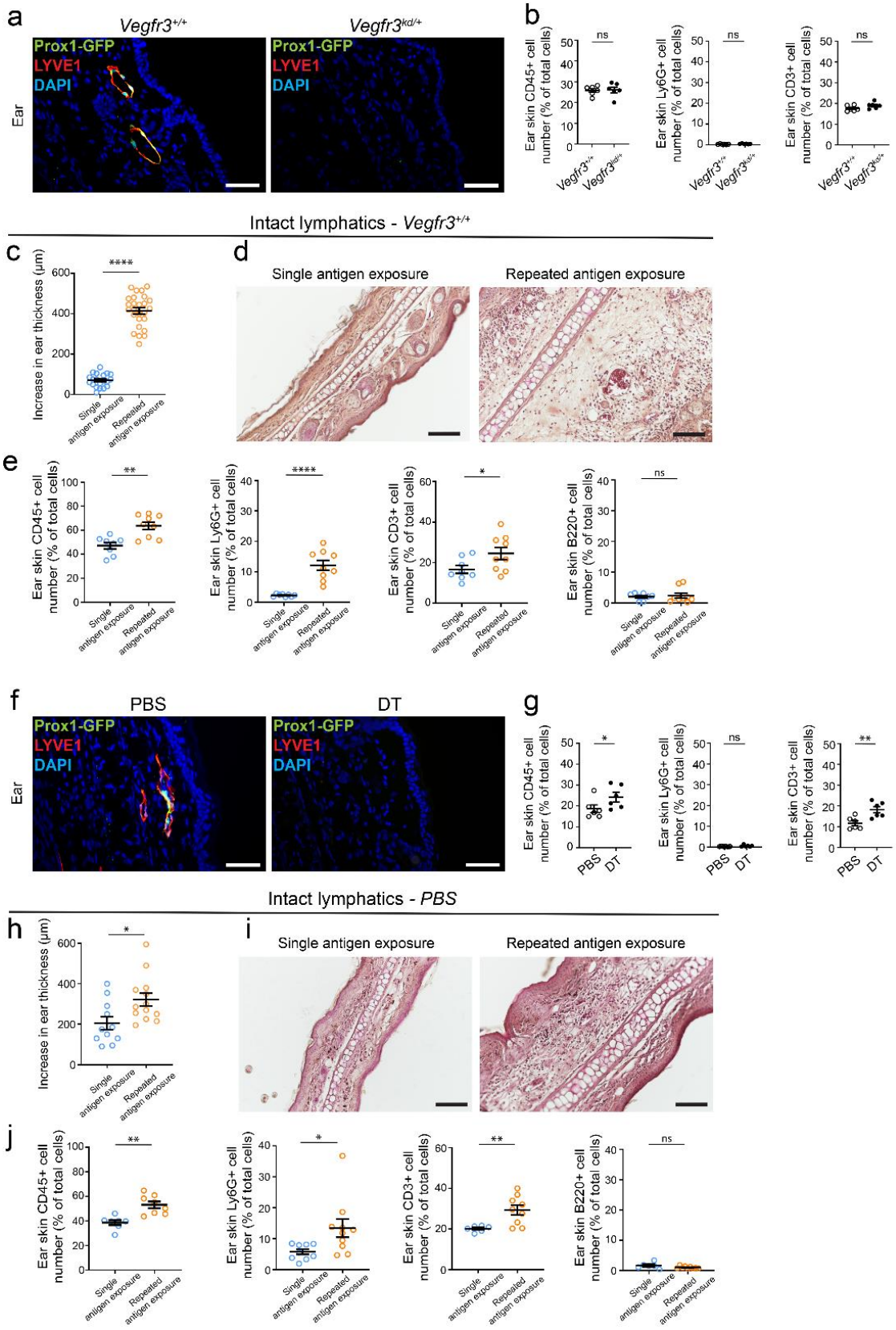

Figure 5. Characterization of contact hypersensitivity in two different mouse models

(a) *Anti-GFP and anti-LYVE1 fluorescent immunostaining of paraffin-based sections of ear of Prox1^{GFP} lymphatic reporter Vegfr3$^{+/+}$ and Vegfr3$^{kd/+}$ mice. Bars: 50 µm; n=4 for each group.* ***(b)*** *Quantitative flow*

cytometry data for immune cells (P=0.8754 for CD45$^+$ cells, P=0.0925 for Ly6G$^+$ cells, P=0.1332 for CD3$^+$ cells and P=0.0654 for B220$^+$ cells; two-tailed, unpaired t-test; mean ± SEM; n=6 mouse ears for each group). ***(c)*** *The increase in ear thickness of mice with intact lymphatics (Vegfr3$^{+/+}$) (P=4,4721×10^{-22}; two-tailed, unpaired t-test; mean ± SEM; n=20 and n=26 for group after single and repeated antigen exposure, respectively).* ***(d)*** *HE histology of ear sections. Bars: 100 µm; n=8 and n=12 for groups with single and repeated antigen exposure, respectively.* ***(e)*** *Quantitative data for immune cells from ear skin by flow cytometry (P=0.0013 for CD45$^+$ cells, P=4.2370×10^{-5} for Ly6G$^+$ cells, P=0.0460 for CD3$^+$ cells; two-tailed, unpaired t-test; mean ± SEM; P=0.4817 for B220$^+$ cells; Mann-Whitney U test; mean ± SEM; n=8 and n=9 for group with single and repeated antigen exposure, respectively).* ***(f)*** *Anti-GFP and anti-LYVE1 fluorescent immunostaining of Prox1^{GFP} lymphatic reporter Flt4-CreER^{T2}; iDTR$^{fl/fl}$ mouse ear 8 days after the first PBS or diphtheria toxin (DT) injection. Bars: 50 µm; n=3 for each group.* ***(g)*** *Immune cells quantified by flow cytometry following the digestion of ears 8 days after the first PBS and DT injection (P=0.0254 for CD45$^+$ cells, P=0.2432 for Ly6G$^+$ cells, P=0.0073 for CD3$^+$ cells and P=0.4028 for B220$^+$ cells; two-tailed, paired t-test; mean ± SEM; n=6 mouse ear for each group).* ***(h)*** *The increase in ear thickness of mice with intact lymphatics (PBS-injected, Flt4-CreER^{T2}; iDTR$^{fl/fl}$) (P=0.0174; two-tailed, unpaired t-test; mean ± SEM; n=11 and n=13 for group after single and repeated antigen exposure, respectively).* ***(i)*** *HE of ear sections with intact lymphatics. Bars: 100 µm; n=11 and n=13 for group with single and repeated antigen exposure, respectively.* ***(j)*** *Quantitative data for immune cell numbers (P=0.0011 for CD45$^+$ cells, P=0.0088 for CD3$^+$ cells, P=0.0950 for B220$^+$ cells; two-tailed, unpaired t-test; mean ± SEM; n=6 and n=9 for groups with single and repeated antigen exposure, respectively; P=0.0101 for Ly6G$^+$ cells; Mann-Whitney U test; mean ± SEM; n=9 and n=10 for groups with single and repeated antigen exposure, respectively) [I].*

4.2. Single antigen exposure does not induce a pronounced inflammatory reaction in cases of lymphatic deficiency

We then examined whether the absence of lymphatics affects the skin's response to single antigen exposure in our mouse models. Mice lacking lymphatics in both phases and only in the elicitation phase displayed a significant increase in ear thickness compared to those with intact lymphatics. Importantly, this increase was not accompanied by notable immune cell infiltration, as observed through anti-CD45 and anti-Gr1 immunostaining. Notably, acetone treatment of the ear as a vehicle control (labelled "no exposure") did not significantly affect ear thickness. Whole-mount and anti-LYVE1 immunostaining revealed the absence of lymphatics after single antigen challenge in mice lacking lymphatic vessels in both phases and only in the elicitation phase (*data not shown*) [I].

Furthermore, we analyzed immune cell populations by flow cytometry in digested single-treated ear samples. Except for an increase in T cell numbers observed solely in mice without lymphatic vessels in the elicitation phase, no significant changes were detected in various immune cell populations. Additionally, cytokine levels in the supernatant of digested ear samples were measured using a cytokine array, indicating no significant differences in cytokine levels in lymphatic-deficient animals after single antigen exposure compared to controls with intact lymphatics (*data not shown*) [I].

In summary, lymphatic deficiency does not significantly alter immune cell composition or cytokine levels in response to single antigen exposure in mouse models.

4.3. Decreased inflammation after repeated antigen treatment in mice lacking lymphatics in both phases of CHS

We employed *Vegfr3*$^{kd/+}$ mice lacking lymphatics in the skin during both the sensitization and elicitation phases of antigen exposure to investigate the pathogenesis of CHS (Figure 6a). These mice exhibited a less pronounced increase in ear thickness after repeated antigen exposure compared to mice with intact lymphatics (Figure 6b). The impact of the second antigen exposure on inflammatory responses was calculated by subtracting the mean of the corresponding control group after single antigen exposure from the individual data points after repeated exposure, as highlighted in purple in the subsequent sections. Additionally, mice lacking lymphatics showed significantly less immune cell infiltration, as demonstrated by HE staining (Figure 6c), and anti-CD45 and anti-Gr1 immunostainings (Figure 6d, e). To comprehensively characterize the infiltrating immune cell populations, flow cytometry was performed. Mice lacking lymphatics in both phases exhibited a significant reduction in the infiltration of CD45-positive leukocytes and Ly6G-positive cells representing neutrophils, while no significant difference was detected in the number of T cells and B cells (Figure 6f). In mice with intact lymphatics, repeated antigen treatment induced several proinflammatory cytokines (including IFN-γ, IL-1β) and chemokines (including IP-10, JE, MCP-5, MIP1α/β, RANTES), contributing to the recruitment of T cells, dendritic cells, and monocytes. However, the levels of most of these cytokines were significantly lower in mice lacking lymphatics (Figure 6g, h). Even after repeated antigen exposure, lymphatic vessels remained absent in the ear with lymphatic deficiency. However, the lymphatic structure in the control ear exhibited significant alterations compared to ears treated with a single application, showing an increased number and dilation under inflammatory conditions (*data not shown*) [I].

Therefore, our results indicate that the absence of lymphatics during both phases of CHS leads to a reduced immune response after repeated antigen exposure.

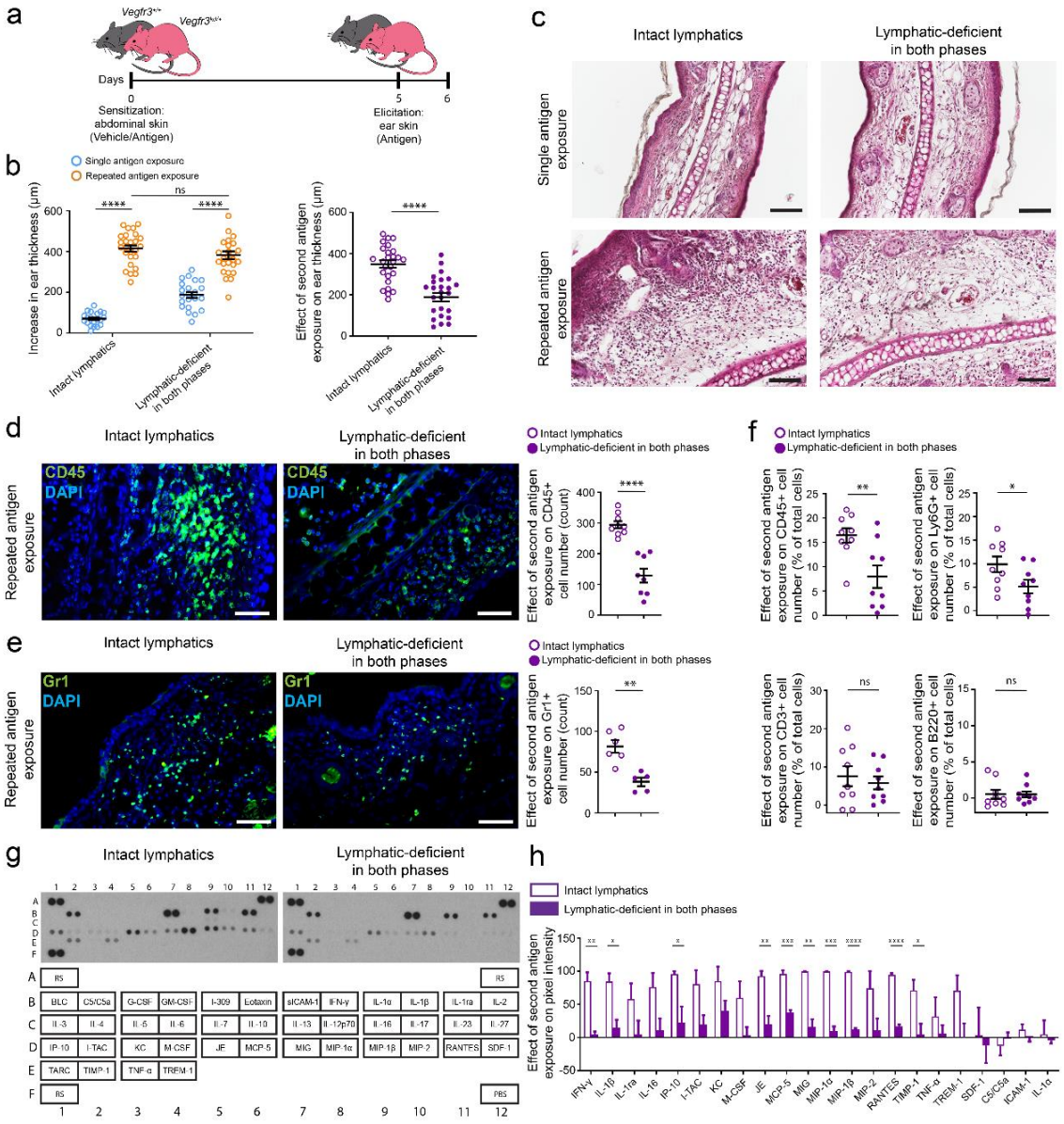

Figure 6. The effect of repeated antigen exposure in mice lacking lymphatic vessels both in the sensitization and elicitation phases

(a) *Mice with normal lymphatics or dermal lymphatic deficiency were treated with antigen on the abdominal skin and 5 days after exposure, antigen treatment was repeated on the ear skin.* ***(b)*** *The increase in ear thickness of mice with intact lymphatics and lymphatic-deficient in both phases (two-way ANOVA with Tukey's multiple comparisons test; mean ± SEM; n=20 and n=26 for group after single and repeated antigen exposure, respectively; normal vs lymphatic deficiency after single exposure: P<0.0001; normal lymphatics after single vs repeated exposure: P<0.0001; normal vs lymphatic deficiency after repeated exposure: P=0.4050; lymphatic deficiency after single vs repeated exposure: P<0.0001). The effect of the repeated antigen exposure on ear thickness is calculated by subtracting the mean of the corresponding control group values after single exposure ($P=2.9813\times10^{-7}$ two-tailed, unpaired t-test; mean ± SEM; n=26 for each group).* ***(c)*** *HE histology of ear skin sections. Bars: 100 µm; n=12 for each group.* ***(d)*** *CD45 expressing immune cells in ear sections visualized by fluorescent microscopy. Bars: 50 µm; $CD45^+$ cell numbers ($P = 7.8594\times10^{-6}$; two-tailed, unpaired t-test; mean ± SEM; n=9 and n=8 for the group with intact and lymphatic deficiency in both phases, respectively).* ***(e)*** *same as (d) for Gr1 expressing cells (P=0.0018; two-tailed, unpaired t-test; mean ± SEM; n=6 and n=5 for group with intact lymphatics and*

lymphatic-deficient in both phases, respectively). ***(f)*** *Quantitative flow cytometry data for immune cell numbers from ears (P=0.0071 for CD45$^+$ cells, P=0.0467 for Ly6G$^+$ cells, and P=0.5720 for CD3$^+$ cells; two-tailed, unpaired t-test; mean ± SEM; n=9 for each group; P=0.6048 for B220$^+$ cells; Mann-Whitney U test; mean ± SEM; n=9 for each group).* ***(g)*** *Cytokine array to detect cytokine expression in ear tissue lysates. The representative images show the arrangement of the cytokines in duplicates (RS: Reference Spot; n=3). Data are shown from a 5-minute exposure to the X-ray film.* ***(h)*** *Summary graph of the quantification of the cytokine array demonstrating the effect of the second antigen exposure over the first (two-tailed, unpaired t-test; mean ± SEM; n=3 for each group) [I].*

4.4 Lacking lymphatics only in the sensitization phase of CHS leads to reduced inflammation after repeated antigen treatment

While previous data suggest the significant contribution of the lymphatic system to inflammation during CHS (110, 147, 150, 151), it remains unclear whether lymphatics play an equally important role during both phases of CHS. To investigate the role of lymphatics in the sensitization phase of CHS, we induced local lymphatic deletion in the hind paw of Flt4-CreER^{T2}; *iDTR$^{fl/fl}$* mice using diphtheria toxin treatment. Specifically, we targeted lymphatic ablation in the hind limbs during the sensitization phase, followed by elicitation in the ear skin (Figure 7a). Confirmation of lymphatic deletion was obtained through anti-LYVE1 and anti-PDPN immunostainings, which induced edema formation in the hind limb (*data not shown*) [I]. Compared to controls, mice lacking lymphatics displayed a less pronounced increase in ear thickness and cell infiltration after repeated antigen exposure in the ear (Figure 7b, c), consistent with findings from our previous mouse model. Notably, these mice also exhibited lower CD45$^+$ and Gr1$^+$ immune cell infiltration (Figure 7d, e). Flow cytometry further confirmed the reduced infiltration of CD45$^+$ and Ly6G$^+$ cells in mice with lymphatic deficiency compared to mice with intact lymphatics, while CD3$^+$ and B220$^+$ cell populations showed no significant difference between these groups (Figure 7f).

Subsequently, we aimed to characterize the regional lymph nodes of the hind limbs where the sensitization phase was induced. The size of the inguinal lymph nodes on the treated side of lymphatic-deficient mice significantly decreased after repeated antigen exposure compared to mice with intact lymphatics (Figure 7g), indicating a diminished immune response in the absence of lymphatic vessels. However, this effect was not detectable on the other, untreated side of the same mouse. No major alterations in the structure of lymphatic and blood vessels could be detected by staining histological slides with anti-LYVE1 and anti-CD31 in the inguinal lymph node (Figure 7g), demonstrating

that despite the DT injection, the lymphatic and blood vessel structures within the lymph node remained intact.

Overall, these findings suggest that skin lymphatics contribute to immunization against antigens during the sensitization phase in the development of CHS.

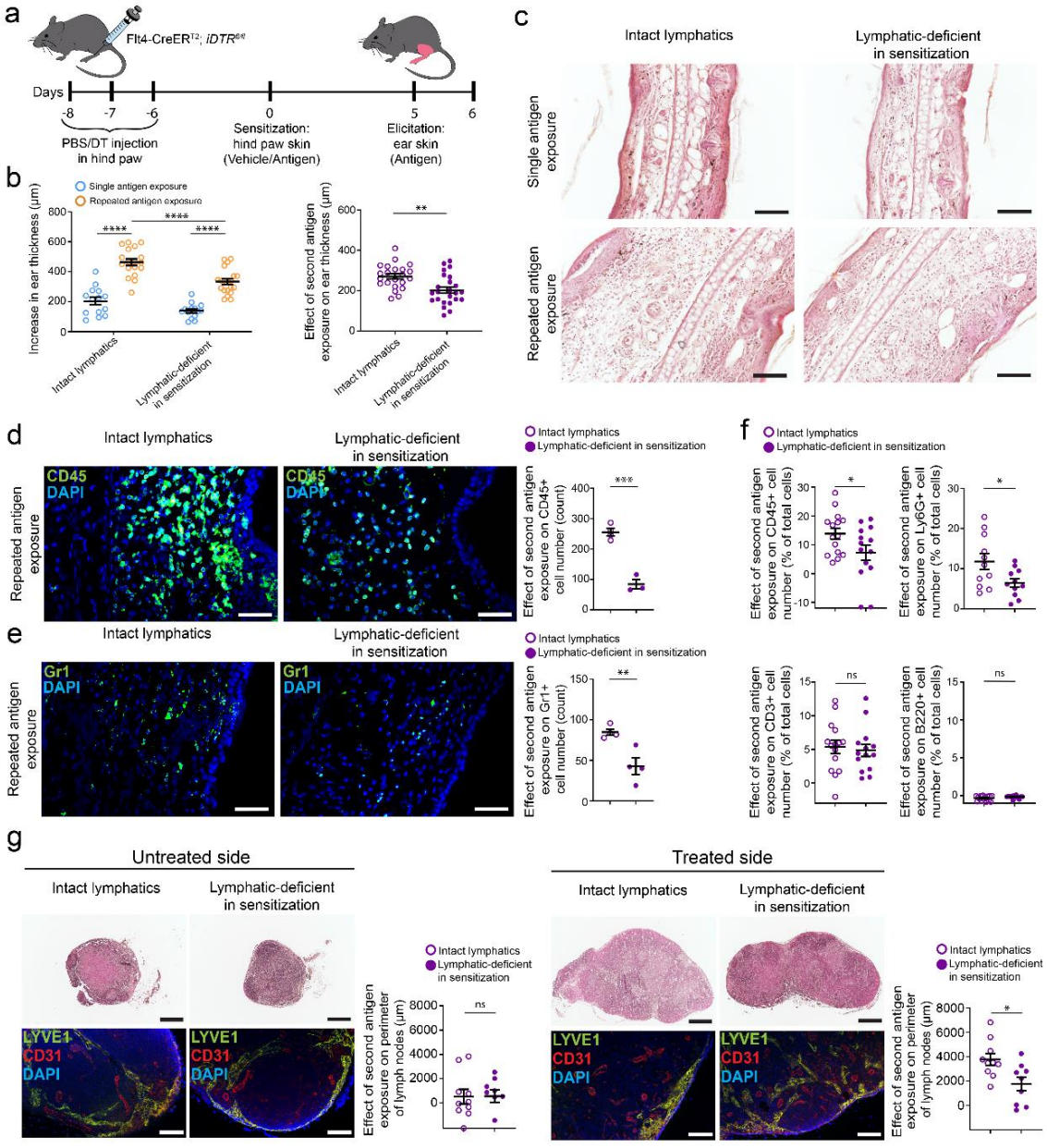

Figure 7. The effect of repeated antigen exposure in mice lacking lymphatic vessels in the sensitization phase

(a) *Flt4-CreER^{T2}; iDTR$^{fl/+}$ mice were injected with DT into the hind paw skin for 3 days. As a control, PBS was administered to a littermate. Mice with normal lymphatics or local lymphatic deficiency in the hind paw were treated with vehicle or antigen on the hind paw skin and 5 days after exposure, antigen treatment was repeated on ear skin.* ***(b)*** *The increase in ear thickness (two-way ANOVA with Tukey's multiple comparisons test; mean ± SEM; n=14 and n=18 for the group after single and repeated exposure; normal*

lymphatics vs lymphatic deficiency after single exposure: P=0.1755; normal lymphatics after single vs repeated exposure: P<0.0001; normal lymphatics vs lymphatic deficiency after repeated exposure: P<0.0001; lymphatic deficiency after single vs repeated exposure: P<0.0001). The effect of the second antigen exposure on ear thickness (P=0.0015; two-tailed, unpaired t-test; mean ± SEM; n=18 and n=20 for the group with intact lymphatics and lymphatic deficiency only in sensitization). ***(c)*** *HE histology of ear skin sections. Bars: 100 µm; n=8 for each group.* ***(d)*** *CD45 expressing cells in ear sections. Bars: 50 µm; n=4 for each group. Number of $CD45^+$ cells in ear quantified based on fluorescent immunohistology (P=0.0003; two-tailed, unpaired t-test; mean ± SEM; n=4 for each group).* ***(e)*** *Same as (d) for Gr1 expressing cells. Bars: 50 µm; n=4 for each group. Number of $Gr1^+$ cells in ear (P=0.0084; two-tailed, unpaired t-test; mean ± SEM; n=4 for each group).* ***(f)*** *The effect of the second antigen exposure to the $CD45^+$, $Ly6G^+$, $CD3^+$ and $B220^+$ immune cells (P=0.0489 for $CD45^+$ cells; P=0.0279 for $Ly6G^+$ cells and P=0.6934 for $CD3^+$ cells; two-tailed, unpaired t-test; mean ± SEM; P=0.2012 for $B220^+$ cells; Mann-Whitney U test; mean ± SEM; n=11 for group with intact lymphatics and n=15 for group with lymphatic deficiency only in elicitation).* ***(g)*** *Mice with normal lymphatics or local lymphatic deficiency in the hind paw were treated with antigen on the hind paw and 5 days after exposure antigen treatment was repeated on ear skin. HE histology of inguinal lymph nodes from the untreated and the treated side of mice with intact lymphatics or lymphatic deficiency. Bars: 500 µm; n=8 for each group. Anti-LYVE1 and anti-CD31 fluorescent immunostaining of the inguinal lymph nodes demonstrating the structure of blood and lymphatic vessels. Bars: 100 µm; n=8 for each group. The effect of repeated antigen exposure to the perimeter of the inguinal lymph node (P=0.9612 for inguinal lymph nodes on untreated side, P=0.0136 for inguinal lymph nodes on the treated side; two-tailed, unpaired t-test; mean ± SEM; n=10 and n=9 for groups with intact lymphatics and lymphatic-deficient, respectively) [I].*

4.5 Lack of lymphatics only in the sensitization phase attenuates naive T cell activation

Antigen-presenting cells play a crucial role in the sensitization phase of CHS by presenting the contact antigen to naive T cells in the lymph nodes, which is associated with increased IFN-γ secretion (74, 176). Hence, we examined whether lymphatic deficiency during the sensitization phase would impact the activation of naive T cells in the regional lymph node by assessing the IFN-γ production of these cells *ex vivo*. Following the initial antigen exposure to hind limbs with intact lymphatics or lymphatic deficiency, lymph nodes were isolated, and suspensions were generated. Subsequently, the cell suspensions were restimulated *in vitro* with the water-soluble antigen in a restimulation assay (passive CHS model). Cells from mice with intact lymphatics exhibited significantly higher IFN-γ production compared to cells from mice lacking lymphatics in the sensitization phase in the passive CHS model (Figure 8).

Hence, our results suggest that besides regulating immunization in the sensitization phase following repeated antigen exposures, lymphatics also contribute to T cell activation in the inguinal lymph nodes.

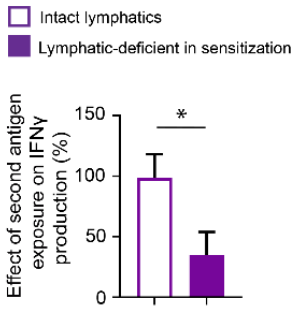

Figure 8. IFN-γ production in the regional lymph nodes of mice lacking lymphatic vessels in the sensitization phase after repeated antigen treatment

Mice with normal lymphatics or local lymphatic deficiency in the hind paw were treated with vehicle or antigen on the hind paw and 5 days after exposure inguinal lymph nodes were collected and prepared into cell cultures. Then the cell cultures were treated with vehicle or with water-soluble antigen and IFN-γ values in the supernatant were measured by ELISA. Normalized concentration of IFN-γ in supernatants after the repeated antigen treatment is shown in cell cultures containing T-cells, collected from lymph nodes of mice with intact lymphatics or lymphatic deficiency in sensitization. The graph demonstrates the effect of the second antigen exposure to the IFN-γ levels calculated by subtracting the mean of corresponding control groups after in vivo antigen exposure (P=0.0205; Mann-Whitney U test; mean ± SEM; n=7 and n=8 for groups of intact lymphatics and lymphatic-deficient, respectively) [I].

4.6 Lacking lymphatics only in the elicitation phase of CHS leads to augmented inflammation after repeated antigen treatments

We then sought to delineate the specific role of lymphatics during the elicitation phase. In a regimen involving repeated antigen exposure, sensitization was initiated by treating the abdomen with intact lymphatics, followed by elicitation induction in the ear with either intact lymphatics or local lymphatic deficiency induced by DT injection (Figure 9a). Surprisingly, mice lacking lymphatics in the elicitation phase exhibited increased ear swelling compared to contralateral ears with intact lymphatics (Figure 9b), accompanied by a higher influx of infiltrating cells (Figure 9c). Extending our observation period to four days after the challenge revealed a consistent increase in ear thickness in both intact lymphatics and lymphatic-deficient groups. However, the severity of inflammation notably escalated each day in mice with lymphatic deficiency (Figure 9d). Immunostaining of ear sections also confirmed increased $CD45^+$ and $Gr1^+$ immune cell infiltration in mice lacking lymphatics in the elicitation phase (Figure 9e, f). Furthermore, flow cytometry analysis of digested ear samples demonstrated a significantly increased $CD45^+$ and $Ly6G^+$ cell count in mice where lymphatics were deleted only in the elicitation phase, a trend that persisted up to day 9. Additionally, no significant difference was

observed in $CD3^+$ and $B220^+$ cell populations after repeated antigen exposure (Figure 9g). The heightened inflammation in mice lacking lymphatics was further corroborated by a cytokine array for ear lysates, showing markedly increased chemokine (MCP-5, MIG, MIP-1α/β, MIP-2, RANTES) and pro-inflammatory cytokine (IFN-γ, IL-1β) production (Figure 9h, i).

In summary, our findings suggest that skin lymphatics play an anti-inflammatory role during the elicitation phase of CHS.

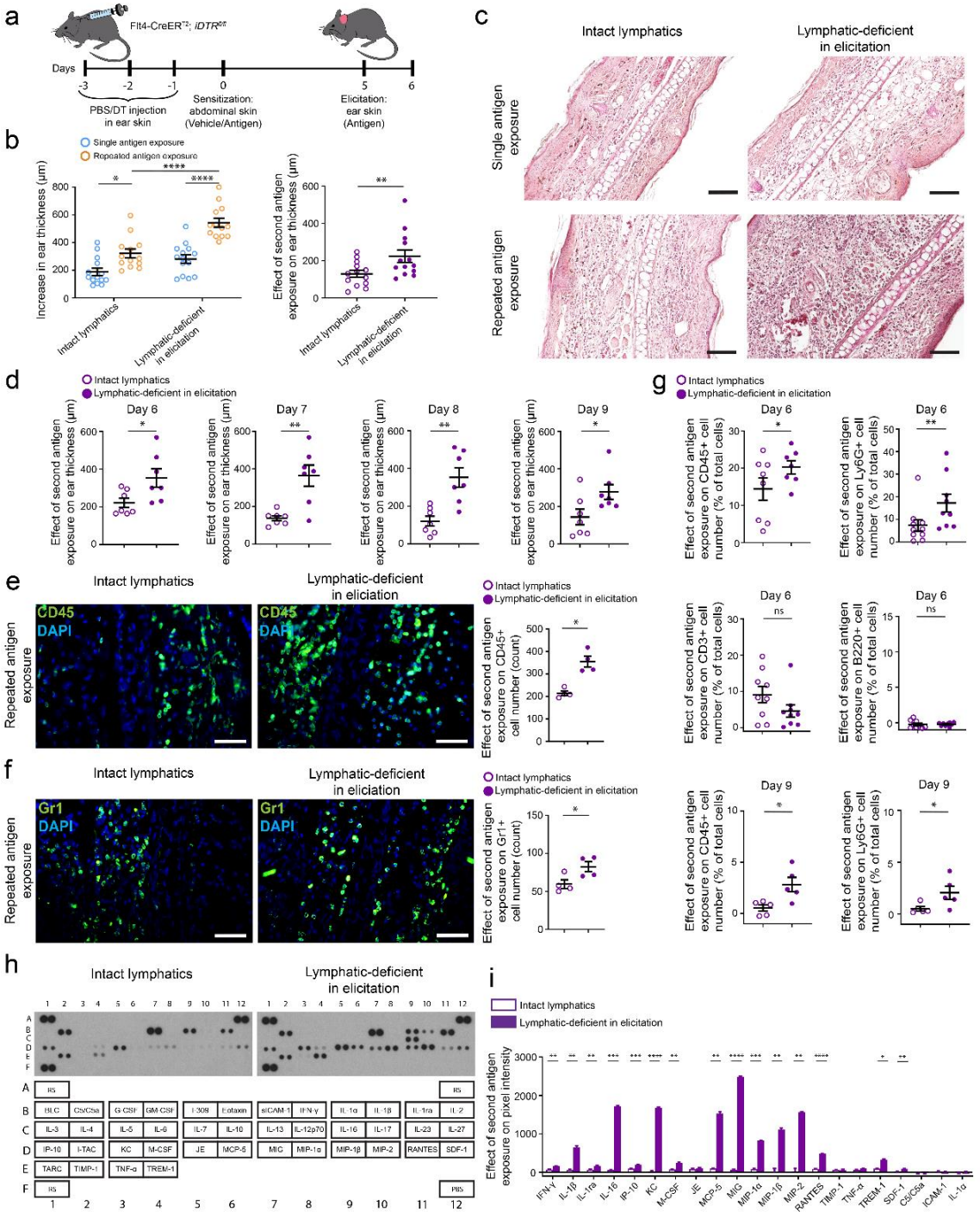

Figure 9. The effect of repeated antigen exposure in mice lacking lymphatic vessels in the elicitation phase

(a) *Flt4-CreER^{T2}; iDTR$^{fl/+}$ mice were injected with DT into ear skin for 3 days. As a control, PBS was administered into contralateral ear of the same animal. Mice with normal lymphatics or local lymphatic deficiency in ear skin were treated with antigen on the abdominal skin and 5 days after exposure, antigen treatment was repeated on ear skin.* ***(b)*** *The increase in ear thickness after antigen exposure (two-way ANOVA with Tukey's multiple comparisons test; mean ± SEM; n=14 for group and n=13 for group after single and repeated exposure respectively; normal lymphatics vs lymphatic deficiency after single exposure: P=0.1339; normal lymphatics after single vs repeated exposure: P=0.0150; normal lymphatics*

vs lymphatic deficiency after repeated exposure: P<0.0001; lymphatic deficiency after single vs repeated exposure: P<0.0001).The effect of the repeated antigen exposure on ear thickness (P=0.0024; Wilcoxon signed-rank test; mean ± SEM; n=13 for each group). ***(c)*** *HE histology of ear sections. Bars: 100 µm; n=13 for each group.* ***(d)*** *The effect of the repeated antigen exposure on ear thickness at different time points (P=0.0469 for day 6, Wilcoxon signed-rank test; P=0.0053 for day 7, two-tailed, paired t-test; P=0.0026 for day 8, two-tailed, paired t-test; P=0.0313 for day 9, Wilcoxon signed-rank test; mean ± SEM; n=7 for each group).* ***(e)*** *CD45 expressing cells are shown in ear sections by fluorescent immunohistology. Bars: 50 µm; n=6 for each group. Number of $CD45^+$ cells quantified based on fluorescent immunohistology. (P=0.0156; two-tailed, paired t-test; mean ± SEM; n=4 for each group).* ***(f)*** *Same as (e) for Gr1 expressing cells. Bars: 50 µm; n=6 for each group. Quantification of $Gr1^+$ cell numbers based on fluorescent immunohistology (P=0.0125, two-tailed, paired t-test; mean ± SEM; n=4 for each group).* ***(g)*** *The effect of the second antigen exposure on immune cell counts from ears at day 6 and 9 (Day 6: P=0.0471 for $CD45^+$ cells, and P=0.9574 for $B220^+$ cells; two-tailed, paired t-test; mean ± SEM; n=9 and n=10 for the group with intact lymphatics and lymphatic deficiency; P=0.0039 for $Ly6G^+$ cells and P=0.1641 for $CD3^+$ cells; Wilcoxon signed-rank test; mean ± SEM; n=9 for each group; Day 9 measured by CytoFLEX: P=0.0176 for $CD45^+$ cells, two-tailed, unpaired t-test; P=0.0317 for $Ly6G^+$ cells, Mann-Whitney U test; mean ± SEM; n=5 for each group).* ***(h)*** *Cytokine array to detect cytokine expression in ear tissue lysates. The representative images show the arrangement of the cytokines in duplicates (RS: Reference Spot; n=3 from each group). Data are shown from a 10-minute exposure to the X-ray film.* ***(i)*** *Summary graph of the quantification of the cytokine array demonstrating the effect of the second antigen exposure over the first (two-tailed, paired t-test; mean ± SEM; n=3 for each group) [I].*

4.7 Neutrophil granulocytes contribute to the exaggerated inflammation in lymphatic deficiency during the elicitation phase

During the elicitation phase, an exacerbated inflammation was observed in the mouse ear lacking lymphatics. Subsequently, we delved into the role of neutrophil granulocytes in this heightened inflammatory response. To address this, we employed an anti-Ly6G antibody to deplete neutrophil granulocytes just prior to the elicitation phase, using an isotype antibody as a control treatment. The CHS model was then induced as previously outlined (Figure 10a). To validate the efficacy of neutrophil depletion, we assessed immune cell counts in peripheral blood before and after antibody administration at three different time points. Neutrophil numbers, which were identified as $Ly6G^+$, $CD45^+$ and $CD11b^+$ cells were significantly reduced approaching zero (Figure 10b), while other immune cells such as B cells remained unaffected (*data not shown*) [I], indicating successful neutrophil depletion. Although a diminished inclination toward inflammatory response was observed in the absence of neutrophils following single antigen exposure, this did not result in a significant reduction in ear thickness or $CD45^+$ and $CD25^+$ immune cell infiltration, despite clear neutrophil depletion (Figure 10c, d).

After the induction of inflammatory skin disease, the increase in ear thickness was markedly attenuated in ears with lymphatic deficiency due to neutrophil absence, reaching levels comparable to ears with intact lymphatics (Figure 10e). This observation

was supported by flow cytometry measurements, which, compared to the lymphatic-deficient isotype control-treated group, showed decreased $CD45^+$ immune cell infiltration in the lymphatic-deficient and Ly6G-depleted group, while neutrophils were clearly depleted (Figure 10f). Additionally, the number of $CD25^+$ regulatory T cells (Treg) notably increased after repeated antigen exposure, particularly in ears with lymphatic deficiency. Notably, regulatory T cell numbers were substantially reduced after neutrophil depletion in the absence of lymphatic vessels, suggesting that their recruitment was influenced by neutrophils (Figure 10f).

In conclusion, these findings indicate that neutrophils play a significant role in the development of exaggerated inflammation in lymphatic deficiency during the elicitation phase. Moreover, these cells also impact regulatory T cells during inflammatory conditions.

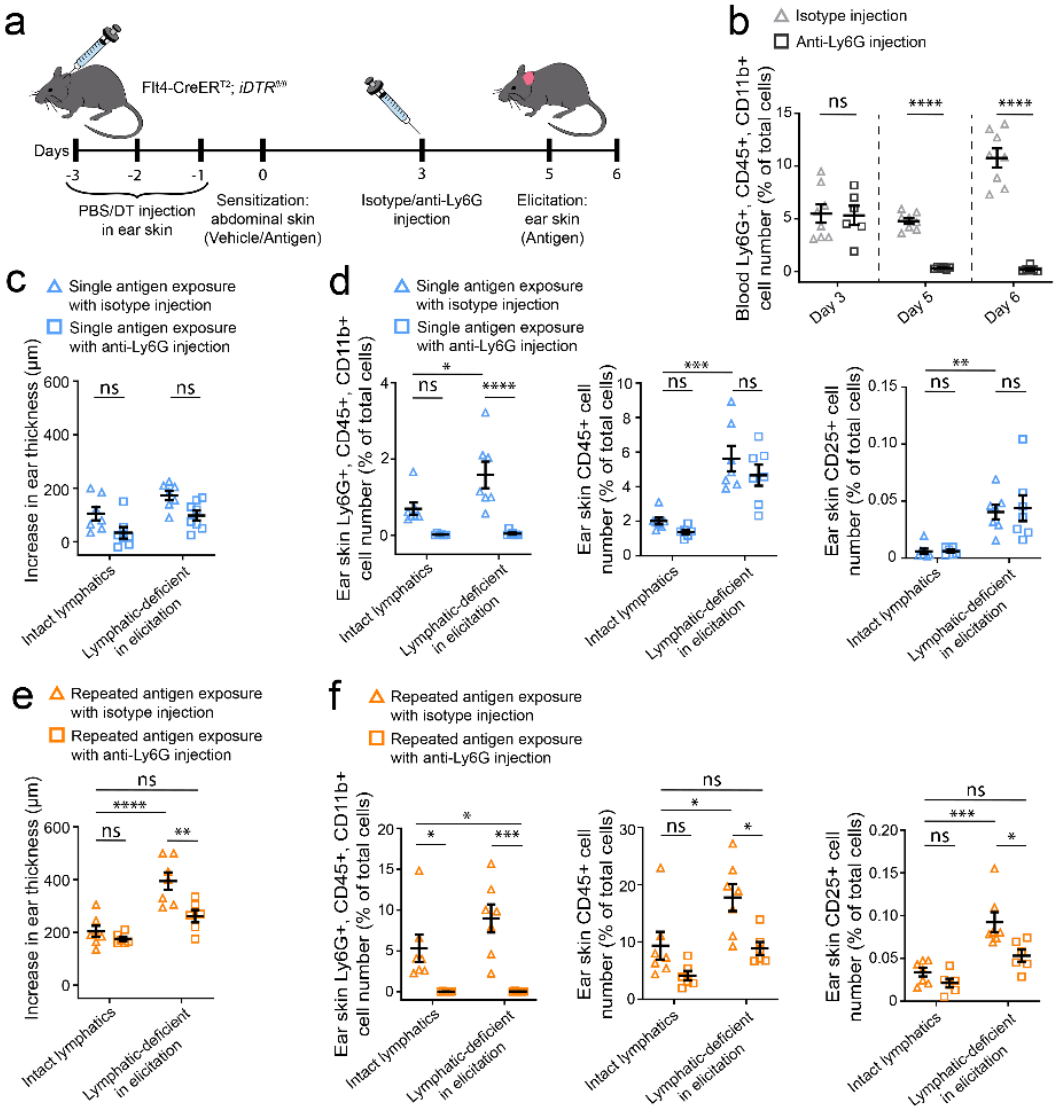

Figure 10. The effect of neutrophil granulocyte depletion in elicitation phase of CHS in the absence of lymphatic vessels

(a) *Flt4-CreER^{T2}; iDTR$^{fl/+}$ mice were injected with PBS or DT into ear skin for 3 days. Three days after the first injection, mice with intact lymphatics or local lymphatic deficiency in ear were treated with vehicle or antigen on the abdominal skin. Five days after exposure, antigen treatment was repeated on ear skin. Two days before the elicitation, the mice received anti-Ly6G injection to eliminate neutrophil granulocytes.* ***(b)*** *Ly6G$^+$ and B220$^+$ immune cell numbers in peripheral blood before and after neutrophil depletion measured by CytoFLEX (Ly6G: P=0.90135 for day 3; P<0.00001 for day 5; P<0.00001 for day 6; B220: P=0.38774 for day 3; P=0.55165 for day 5; P=0.46292 for day 6; two-tailed, unpaired t-test; mean ± SEM; n=8 and n=7 for groups of isotype control and anti-Ly6G, respectively).* ***(c)*** *The increase in ear thickness of mice with intact lymphatics and lymphatic-deficient in elicitation phase followed by neutrophil depletion after single exposure (two-way ANOVA with Tukey's multiple comparisons test; mean ± SEM; n=8 for group with lymphatic deficiency after neutrophil depletion and n=7 for the remaining group; normal lymphatics with isotype injection vs neutrophil depletion: P=0.1022; lymphatics deficiency with isotype injection vs neutrophil depletion: P=0.0690).* ***(d)*** *Ly6G$^+$, CD45$^+$ and CD25$^+$ immune cell numbers after single antigen exposure measured by CytoFLEX (two-way ANOVA with Tukey's multiple comparisons test; mean ± SEM; n=7 for each group; Ly6G: normal lymphatics with isotype injection vs neutrophil depletion: P=0.1195; lymphatics deficiency with isotype injection vs neutrophil depletion: P<0.0001; normal lymphatics vs lymphatics deficiency with isotype injection: P=0.0199;CD45: normal lymphatics with isotype injection vs*

neutrophil depletion: P=0.8285; lymphatics deficiency after single exposure with isotype injection vs neutrophil depletion: P=0.5319; normal lymphatics vs lymphatics deficiency with isotype injection: P=0.0002; CD25$^+$: normal lymphatics with isotype injection vs neutrophil depletion: P>0.9999; normal lymphatics vs lymphatic deficiency with isotype injection: P=0.0053; lymphatics deficiency with isotype injection vs neutrophil depletion: P=0.9849; normal lymphatics vs lymphatic deficiency with isotype injection: P=0.0081). ***(e)*** *The increase in ear thickness of mice with intact lymphatics and lymphatic-deficient in elicitation phase followed by neutrophil depletion after repeated exposure (two-way ANOVA with Tukey's multiple comparisons test; mean ± SEM; n=7 and n=6 for group with isotype injection and neutrophil depletion respectively; normal lymphatics with isotype injection vs neutrophil depletion: P=0.8482; lymphatics deficiency with isotype injection vs neutrophil depletion: P=0.0042; normal lymphatics with isotype injection vs lymphatics deficiency with neutrophil depletion: P=0.3472; normal lymphatics vs lymphatics deficiency with isotype injection: P<0.0001).* ***(f)*** *Ly6G$^+$, CD45$^+$ and CD25$^+$ immune cell numbers after single repeated exposure measured by CytoFLEX (two-way ANOVA with Tukey's multiple comparisons test; mean ± SEM; n=7 for each group; Ly6G: normal lymphatics with isotype injection vs neutrophil depletion: P=0.0431; lymphatics deficiency with isotype injection vs neutrophil depletion: P=0.0004; normal lymphatics with isotype injection vs lymphatics deficiency with neutrophil depletion after repeated exposure: P=0.0433; CD45: normal lymphatics with isotype injection vs neutrophil depletion: P=0.2583; lymphatics deficiency with isotype injection vs neutrophil depletion: P=0.0186; normal lymphatics vs lymphatic deficiency with isotype injection: P=0.0213; normal lymphatics with isotype injection vs lymphatics deficiency with neutrophil depletion after repeated exposure: P=0.9977; CD25: normal lymphatics with isotype injection vs neutrophil depletion: P=0.6791; lymphatics deficiency with isotype injection vs neutrophil depletion: P=0.0103; normal lymphatics vs lymphatic deficiency with isotype injection: P=0.0001; normal lymphatics with isotype injection vs lymphatics deficiency with neutrophil depletion after repeated exposure: P=0.3332) [I].*

4.8 The essential role of FcRγ ITAM tyrosines in neutrophil activation

The previously shown experiment underscored the crucial role of neutrophils in driving exacerbated skin inflammation when lymphatics are absent. However, the precise molecular mechanisms underlying these processes remain elusive. To gain deeper insights into neutrophil mediated tissue damage in another disease model, we employed a genetic approach to delineate the potential involvement of FcRγ ITAM tyrosines in neutrophils and autoimmune arthritis *in vivo*.

The functional significance of FcRγ ITAM tyrosines in neutrophils and neutrophil-dependent autoimmune diseases has not been directly assessed previously. For our *in vivo* investigations, we generated bone marrow irradiated chimeras alongside intact mice to explore the contribution of FcRγ ITAM tyrosines in the myeloid compartment. Utilizing FcRγ-deficient mice expressing either wild type (WT γ) or ITAM tyrosine mutant FcRγ (YF γ; where tyrosine at positions 65 and 76 were substituted with phenylalanine), we crossed these animals with FcRγ KO mice ($\gamma^{-/-}$), yielding $\gamma^{-/-}$ WT γ Tg and $\gamma^{-/-}$ YF γ Tg animals. To enhance the expression of the transgenic wild type and mutant FcRγ chain, we further crossed mice to generate homozygous, double transgenic animals.

Overall, we observed no substantial differences in the arthritic phenotypes between intact animals and bone marrow chimeras. Upon administration of K/BxN serum, $\gamma^{-/-}$ WT γ Tg mice displayed a discernible but modest inflammation on the hind limbs, a response notably absent in $\gamma^{-/-}$ YF γ Tg animals (*data not shown*) [II].

However, we did not observe any changes in ankle thickness in the $\gamma^{-/-}$ WT γ Tg mice as seen similarly in $\gamma^{-/-}$ mice, even though they exhibited an increase in clinical score (*data not shown*) [II]. Upon histological examination, we observed a significant influx of leukocytes into the joints of the $\gamma^{-/-}$ WT γ Tg mice, confirming the presence of inflammation externally. Despite the substantial infiltration of immune cells and joint damage observed in wild type mice following arthritis serum administration, the ankle tissue of $\gamma^{-/-}$ and $\gamma^{-/-}$ YF γ Tg mice remained intact and healthy (Figure 11).

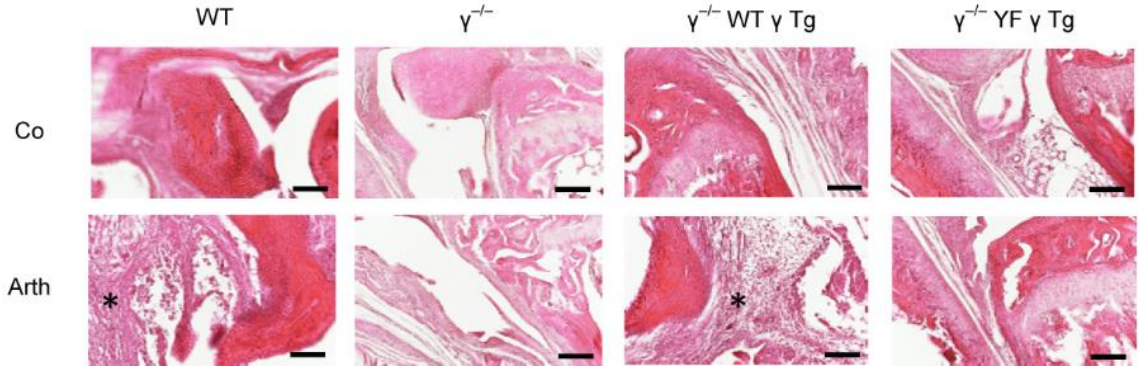

Figure 11. Massive leukocyte infiltration was detected in $\gamma^{-/-}$ WT γ transgenic mice in contrast to $\gamma^{-/-}$ YF γ transgenic mice

Leukocyte infiltration was visualized by microscopy after HE staining (scale bars: 100 µm; asterisks point at infiltrating leukocytes) [II].

Taken together, these findings underscore the critical role of FcRγ ITAM tyrosines in driving neutrophil effector responses and the initiation and progression of autoantibody-induced experimental arthritis *in vivo*. This suggests a signaling function of the molecule beyond its role in receptor stabilization.

5. Discussion

The above detailed literature underscores the importance of lymphatic- and neutrophil-dependent processes in inflammatory diseases. First, in this investigation, we delineated the immunomodulatory impacts of local lymphatics during the sensitization and elicitation phases of TNCB-induced contact hypersensitivity. To achieve this, we utilized genetic mouse models characterized by the absence and inducible elimination of local skin lymphatics. The *Vegfr3*$^{kd/+}$ model displayed a complete absence of lymphatics in the skin, as evidenced by observations in the ear and back skin (Figure 5a, b; (*data not shown*) [I]), consistent with previous reports (66, 177). Although conventional paraffin-based histology did not unveil alterations in the lymphatic structures of internal organs, including the lungs and small intestine (*data not shown*) [I], a more intricate visualization of lymphatic morphology was required in our recent study to detect changes in these internal organs (177). In the Flt4-CreER^{T2}; *iDTR*$^{fl/fl}$ model, localized administration of diphtheria toxin effectively eradicated skin lymphatics in a site-dependent manner compared to PBS-treated control animals (Figure 5; (*data not shown*) [I]), in line with previous findings (163, 168), while other organs remained unaffected.

As previously noted (163), we observed a modest yet notable increase in the infiltration of CD45$^+$ immune cells and CD3$^+$ T cells at the site of diphtheria toxin injection, even without additional antigen treatments (Figure 5f-g). Interestingly, despite the chronic contact hypersensitivity being characterized as a T cell-mediated inflammatory skin disease, the heightened T cell count did not impact the progression of CHS (Figure 9g). This elevation in T cell infiltration was not evident in the skin of *Vegfr3*$^{kd/+}$ mice lacking lymphatics throughout the skin compared to *Vegfr3*$^{+/+}$ animals (Figure 5b). It's noteworthy that neither single nor repeated antigen exposures induced the regeneration of lymphatics in our lymphatic-deficient animal models. Conversely, inflammation-driven morphological alterations in skin lymphatics were observable in mice with intact lymphatics following repeated antigen treatment, including dilation and an increased number of lymphatic vessels (*data not shown*) [I]. The two mouse strains employed serve as optimal models for dissecting the distinct roles of lymphatic vessels in the two phases of contact hypersensitivity. We verified that our CHS protocol, entailing repeated antigen exposure, triggers ear thickening and immune cell infiltration in mice on both the NMRI (*Vegfr3*$^{+/+}$) and C57Bl/6 (Flt4-CreER^{T2}; *iDTR*$^{fl/fl}$ treated with PBS)

genetic backgrounds (Figures 5c-e and 5h-j), indicating the robustness, stability, and comparability of the applied disease model, as outlined in the previously described protocol (142).

The impact of lymphatic deficiency following a single antigen exposure has not been extensively elucidated previously. Upon administering single antigen treatment, we observed increased thickening of the lymphatic-deficient ears compared to ears with intact lymphatics, yet no significant disparity in immune cell infiltration or local cytokine release was evident (*data not shown*) [I]. This suggests a propensity toward local edema formation in the treated skin of our models lacking skin lymphatics, which, however, did not translate into a significant inflammatory response following single antigen exposure.

While it's well-established that lymphatics serve as conduits for immune cells, including professional antigen-presenting cells, to migrate to local lymph nodes, their importance in initiating the sensitization phase of CHS has not been thoroughly explored (178). Our study demonstrates that the absence of lymphatics in the sensitization phase leads to a less pronounced immune response, demonstrating reduced cytokine/chemokine production and diminished immune cell infiltration. This indicates the critical role of lymphatic-dependent mechanisms in effectively inducing CHS pathogenesis during this phase (Figure 6, 7 and 8).

Conversely, the inflammatory response was markedly enhanced when lymphatics were absent during the elicitation phase, highlighting that after repeated antigen challenge, the immunomodulatory effect of lymphatics is primarily discernible (Figure 9). Previous studies employing antibody-mediated blockade of VEGFR3 signaling in an acute manner in CHS have also shown an accelerated immune response (147, 151). Similarly, investigations using VEGFR3 blocking antibodies in inflammatory bowel disease and transgenic TNF mouse models of rheumatoid arthritis have demonstrated increased inflammation (147, 179-181). Further research is warranted to elucidate how lymphatics and VEGFR3 signaling modulate the immune response in CHS and other inflammatory diseases.

It's crucial to note that applying a VEGFR3 blocking antibody may not be directly comparable to an experimental model where lymphatics are absent in the skin, as utilized in our study. Although both strategies can modulate the immune response, their mechanisms of action may differ significantly. Additionally, the acute and/or

uncontrolled effects of VEGF-C – VEGFR3 axis stimulation in disease models using adenovirus and transgenic strategies present inherent limitations. Moreover, these approaches may influence not only lymphatics but also other cell types, including immune cells. Notably, these strategies have been commonly employed in previous studies to understand the role of skin lymphatics in CHS and other inflammatory diseases (110, 147, 151, 182).

In line with this, the VEGF-C – VEGFR3 axis serves as a crucial therapeutic target in inflammatory diseases (183, 184). Our findings describing a more robust inflammatory reaction in the elicitation phase of CHS in the absence of lymphatics are consistent with previous reports indicating the challenges in managing patients with a combination of allergic contact dermatitis and lymphedema (185).

Previously, the pivotal role of neutrophil granulocytes in both the sensitization and elicitation phases of CHS was established (142). However, the precise mechanisms underlying the interplay between neutrophils and lymphatics in inflammation remained elusive. *In vivo* labeling technologies have enabled the tracking of neutrophils as they migrate from the periphery to draining lymph nodes and interact with lymphatic vessels in mice (186, 187). Neutrophils are rapidly recruited to inflamed skin in response to both microbial and sterile challenges but migrate to draining lymph nodes via lymphatic vessels only in the presence of an infectious stimulus, not a sterile one (186). This implies that neutrophil egress from inflamed tissues may be influenced by neutrophil phenotype and tissue environment. Further factors promoting neutrophil entry into lymphatic vessels remain to be defined. Neutrophils penetrate the lymphatic endothelium through a process called transmigration (188). Instead of migrating through established cellular junctions in the lymphatic endothelium, neutrophils induce enzymatic degradation and retraction of lymphatic endothelial cells (189). This creates large openings that allow rapid passage of neutrophils through the endothelium, providing a route for further neutrophil migration. This mechanism may explain the rapid kinetics of neutrophil recruitment in the lymph nodes, peaking 4–6 hours after administration of an inflammatory stimulus (118). In our experimental conditions, crosstalk between neutrophils and lymphatics in modulating immune responses is hindered due to the absence of lymphatics, leading to accumulation of neutrophils in the inflamed skin, exacerbating inflammation (Figure 9).

Regulatory T cells, a subpopulation of $CD4^+$ T cells, are integral to the adaptive immune system, regulating immune responses and maintaining immune homeostasis (190). Previous studies have demonstrated the crucial role of Tregs in controlling CHS by suppressing $CD8^+$ T cells in both the sensitization and elicitation phases (132, 191). It was also shown that Tregs can induce neutrophil recruitment by producing of CXCL8 (192). Additionally, Treg depletion and IL-10 neutralization have been shown to affect the leukemia microenvironment, partially restoring neutrophil homeostasis (193). Our findings now elucidate the crucial involvement of neutrophil granulocytes in driving the exaggerated immune response in the absence of lymphatics, significantly affecting the recruitment of regulatory T cells (Figure 10). While previous studies have revealed the intricate crosstalk between regulatory T cells and neutrophils (194), further investigations are warranted to elucidate their role within the context of the lymphatic system. Regulatory T cell trafficking between lymph nodes and inflamed tissue is vital for optimal immune suppression (195). However, impaired regulatory T cell homing and recirculation may occur due to the lack of interaction with lymphatic endothelial cells during acute inflammation (196) causing an increased number of Treg cells in the inflamed tissue site (Figure 10f).

In summary, we delineated the contribution of the skin lymphatic density to the progression of CHS using genetic mouse models lacking lymphatics in either the sensitization, elicitation, or both phases of CHS. Our experimental evidence suggests that lymphatics modulate both phases of CHS: during the sensitization phase, lymphatics contribute to the development of antigen-induced immunization, whereas in the elicitation phase, these structures moderate the inflammatory response and immune cell infiltration. Our findings imply that distinct strategies are necessary during the two phases of CHS to modulate the disease's progression.

As previously discussed, recent research has increasingly supported the notion of neutrophils exerting both direct and indirect regulatory effects on adaptive immunity over the past few decades, including their significant interaction with the lymphatic system (116). At the same time, there is a growing recognition of the involvement of neutrophils in various inflammatory conditions, such as rheumatoid arthritis, attributed to a better understanding of their proinflammatory and immunomodulatory characteristics.

Neutrophils are rapidly recruited from the bloodstream to sites of tissue damage, exacerbating inflammation; however, the specific immunological mechanisms underlying these effects remain poorly understood.

In vivo investigations utilizing murine wild type and ITAM tyrosine mutant transgenes have indicated the involvement of ITAM tyrosines in various mast cell functions, including degranulation, cytokine production, prostaglandin synthesis, and passive systemic anaphylaxis (166). While initial *in vivo* studies on the NOTAM mouse model carrying ITAM mutant FcRγ suggested the ITAM-dependence of cytotoxicity, immune complex uptake, and antigen cross-presentation in dendritic cells (197, 198), recent research has shown that daratumumab-induced cancer cell death (via FcγRIIB blockade) and splenic dendritic cell-mediated activation of $CD4^+$ and $CD8^+$ T cells occur independently of FcRγ ITAM signaling (199, 200). Our own *in vivo* investigations corroborate the essential role of FcRγ ITAM signaling in the initiation and progression of autoimmune arthritis. However, further studies are warranted to explore whether, similarly to dendritic cells, ITAM-independent signaling mechanisms are implicated in other *in vivo* functions of neutrophils. Additionally, evaluating the role of FcRγ ITAM tyrosines in other autoantibody-mediated disease models in the future would further bolster our findings with experimental arthritis.

Comparative analysis revealed that double transgenic wild type FcRγ mice could more effectively reverse the development of autoimmune arthritis on the FcRγ-deficient background compared to hemizygous animals, underscoring the significant impact of the signaling molecule level on disease progression. This observation may elucidate why ankle thickness remained largely unchanged, despite the substantial increase in the more sensitive clinical score and robust immune cell infiltration observed in single $\gamma^{-/-}$ WT γ Tg animals. Furthermore, to assess the degree of inflammation more effectively in arthritic ankles and gain a deeper understanding of arthritis pathogenesis, it is essential to overcome the challenge of preparing high-quality histology from bone-containing tissue (Figure 11). In our laboratory, we have developed a reliable method for processing ankle tissue while preserving its quality.

Given that the absence of FcRγ was not necessary for neutrophil migration in experimental arthritis, it can be inferred that FcRγ ITAM tyrosines likely do not participate in neutrophil accumulation at the site of inflammation (201). Instead, these

tyrosines may play a role in mediating immune complex-triggered responses at the site of tissue damage, as evidenced by our findings in conjunction with several other molecules involved in neutrophil Fcγ receptor signaling (157, 202, 203).

To sum up, our studies unveil the critical role of FcRγ ITAM tyrosines in immune complex-mediated activation of neutrophils and the development and progression of autoantibody-induced autoimmune arthritis. Importantly, our research furnishes the first direct *in vivo* evidence for the involvement of FcRγ ITAM tyrosines in a neutrophil-dependent arthritis model. Understanding these molecular mechanisms may pave the way for novel therapeutic targets in the treatment of certain autoimmune diseases in the future, such as the development of molecules capable of masking the ITAM tyrosines of FcRγ.

6. Conclusions

Inflammation is a complex response to predominantly local tissue alteration underlying a wide variety of physiological and pathological processes. However, the events that lead to localized chronic inflammation are partly understood. Chronic inflammatory diseases have been identified as the most significant cause of death worldwide today (204). Therefore, our aim was to extensively investigate the lymphatics- and neutrophil-dependent processes in inflammatory conditions using different disease models, like allergic skin disease or autoimmune arthritis.

Firstly, we characterized various genetic mouse models to elucidate the role of lymphatics in different phases of contact hypersensitivity. Our findings provide valuable insights into the complex interplay between lymphatics and immune responses during the sensitization and elicitation phases of CHS. Using different mouse strains and experimental strategies, we demonstrated distinct inflammatory responses to single and repeated antigen exposures in the presence or absence of lymphatics. Our results showed that single antigen exposure did not induce significant inflammation in mice lacking lymphatics, indicating that lymphatic vessels play a minor role in the immune response under these conditions.

Furthermore, our study revealed that lymphatic deficiency during both phases of CHS resulted in reduced inflammation after repeated antigen exposure. This suggests that lymphatics contribute to the initiation and progression of inflammation during CHS. Corresponding with the previous results, when lymphatics were absent only during the sensitization phase, we observed diminished inflammation following repeated antigen exposure as well. This indicates that lymphatics play a crucial role in mediating the immune response during the sensitization phase of CHS. Interestingly, when the lymphatic vessels were lacking only during the elicitation phase, an augmented inflammation was measured after the second antigen treatment, indicating that the skin lymphatics play an anti-inflammatory role during the elicitation phase of CHS.

Additionally, we investigated the impact of neutrophil granulocytes on inflammation in the absence of lymphatics during the elicitation phase. Our results showed that neutrophils significantly contribute to exaggerated inflammation in lymphatic-deficient mice during this phase, highlighting the intricate crosstalk between neutrophils and lymphatics in modulating immune responses.

Besides the neutrophils' crucial role in inflammatory skin conditions, they are also involved in the pathogenesis of various autoimmune diseases. Our research elucidates the pivotal function of FcRγ ITAM tyrosines in activating neutrophils in response to immune complexes, as well as in the pathogenesis and advancement of autoantibody-triggered autoimmune arthritis. Importantly, our findings represent the initial direct *in vivo* demonstration of the involvement of FcRγ ITAM tyrosines in a model of neutrophil-dependent arthritis.

As a conclusion, our new findings are the followings:

1. We characterized reliable mouse models in which the two phases of CHS could be investigated separately.
2. Skin lymphatics have distinct but important roles in the two phases of contact hypersensitivity: during the sensitization phase, they aid in the sensitization process of immune cells.
3. In contrast, during the elicitation phase, they downregulate the inflammatory response and the infiltration of immune cells.
4. Neutrophils are crucial to create exaggerated inflammation in the absence of lymphatics in the elicitation phase influencing the regulatory T cell numbers.
5. FcRγ ITAM tyrosines play a critical role in the induction of neutrophil effector responses.

In conclusion, our studies underscore the importance of lymphatic vessels in immune responses during cutaneous inflammation and provide comprehensive insights into the important role of neutrophils in autoimmune pathologic conditions. Exploring the role of lymphatics in CHS pathogenesis and understanding the detailed molecular pathways of neutrophils may offer valuable insights for developing innovative therapeutic strategies in the future.

7. Summary

Inflammation is a crucial part of the body's defense process, by which the immune system recognizes and eliminates the harmful stimuli followed by the healing process. Inflammation involves a coordinated interaction between immune cells, blood and lymphatic vessels, and molecular mediators to restore tissue homeostasis. However, shifts in the inflammatory response from short- to long-lived can disrupt immune tolerance and result in significant changes across all tissues and organs elevating the susceptibility to various non-communicable diseases. Chronic inflammatory conditions, such as diabetes, autoimmune diseases, or allergy represent the most substantial risk to human health according to the World Health Organization.

Our study aimed to better understand the neutrophil- and lymphatic-dependent processes in tissue injury of different inflammatory diseases such as autoimmune rheumatoid arthritis and contact hypersensitivity.

In our study, we were able to investigate the lymphatic vessels separately during the two distinct phases of contact hypersensitivity using various mouse models. Our findings showed reduced inflammation and neutrophil infiltration in mice lacking lymphatics during both sensitization and elicitation phases, with a similar attenuated phenotype with lymphatic deficiency only during sensitization. Conversely, mice deficient in lymphatics only during elicitation exhibited heightened inflammation driven by increased neutrophil infiltration.

Moreover, re-expression of wild-type and ITAM tyrosine mutant (Y65F/Y76F) FcRγ restored activating Fcγ receptor expression in FcRγ-deficient neutrophils. However, only the wild-type transgenic form mediated Fcγ receptor-dependent effector functions, leading to autoimmune arthritis in mice.

In conclusion, skin lymphatics play a crucial but distinct role in CHS's sensitization and elicitation phases. They contribute to immune cell sensitization during the first phase while they can moderate the inflammation and immune cell infiltration during elicitation. Additionally, our findings underscore the critical role of FcRγ ITAM tyrosines in inducing neutrophil effector responses and driving the initiation and progression of autoantibody-induced experimental arthritis *in vivo*, suggesting a signaling function beyond receptor stabilization.

8. References

1. Choi I, Lee S, Hong YK. The new era of the lymphatic system: no longer secondary to the blood vascular system. Cold Spring Harb Perspect Med. 2012;2(4):1-23.

2. Adamczyk LA, Gordon K, Kholova I, Meijer-Jorna LB, Telinius N, Gallagher PJ, van der Wal AC, Baandrup U. Lymph vessels: the forgotten second circulation in health and disease. Virchows Arch. 2016;469(1):3-17.

3. Suy R, Thomis S, Fourneau I. The discovery of the lymphatics in the seventeenth century. Part iii: the dethroning of the liver. Acta Chir Belg. 2016;116(6):390-7.

4. Ambrose CT. Immunology's first priority dispute--an account of the 17th-century Rudbeck-Bartholin feud. Cell Immunol. 2006;242(1):1-8.

5. Alitalo K, Tammela T, Petrova TV. Lymphangiogenesis in development and human disease. Nature. 2005;438(7070):946-53.

6. Tammela T, Alitalo K. Lymphangiogenesis: Molecular mechanisms and future promise. Cell. 2010;140(4):460-76.

7. Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. Cell. 1999;98(6):769-78.

8. Wigle JT, Harvey N, Detmar M, Lagutina I, Grosveld G, Gunn MD, Jackson DG, Oliver G. An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. EMBO J. 2002;21(7):1505-13.

9. Francois M, Caprini A, Hosking B, Orsenigo F, Wilhelm D, Browne C, Paavonen K, Karnezis T, Shayan R, Downes M, Davidson T, Tutt D, Cheah KS, Stacker SA, Muscat GE, Achen MG, Dejana E, Koopman P. Sox18 induces development of the lymphatic vasculature in mice. Nature. 2008;456(7222):643-7.

10. Petrova TV, Makinen T, Makela TP, Saarela J, Virtanen I, Ferrell RE, Finegold DN, Kerjaschki D, Yla-Herttuala S, Alitalo K. Lymphatic endothelial reprogramming of

vascular endothelial cells by the Prox-1 homeobox transcription factor. EMBO J. 2002;21(17):4593-9.

11. Hong YK, Harvey N, Noh YH, Schacht V, Hirakawa S, Detmar M, Oliver G. Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate. Dev Dyn. 2002;225(3):351-7.

12. Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol. 2007;8(6):464-78.

13. Haiko P, Makinen T, Keskitalo S, Taipale J, Karkkainen MJ, Baldwin ME, Stacker SA, Achen MG, Alitalo K. Deletion of vascular endothelial growth factor C (VEGF-C) and VEGF-D is not equivalent to VEGF receptor 3 deletion in mouse embryos. Mol Cell Biol. 2008;28(15):4843-50.

14. Zhang L, Zhou F, Han W, Shen B, Luo J, Shibuya M, He Y. VEGFR-3 ligand-binding and kinase activity are required for lymphangiogenesis but not for angiogenesis. Cell Res. 2010;20(12):1319-31.

15. Dumont DJ, Jussila L, Taipale J, Lymboussaki A, Mustonen T, Pajusola K, Breitman M, Alitalo K. Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. Science. 1998;282(5390):946-9.

16. Srinivasan RS, Escobedo N, Yang Y, Interiano A, Dillard ME, Finkelstein D, Mukatira S, Gil HJ, Nurmi H, Alitalo K, Oliver G. The Prox1-Vegfr3 feedback loop maintains the identity and the number of lymphatic endothelial cell progenitors. Genes Dev. 2014;28(19):2175-87.

17. Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci U S A. 1998;95(2):548-53.

18. Baldwin ME, Halford MM, Roufail S, Williams RA, Hibbs ML, Grail D, Kubo H, Stacker SA, Achen MG. Vascular endothelial growth factor D is dispensable for development of the lymphatic system. Mol Cell Biol. 2005;25(6):2441-9.

19. Schacht V, Ramirez MI, Hong YK, Hirakawa S, Feng D, Harvey N, Williams M, Dvorak AM, Dvorak HF, Oliver G, Detmar M. T1alpha/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. EMBO J. 2003;22(14):3546-56.

20. Uhrin P, Zaujec J, Breuss JM, Olcaydu D, Chrenek P, Stockinger H, Fuertbauer E, Moser M, Haiko P, Fassler R, Alitalo K, Binder BR, Kerjaschki D. Novel function for blood platelets and podoplanin in developmental separation of blood and lymphatic circulation. Blood. 2010;115(19):3997-4005.

21. Bertozzi CC, Schmaier AA, Mericko P, Hess PR, Zou Z, Chen M, Chen CY, Xu B, Lu MM, Zhou D, Sebzda E, Santore MT, Merianos DJ, Stadtfeld M, Flake AW, Graf T, Skoda R, Maltzman JS, Koretzky GA, Kahn ML. Platelets regulate lymphatic vascular development through CLEC-2-SLP-76 signaling. Blood. 2010;116(4):661-70.

22. Suzuki-Inoue K, Inoue O, Ding G, Nishimura S, Hokamura K, Eto K, Kashiwagi H, Tomiyama Y, Yatomi Y, Umemura K, Shin Y, Hirashima M, Ozaki Y. Essential in vivo roles of the C-type lectin receptor CLEC-2: embryonic/neonatal lethality of CLEC-2-deficient mice by blood/lymphatic misconnections and impaired thrombus formation of CLEC-2-deficient platelets. J Biol Chem. 2010;285(32):24494-507.

23. Abtahian F, Guerriero A, Sebzda E, Lu MM, Zhou R, Mocsai A, Myers EE, Huang B, Jackson DG, Ferrari VA, Tybulewicz V, Lowell CA, Lepore JJ, Koretzky GA, Kahn ML. Regulation of blood and lymphatic vascular separation by signaling proteins SLP-76 and Syk. Science. 2003;299(5604):247-51.

24. Ichise H, Ichise T, Ohtani O, Yoshida N. Phospholipase Cgamma2 is necessary for separation of blood and lymphatic vasculature in mice. Development. 2009;136(2):191-5.

25. Suzuki-Inoue K, Fuller GL, Garcia A, Eble JA, Pohlmann S, Inoue O, Gartner TK, Hughan SC, Pearce AC, Laing GD, Theakston RD, Schweighoffer E, Zitzmann N, Morita T, Tybulewicz VL, Ozaki Y, Watson SP. A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. Blood. 2006;107(2):542-9.

26. Suzuki-Inoue K, Kato Y, Inoue O, Kaneko MK, Mishima K, Yatomi Y, Yamazaki Y, Narimatsu H, Ozaki Y. Involvement of the snake toxin receptor CLEC-2, in podoplanin-mediated platelet activation, by cancer cells. J Biol Chem. 2007;282(36):25993-6001.

27. Chen CY, Bertozzi C, Zou Z, Yuan L, Lee JS, Lu M, Stachelek SJ, Srinivasan S, Guo L, Vicente A, Mericko P, Levy RJ, Makinen T, Oliver G, Kahn ML. Blood flow reprograms lymphatic vessels to blood vessels. J Clin Invest. 2012;122(6):2006-17.

28. Welsh JD, Kahn ML, Sweet DT. Lymphovenous hemostasis and the role of platelets in regulating lymphatic flow and lymphatic vessel maturation. Blood. 2016;128(9):1169-73.

29. Francois M, Short K, Secker GA, Combes A, Schwarz Q, Davidson TL, Smyth I, Hong YK, Harvey NL, Koopman P. Segmental territories along the cardinal veins generate lymph sacs via a ballooning mechanism during embryonic lymphangiogenesis in mice. Dev Biol. 2012;364(2):89-98.

30. Dagenais SL, Hartsough RL, Erickson RP, Witte MH, Butler MG, Glover TW. Foxc2 is expressed in developing lymphatic vessels and other tissues associated with lymphedema-distichiasis syndrome. Gene Expr Patterns. 2004;4(6):611-9.

31. Petrova TV, Karpanen T, Norrmen C, Mellor R, Tamakoshi T, Finegold D, Ferrell R, Kerjaschki D, Mortimer P, Yla-Herttuala S, Miura N, Alitalo K. Defective valves and abnormal mural cell recruitment underlie lymphatic vascular failure in lymphedema distichiasis. Nat Med. 2004;10(9):974-81.

32. Srinivasan RS, Dillard ME, Lagutin OV, Lin FJ, Tsai S, Tsai MJ, Samokhvalov IM, Oliver G. Lineage tracing demonstrates the venous origin of the mammalian lymphatic vasculature. Genes Dev. 2007;21(19):2422-32.

33. Klotz L, Norman S, Vieira JM, Masters M, Rohling M, Dube KN, Bollini S, Matsuzaki F, Carr CA, Riley PR. Cardiac lymphatics are heterogeneous in origin and respond to injury. Nature. 2015;522(7554):62-7.

34. Martinez-Corral I, Ulvmar MH, Stanczuk L, Tatin F, Kizhatil K, John SW, Alitalo K, Ortega S, Makinen T. Nonvenous origin of dermal lymphatic vasculature. Circ Res. 2015;116(10):1649-54.

35. Kim KE, Sung HK, Koh GY. Lymphatic development in mouse small intestine. Dev Dyn. 2007;236(7):2020-5.

36. Jakic B, Kerjaschki D, Wick G. Lymphatic Capillaries in Aging. Gerontology. 2020;66(5):419-26.

37. Zolla V, Nizamutdinova IT, Scharf B, Clement CC, Maejima D, Akl T, Nagai T, Luciani P, Leroux JC, Halin C, Stukes S, Tiwari S, Casadevall A, Jacobs WR, Jr., Entenberg D, Zawieja DC, Condeelis J, Fooksman DR, Gashev AA, Santambrogio L. Aging-related anatomical and biochemical changes in lymphatic collectors impair lymph transport, fluid homeostasis, and pathogen clearance. Aging Cell. 2015;14(4):582-94.

38. Ocskay Z, Balint L, Christ C, Kahn ML, Jakus Z. CCBE1 regulates the development and prevents the age-dependent regression of meningeal lymphatics. Biomed Pharmacother. 2024;170(1):1-13.

39. Weitman E, Cuzzone D, Mehrara BJ. Tissue engineering and regeneration of lymphatic structures. Future Oncol. 2013;9(9):1365-74.

40. Swartz MA, Skobe M. Lymphatic function, lymphangiogenesis, and cancer metastasis. Microsc Res Tech. 2001;55(2):92-9.

41. Baluk P, Fuxe J, Hashizume H, Romano T, Lashnits E, Butz S, Vestweber D, Corada M, Molendini C, Dejana E, McDonald DM. Functionally specialized junctions between endothelial cells of lymphatic vessels. J Exp Med. 2007;204(10):2349-62.

42. Leak LV, Burke JF. Ultrastructural studies on the lymphatic anchoring filaments. J Cell Biol. 1968;36(1):129-49.

43. Trzewik J, Mallipattu SK, Artmann GM, Delano FA, Schmid-Schonbein GW. Evidence for a second valve system in lymphatics: endothelial microvalves. FASEB J. 2001;15(10):1711-7.

44. Bazigou E, Xie S, Chen C, Weston A, Miura N, Sorokin L, Adams R, Muro AF, Sheppard D, Makinen T. Integrin-alpha9 is required for fibronectin matrix assembly during lymphatic valve morphogenesis. Dev Cell. 2009;17(2):175-86.

45. Koltowska K, Betterman KL, Harvey NL, Hogan BM. Getting out and about: the emergence and morphogenesis of the vertebrate lymphatic vasculature. Development. 2013;140(9):1857-70.

46. Maby-El Hajjami H, Petrova TV. Developmental and pathological lymphangiogenesis: from models to human disease. Histochem Cell Biol. 2008;130(6):1063-78.

47. Girard JP, Moussion C, Forster R. HEVs, lymphatics and homeostatic immune cell trafficking in lymph nodes. Nat Rev Immunol. 2012;12(11):762-73.

48. von Andrian UH, Mempel TR. Homing and cellular traffic in lymph nodes. Nat Rev Immunol. 2003;3(11):867-78.

49. Levick JR, Michel CC. Microvascular fluid exchange and the revised Starling principle. Cardiovasc Res. 2010;87(2):198-210.

50. Randolph GJ, Ivanov S, Zinselmeyer BH, Scallan JP. The Lymphatic System: Integral Roles in Immunity. Annu Rev Immunol. 2017;35(1):31-52.

51. Jiang X, Nicolls MR, Tian W, Rockson SG. Lymphatic Dysfunction, Leukotrienes, and Lymphedema. Annu Rev Physiol. 2018;80:49-70.

52. Nanjee MN, Cooke CJ, Garvin R, Semeria F, Lewis G, Olszewski WL, Miller NE. Intravenous apoA-I/lecithin discs increase pre-beta-HDL concentration in tissue fluid and stimulate reverse cholesterol transport in humans. J Lipid Res. 2001;42(10):1586-93.

53. Martel C, Li W, Fulp B, Platt AM, Gautier EL, Westerterp M, Bittman R, Tall AR, Chen SH, Thomas MJ, Kreisel D, Swartz MA, Sorci-Thomas MG, Randolph GJ. Lymphatic vasculature mediates macrophage reverse cholesterol transport in mice. J Clin Invest. 2013;123(4):1571-9.

54. Lim HY, Thiam CH, Yeo KP, Bisoendial R, Hii CS, McGrath KC, Tan KW, Heather A, Alexander JS, Angeli V. Lymphatic vessels are essential for the removal of cholesterol from peripheral tissues by SR-BI-mediated transport of HDL. Cell Metab. 2013;17(5):671-84.

55. Machnik A, Neuhofer W, Jantsch J, Dahlmann A, Tammela T, Machura K, Park JK, Beck FX, Muller DN, Derer W, Goss J, Ziomber A, Dietsch P, Wagner H, van Rooijen N, Kurtz A, Hilgers KF, Alitalo K, Eckardt KU, Luft FC, Kerjaschki D, Titze J. Macrophages regulate salt-dependent volume and blood pressure by a vascular endothelial growth factor-C-dependent buffering mechanism. Nat Med. 2009;15(5):545-52.

56. Wiig H, Schroder A, Neuhofer W, Jantsch J, Kopp C, Karlsen TV, Boschmann M, Goss J, Bry M, Rakova N, Dahlmann A, Brenner S, Tenstad O, Nurmi H, Mervaala E, Wagner H, Beck FX, Muller DN, Kerjaschki D, Luft FC, Harrison DG, Alitalo K, Titze J. Immune cells control skin lymphatic electrolyte homeostasis and blood pressure. J Clin Invest. 2013;123(7):2803-15.

57. Szotak-Ajtay K, Szoke D, Kovacs G, Andreka J, Brenner GB, Giricz Z, Penninger J, Kahn ML, Jakus Z. Reduced Prenatal Pulmonary Lymphatic Function Is Observed in Clp1 (K/K) Embryos With Impaired Motor Functions Including Fetal Breathing Movements in Preparation of the Developing Lung for Inflation at Birth. Front Bioeng Biotechnol. 2020;8(1):1-15.

58. Jakus Z, Gleghorn JP, Enis DR, Sen A, Chia S, Liu X, Rawnsley DR, Yang Y, Hess PR, Zou Z, Yang J, Guttentag SH, Nelson CM, Kahn ML. Lymphatic function is required prenatally for lung inflation at birth. J Exp Med. 2014;211(5):815-26.

59. Karaman S, Hollmen M, Robciuc MR, Alitalo A, Nurmi H, Morf B, Buschle D, Alkan HF, Ochsenbein AM, Alitalo K, Wolfrum C, Detmar M. Blockade of VEGF-C and VEGF-D modulates adipose tissue inflammation and improves metabolic parameters under high-fat diet. Mol Metab. 2015;4(2):93-105.

60. Swartz MA, Lund AW. Lymphatic and interstitial flow in the tumour microenvironment: linking mechanobiology with immunity. Nat Rev Cancer. 2012;12(3):210-9.

61. Kim H, Kataru RP, Koh GY. Inflammation-associated lymphangiogenesis: a double-edged sword? J Clin Invest. 2014;124(3):936-42.

62. Warren AG, Brorson H, Borud LJ, Slavin SA. Lymphedema: a comprehensive review. Ann Plast Surg. 2007;59(4):464-72.

63. Brix B, Sery O, Onorato A, Ure C, Roessler A, Goswami N. Biology of Lymphedema. Biology (Basel). 2021;10(4):1-20.

64. Aspelund A, Robciuc MR, Karaman S, Makinen T, Alitalo K. Lymphatic System in Cardiovascular Medicine. Circ Res. 2016;118(3):515-30.

65. Gordon K, Spiden SL, Connell FC, Brice G, Cottrell S, Short J, Taylor R, Jeffery S, Mortimer PS, Mansour S, Ostergaard P. FLT4/VEGFR3 and Milroy disease: novel mutations, a review of published variants and database update. Hum Mutat. 2013;34(1):23-31.

66. Karkkainen MJ, Saaristo A, Jussila L, Karila KA, Lawrence EC, Pajusola K, Bueler H, Eichmann A, Kauppinen R, Kettunen MI, Yla-Herttuala S, Finegold DN, Ferrell RE, Alitalo K. A model for gene therapy of human hereditary lymphedema. Proc Natl Acad Sci U S A. 2001;98(22):12677-82.

67. Yuan Y, Arcucci V, Levy SM, Achen MG. Modulation of Immunity by Lymphatic Dysfunction in Lymphedema. Front Immunol. 2019;10(76):1-6.

68. Kotas ME, Medzhitov R. Homeostasis, inflammation, and disease susceptibility. Cell. 2015;160(5):816-27.

69. Medzhitov R. Origin and physiological roles of inflammation. Nature. 2008;454(7203):428-35.

70. Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. Nat Rev Immunol. 2007;7(10):803-15.

71. Serhan CN, Savill J. Resolution of inflammation: the beginning programs the end. Nat Immunol. 2005;6(12):1191-7.

72. Drayton DL, Liao S, Mounzer RH, Ruddle NH. Lymphoid organ development: from ontogeny to neogenesis. Nat Immunol. 2006;7(4):344-53.

73. Pagan AJ, Ramakrishnan L. The Formation and Function of Granulomas. Annu Rev Immunol. 2018;36(1):639-65.

74. Kabashima K, Honda T, Ginhoux F, Egawa G. The immunological anatomy of the skin. Nat Rev Immunol. 2019;19(1):19-30.

75. Hampton HR, Chtanova T. Lymphatic Migration of Immune Cells. Front Immunol. 2019;10(1168):1-10.

76. Kunstfeld R, Hirakawa S, Hong YK, Schacht V, Lange-Asschenfeldt B, Velasco P, Lin C, Fiebiger E, Wei X, Wu Y, Hicklin D, Bohlen P, Detmar M. Induction of cutaneous delayed-type hypersensitivity reactions in VEGF-A transgenic mice results in chronic skin inflammation associated with persistent lymphatic hyperplasia. Blood. 2004;104(4):1048-57.

77. Henno A, Blacher S, Lambert C, Colige A, Seidel L, Noel A, Lapiere C, de la Brassinne M, Nusgens BV. Altered expression of angiogenesis and lymphangiogenesis markers in the uninvolved skin of plaque-type psoriasis. Br J Dermatol. 2009;160(3):581-90.

78. Jackson DG. Leucocyte Trafficking via the Lymphatic Vasculature- Mechanisms and Consequences. Front Immunol. 2019;10(471):1-19.

79. Worbs T, Hammerschmidt SI, Forster R. Dendritic cell migration in health and disease. Nat Rev Immunol. 2017;17(1):30-48.

80. Liao S, von der Weid PY. Lymphatic system: an active pathway for immune protection. Semin Cell Dev Biol. 2015;38(1):83-9.

81. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, Lipp M. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell. 1999;99(1):23-33.

82. Ivanov S, Scallan JP, Kim KW, Werth K, Johnson MW, Saunders BT, Wang PL, Kuan EL, Straub AC, Ouhachi M, Weinstein EG, Williams JW, Briseno C, Colonna M, Isakson BE, Gautier EL, Forster R, Davis MJ, Zinselmeyer BH, Randolph GJ. CCR7 and IRF4-dependent dendritic cells regulate lymphatic collecting vessel permeability. J Clin Invest. 2016;126(4):1581-91.

83. Tal O, Lim HY, Gurevich I, Milo I, Shipony Z, Ng LG, Angeli V, Shakhar G. DC mobilization from the skin requires docking to immobilized CCL21 on lymphatic endothelium and intralymphatic crawling. J Exp Med. 2011;208(10):2141-53.

84. Farnsworth RH, Karnezis T, Maciburko SJ, Mueller SN, Stacker SA. The Interplay Between Lymphatic Vessels and Chemokines. Front Immunol. 2019;10(518):1-14.

85. Johnson LA, Banerji S, Lawrance W, Gileadi U, Prota G, Holder KA, Roshorm YM, Hanke T, Cerundolo V, Gale NW, Jackson DG. Dendritic cells enter lymph vessels by hyaluronan-mediated docking to the endothelial receptor LYVE-1. Nat Immunol. 2017;18(7):762-70.

86. Thomas SN, Rutkowski JM, Pasquier M, Kuan EL, Alitalo K, Randolph GJ, Swartz MA. Impaired humoral immunity and tolerance in K14-VEGFR-3-Ig mice that lack dermal lymphatic drainage. J Immunol. 2012;189(5):2181-90.

87. Albanesi C, Scarponi C, Giustizieri ML, Girolomoni G. Keratinocytes in inflammatory skin diseases. Curr Drug Targets Inflamm Allergy. 2005;4(3):329-34.

88. Gunn MD, Kyuwa S, Tam C, Kakiuchi T, Matsuzawa A, Williams LT, Nakano H. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. J Exp Med. 1999;189(3):451-60.

89. Itano AA, Jenkins MK. Antigen presentation to naive CD4 T cells in the lymph node. Nat Immunol. 2003;4(8):733-9.

90. Angeli V, Ginhoux F, Llodra J, Quemeneur L, Frenette PS, Skobe M, Jessberger R, Merad M, Randolph GJ. B cell-driven lymphangiogenesis in inflamed lymph nodes enhances dendritic cell mobilization. Immunity. 2006;24(2):203-15.

91. Card CM, Yu SS, Swartz MA. Emerging roles of lymphatic endothelium in regulating adaptive immunity. J Clin Invest. 2014;124(3):943-52.

92. Tewalt EF, Cohen JN, Rouhani SJ, Engelhard VH. Lymphatic endothelial cells - key players in regulation of tolerance and immunity. Front Immunol. 2012;3(305):1-6.

93. Johnson LA, Clasper S, Holt AP, Lalor PF, Baban D, Jackson DG. An inflammation-induced mechanism for leukocyte transmigration across lymphatic vessel endothelium. J Exp Med. 2006;203(12):2763-77.

94. Rouhani SJ, Eccles JD, Riccardi P, Peske JD, Tewalt EF, Cohen JN, Liblau R, Makinen T, Engelhard VH. Roles of lymphatic endothelial cells expressing peripheral tissue antigens in CD4 T-cell tolerance induction. Nat Commun. 2015;6(6771):1-13.

95. Kataru RP, Kim H, Jang C, Choi DK, Koh BI, Kim M, Gollamudi S, Kim YK, Lee SH, Koh GY. T lymphocytes negatively regulate lymph node lymphatic vessel formation. Immunity. 2011;34(1):96-107.

96. Yeo KP, Angeli V. Bidirectional Crosstalk between Lymphatic Endothelial Cell and T Cell and Its Implications in Tumor Immunity. Front Immunol. 2017;8(83):1-13.

97. Hunter MC, Teijeira A, Montecchi R, Russo E, Runge P, Kiefer F, Halin C. Dendritic Cells and T Cells Interact Within Murine Afferent Lymphatic Capillaries. Front Immunol. 2019;10(520):1-11.

98. Kerjaschki D. The crucial role of macrophages in lymphangiogenesis. J Clin Invest. 2005;115(9):2316-9.

99. Maruyama K, Ii M, Cursiefen C, Jackson DG, Keino H, Tomita M, Van Rooijen N, Takenaka H, D'Amore PA, Stein-Streilein J, Losordo DW, Streilein JW. Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. J Clin Invest. 2005;115(9):2363-72.

100. Baluk P, Yao LC, Feng J, Romano T, Jung SS, Schreiter JL, Yan L, Shealy DJ, McDonald DM. TNF-alpha drives remodeling of blood vessels and lymphatics in sustained airway inflammation in mice. J Clin Invest. 2009;119(10):2954-64.

101. Makinen T, Veikkola T, Mustjoki S, Karpanen T, Catimel B, Nice EC, Wise L, Mercer A, Kowalski H, Kerjaschki D, Stacker SA, Achen MG, Alitalo K. Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. EMBO J. 2001;20(17):4762-73.

102. Ji RC. Macrophages are important mediators of either tumor- or inflammation-induced lymphangiogenesis. Cell Mol Life Sci. 2012;69(6):897-914.

103. Ristimaki A, Narko K, Enholm B, Joukov V, Alitalo K. Proinflammatory cytokines regulate expression of the lymphatic endothelial mitogen vascular endothelial growth factor-C. J Biol Chem. 1998;273(14):8413-8.

104. Chaitanya GV, Franks SE, Cromer W, Wells SR, Bienkowska M, Jennings MH, Ruddell A, Ando T, Wang Y, Gu Y, Sapp M, Mathis JM, Jordan PA, Minagar A, Alexander JS. Differential cytokine responses in human and mouse lymphatic endothelial cells to cytokines in vitro. Lymphat Res Biol. 2010;8(3):155-64.

105. Breslin JW, Yuan SY, Wu MH. VEGF-C alters barrier function of cultured lymphatic endothelial cells through a VEGFR-3-dependent mechanism. Lymphat Res Biol. 2007;5(2):105-13.

106. Scallan JP, Zawieja SD, Castorena-Gonzalez JA, Davis MJ. Lymphatic pumping: mechanics, mechanisms and malfunction. J Physiol. 2016;594(20):5749-68.

107. Gasheva OY, Zawieja DC, Gashev AA. Contraction-initiated NO-dependent lymphatic relaxation: a self-regulatory mechanism in rat thoracic duct. J Physiol. 2006;575(3):821-32.

108. Nizamutdinova IT, Maejima D, Nagai T, Bridenbaugh E, Thangaswamy S, Chatterjee V, Meininger CJ, Gashev AA. Involvement of histamine in endothelium-dependent relaxation of mesenteric lymphatic vessels. Microcirculation. 2014;21(7):640-8.

109. Breslin JW, Gaudreault N, Watson KD, Reynoso R, Yuan SY, Wu MH. Vascular endothelial growth factor-C stimulates the lymphatic pump by a VEGF receptor-3-dependent mechanism. Am J Physiol Heart Circ Physiol. 2007;293(1):709-18.

110. Huggenberger R, Detmar M. The cutaneous vascular system in chronic skin inflammation. J Investig Dermatol Symp Proc. 2011;15(1):24-32.

111. Baluk P, Tammela T, Ator E, Lyubynska N, Achen MG, Hicklin DJ, Jeltsch M, Petrova TV, Pytowski B, Stacker SA, Yla-Herttuala S, Jackson DG, Alitalo K, McDonald DM. Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. J Clin Invest. 2005;115(2):247-57.

112. Alitalo K. The lymphatic vasculature in disease. Nat Med. 2011;17(11):1371-80.

113. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol. 2013;13(3):159-75.

114. Futosi K, Fodor S, Mocsai A. Neutrophil cell surface receptors and their intracellular signal transduction pathways. Int Immunopharmacol. 2013;17(3):638-50.

115. Selvaraj P, Fifadara N, Nagarajan S, Cimino A, Wang G. Functional regulation of human neutrophil Fc gamma receptors. Immunol Res. 2004;29(1-3):219-30.

116. Rosales C. Neutrophils at the crossroads of innate and adaptive immunity. J Leukoc Biol. 2020;108(1):377-96.

117. Bogoslowski A, Wijeyesinghe S, Lee WY, Chen CS, Alanani S, Jenne C, Steeber DA, Scheiermann C, Butcher EC, Masopust D, Kubes P. Neutrophils Recirculate through Lymph Nodes to Survey Tissues for Pathogens. J Immunol. 2020;204(9):2552-61.

118. Beauvillain C, Cunin P, Doni A, Scotet M, Jaillon S, Loiry ML, Magistrelli G, Masternak K, Chevailler A, Delneste Y, Jeannin P. CCR7 is involved in the migration of neutrophils to lymph nodes. Blood. 2011;117(4):1196-204.

119. Arokiasamy S, Zakian C, Dilliway J, Wang W, Nourshargh S, Voisin MB. Endogenous TNFalpha orchestrates the trafficking of neutrophils into and within lymphatic vessels during acute inflammation. Sci Rep. 2017;7(44189):1-17.

120. Vono M, Lin A, Norrby-Teglund A, Koup RA, Liang F, Lore K. Neutrophils acquire the capacity for antigen presentation to memory CD4(+) T cells in vitro and ex vivo. Blood. 2017;129(14):1991-2001.

121. Beauvillain C, Delneste Y, Scotet M, Peres A, Gascan H, Guermonprez P, Barnaba V, Jeannin P. Neutrophils efficiently cross-prime naive T cells in vivo. Blood. 2007;110(8):2965-73.

122. Lok LSC, Dennison TW, Mahbubani KM, Saeb-Parsy K, Chilvers ER, Clatworthy MR. Phenotypically distinct neutrophils patrol uninfected human and mouse lymph nodes. Proc Natl Acad Sci U S A. 2019;116(38):19083-9.

123. Vocanson M, Hennino A, Rozieres A, Poyet G, Nicolas JF. Effector and regulatory mechanisms in allergic contact dermatitis. Allergy. 2009;64(12):1699-714.

124. Novak-Bilic G, Vucic M, Japundzic I, Mestrovic-Stefekov J, Stanic-Duktaj S, Lugovic-Mihic L. Irritant and Allergic Contact Dermatitis - Skin Lesion Characteristics. Acta Clin Croat. 2018;57(4):713-20.

125. Peiser M, Tralau T, Heidler J, Api AM, Arts JH, Basketter DA, English J, Diepgen TL, Fuhlbrigge RC, Gaspari AA, Johansen JD, Karlberg AT, Kimber I, Lepoittevin JP, Liebsch M, Maibach HI, Martin SF, Merk HF, Platzek T, Rustemeyer T, Schnuch A, Vandebriel RJ, White IR, Luch A. Allergic contact dermatitis: epidemiology, molecular mechanisms, in vitro methods and regulatory aspects. Current knowledge assembled at an international workshop at BfR, Germany. Cell Mol Life Sci. 2012;69(5):763-81.

126. Haluk Akar H, Adatepe S, Tahan F, Solmaz I. Hair dyes and temporary tattoos are a real hazard for adolescents? Eur Ann Allergy Clin Immunol. 2014;46(1):35-7.

127. Nassau S, Fonacier L. Allergic Contact Dermatitis. Med Clin North Am. 2020;104(1):61-76.

128. Friis UF, Menne T, Schwensen JF, Flyvholm MA, Bonde JP, Johansen JD. Occupational irritant contact dermatitis diagnosed by analysis of contact irritants and allergens in the work environment. Contact Dermatitis. 2014;71(6):364-70.

129. Reduta T, Bacharewicz J, Pawlos A. Patch test results in patients with allergic contact dermatitis in the Podlasie region. Postepy Dermatol Alergol. 2013;30(6):350-7.

130. Suuronen K, Pesonen M, Henriks-Eckerman ML, Aalto-Korte K. Triphenyl phosphite, a new allergen in polyvinylchloride gloves. Contact Dermatitis. 2013;68(1):42-9.

131. Olusegun OA, Martincigh BS. Allergic contact dermatitis: a significant environmental and occupational skin disease. Int J Dermatol. 2021;60(9):1082-91.

132. Honda T, Egawa G, Grabbe S, Kabashima K. Update of immune events in the murine contact hypersensitivity model: toward the understanding of allergic contact dermatitis. J Invest Dermatol. 2013;133(2):303-15.

133. Lepoittevin JP. Metabolism versus chemical transformation or pro- versus prehaptens? Contact Dermatitis. 2006;54(2):73-4.

134. Cumberbatch M, Dearman RJ, Kimber I. Langerhans cells require signals from both tumour necrosis factor-alpha and interleukin-1 beta for migration. Immunology. 1997;92(3):388-95.

135. Schmidt M, Goebeler M. Immunology of metal allergies. J Dtsch Dermatol Ges. 2015;13(7):653-60.

136. Martin SF, Esser PR, Weber FC, Jakob T, Freudenberg MA, Schmidt M, Goebeler M. Mechanisms of chemical-induced innate immunity in allergic contact dermatitis. Allergy. 2011;66(9):1152-63.

137. Weltzien HU, Moulon C, Martin S, Padovan E, Hartmann U, Kohler J. T cell immune responses to haptens. Structural models for allergic and autoimmune reactions. Toxicology. 1996;107(2):141-51.

138. Martin SF. T lymphocyte-mediated immune responses to chemical haptens and metal ions: implications for allergic and autoimmune disease. Int Arch Allergy Immunol. 2004;134(3):186-98.

139. Martin SF. Immunological mechanisms in allergic contact dermatitis. Curr Opin Allergy Clin Immunol. 2015;15(2):124-30.

140. Dudeck A, Dudeck J, Scholten J, Petzold A, Surianarayanan S, Kohler A, Peschke K, Vohringer D, Waskow C, Krieg T, Muller W, Waisman A, Hartmann K, Gunzer M, Roers A. Mast cells are key promoters of contact allergy that mediate the adjuvant effects of haptens. Immunity. 2011;34(6):973-84.

141. Wang M, Qin X, Mudgett JS, Ferguson TA, Senior RM, Welgus HG. Matrix metalloproteinase deficiencies affect contact hypersensitivity: stromelysin-1 deficiency prevents the response and gelatinase B deficiency prolongs the response. Proc Natl Acad Sci U S A. 1999;96(12):6885-9.

142. Weber FC, Nemeth T, Csepregi JZ, Dudeck A, Roers A, Ozsvari B, Oswald E, Puskas LG, Jakob T, Mocsai A, Martin SF. Neutrophils are required for both the sensitization and elicitation phase of contact hypersensitivity. J Exp Med. 2015;212(1):15-22.

143. Azeem M, Kader H, Kerstan A, Hetta HF, Serfling E, Goebeler M, Muhammad K. Intricate Relationship Between Adaptive and Innate Immune System in Allergic Contact Dermatitis. Yale J Biol Med. 2020;93(5):699-709.

144. Gocinski BL, Tigelaar RE. Roles of CD4+ and CD8+ T cells in murine contact sensitivity revealed by in vivo monoclonal antibody depletion. J Immunol. 1990;144(11):4121-8.

145. Raphael I, Nalawade S, Eagar TN, Forsthuber TG. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. Cytokine. 2015;74(1):5-17.

146. Natsuaki Y, Kabashima K. Inducible lymphoid clusters, iSALTs, in contact dermatitis: a new concept of acquired cutaneous immune responses. Med Mol Morphol. 2016;49(3):127-32.

147. Schwager S, Detmar M. Inflammation and Lymphatic Function. Front Immunol. 2019;10(308):1-11.

148. Vranova M, Halin C. Lymphatic Vessels in Inflammation. Journal of Clinical & Cellular Immunology. 2014;5(4):1-17.

149. Zhang Y, Lu Y, Ma L, Cao X, Xiao J, Chen J, Jiao S, Gao Y, Liu C, Duan Z, Li D, He Y, Wei B, Wang H. Activation of vascular endothelial growth factor receptor-3 in macrophages restrains TLR4-NF-kappaB signaling and protects against endotoxin shock. Immunity. 2014;40(4):501-14.

150. Hagura A, Asai J, Maruyama K, Takenaka H, Kinoshita S, Katoh N. The VEGF-C/VEGFR3 signaling pathway contributes to resolving chronic skin inflammation by activating lymphatic vessel function. J Dermatol Sci. 2014;73(2):135-41.

151. Huggenberger R, Ullmann S, Proulx ST, Pytowski B, Alitalo K, Detmar M. Stimulation of lymphangiogenesis via VEGFR-3 inhibits chronic skin inflammation. J Exp Med. 2010;207(10):2255-69.

152. Rudan I, Sidhu S, Papana A, Meng SJ, Xin-Wei Y, Wang W, Campbell-Page RM, Demaio AR, Nair H, Sridhar D, Theodoratou E, Dowman B, Adeloye D, Majeed A, Car J, Campbell H, Wang W, Chan KY, Global Health Epidemiology Reference G. Prevalence of rheumatoid arthritis in low- and middle-income countries: A systematic review and analysis. J Glob Health. 2015;5(1):1-10.

153. Jang S, Kwon EJ, Lee JJ. Rheumatoid Arthritis: Pathogenic Roles of Diverse Immune Cells. Int J Mol Sci. 2022;23(2):1-15.

154. Weiss SJ. Tissue destruction by neutrophils. N Engl J Med. 1989;320(6):365-76.

155. Meissner K, Weissenhofer W. Massive hemorrhage caused by acute solitary gastric erosion Dieulafoy. Change of prognosis due to preoperative endoscopy. Langenbecks Arch Chir. 1976;341(2):135-40.

156. Bruhns P, Jonsson F. Mouse and human FcR effector functions. Immunol Rev. 2015;268(1):25-51.

157. Jakus Z, Simon E, Frommhold D, Sperandio M, Mocsai A. Critical role of phospholipase Cgamma2 in integrin and Fc receptor-mediated neutrophil functions and the effector phase of autoimmune arthritis. J Exp Med. 2009;206(3):577-93.

158. Bruhns P. Properties of mouse and human IgG receptors and their contribution to disease models. Blood. 2012;119(24):5640-9.

159. Jakus Z, Nemeth T, Verbeek JS, Mocsai A. Critical but overlapping role of FcgammaRIII and FcgammaRIV in activation of murine neutrophils by immobilized immune complexes. J Immunol. 2008;180(1):618-29.

160. Takai T, Li M, Sylvestre D, Clynes R, Ravetch JV. FcR gamma chain deletion results in pleiotrophic effector cell defects. Cell. 1994;76(3):519-29.

161. Corr M, Crain B. The role of FcgammaR signaling in the K/B x N serum transfer model of arthritis. J Immunol. 2002;169(11):6604-9.

162. Buch T, Heppner FL, Tertilt C, Heinen TJ, Kremer M, Wunderlich FT, Jung S, Waisman A. A Cre-inducible diphtheria toxin receptor mediates cell lineage ablation after toxin administration. Nat Methods. 2005;2(6):419-26.

163. Gardenier JC, Hespe GE, Kataru RP, Savetsky IL, Torrisi JS, Nores GDG, Dayan JJ, Chang D, Zampell J, Martinez-Corral I, Ortega S, Mehrara BJ. Diphtheria toxin-mediated ablation of lymphatic endothelial cells results in progressive lymphedema. JCI Insight. 2016;1(15):1-15.

164. Martinez-Corral I, Stanczuk L, Frye M, Ulvmar MH, Dieguez-Hurtado R, Olmeda D, Makinen T, Ortega S. Vegfr3-CreER (T2) mouse, a new genetic tool for targeting the lymphatic system. Angiogenesis. 2016;19(3):433-45.

165. Choi I, Chung HK, Ramu S, Lee HN, Kim KE, Lee S, Yoo J, Choi D, Lee YS, Aguilar B, Hong YK. Visualization of lymphatic vessels by Prox1-promoter directed GFP reporter in a bacterial artificial chromosome-based transgenic mouse. Blood. 2011;117(1):362-5.

166. Sakurai D, Yamasaki S, Arase K, Park SY, Arase H, Konno A, Saito T. Fc epsilon RI gamma-ITAM is differentially required for mast cell function in vivo. J Immunol. 2004;172(4):2374-81.

167. Kouskoff V, Korganow AS, Duchatelle V, Degott C, Benoist C, Mathis D. Organ-specific disease provoked by systemic autoimmunity. Cell. 1996;87(5):811-22.

168. Szoke D, Kovacs G, Kemecsei E, Balint L, Szotak-Ajtay K, Aradi P, Styevkone Dinnyes A, Mui BL, Tam YK, Madden TD, Kariko K, Kataru RP, Hope MJ, Weissman D, Mehrara BJ, Pardi N, Jakus Z. Nucleoside-modified VEGFC mRNA induces organ-specific lymphatic growth and reverses experimental lymphedema. Nat Commun. 2021;12(1):1-18.

169. Martin SF. Induction of contact hypersensitivity in the mouse model. Methods Mol Biol. 2013;961(1):325-35.

170. Scheiermann C, Kunisaki Y, Frenette PS. Circadian control of the immune system. Nat Rev Immunol. 2013;13(3):190-8.

171. Christianson CA, Corr M, Yaksh TL, Svensson CI. K/BxN serum transfer arthritis as a model of inflammatory joint pain. Methods Mol Biol. 2012;851:249-60.

172. Nemeth T, Futosi K, Szabo M, Aradi P, Saito T, Mocsai A, Jakus Z. Importance of Fc Receptor gamma-Chain ITAM Tyrosines in Neutrophil Activation and in vivo Autoimmune Arthritis. Front Immunol. 2019;10(252):1-11.

173. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. Fiji: an open-source platform for biological-image analysis. Nature Methods. 2012;9(7):676-82.

174. Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D, Breitman M, Alitalo K. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci U S A. 1995;92(8):3566-70.

175. Rauniyar K, Jha SK, Jeltsch M. Biology of Vascular Endothelial Growth Factor C in the Morphogenesis of Lymphatic Vessels. Front Bioeng Biotechnol. 2018;6(7):1-12.

176. Kaplan DH, Igyarto BZ, Gaspari AA. Early immune events in the induction of allergic contact dermatitis. Nat Rev Immunol. 2012;12(2):114-24.

177. Christ C, Jakus Z. Visualization of Organ-Specific Lymphatic Growth: An Efficient Approach to Labeling Molecular Markers in Cleared Tissues. Int J Mol Sci. 2023;24(6):1-17.

178. Collado-Diaz V, Medina-Sanchez JD, Gkountidi AO, Halin C. Imaging leukocyte migration through afferent lymphatics. Immunol Rev. 2022;306(1):43-57.

179. D'Alessio S, Correale C, Tacconi C, Gandelli A, Pietrogrande G, Vetrano S, Genua M, Arena V, Spinelli A, Peyrin-Biroulet L, Fiocchi C, Danese S. VEGF-C-dependent stimulation of lymphatic function ameliorates experimental inflammatory bowel disease. J Clin Invest. 2014;124(9):3863-78.

180. Guo R, Zhou Q, Proulx ST, Wood R, Ji RC, Ritchlin CT, Pytowski B, Zhu Z, Wang YJ, Schwarz EM, Xing L. Inhibition of lymphangiogenesis and lymphatic drainage via vascular endothelial growth factor receptor 3 blockade increases the severity of inflammation in a mouse model of chronic inflammatory arthritis. Arthritis Rheum. 2009;60(9):2666-76.

181. Jurisic G, Sundberg JP, Detmar M. Blockade of VEGF receptor-3 aggravates inflammatory bowel disease and lymphatic vessel enlargement. Inflamm Bowel Dis. 2013;19(9):1983-9.

182. Kataru RP, Jung K, Jang C, Yang H, Schwendener RA, Baik JE, Han SH, Alitalo K, Koh GY. Critical role of CD11b+ macrophages and VEGF in inflammatory lymphangiogenesis, antigen clearance, and inflammation resolution. Blood. 2009;113(22):5650-9.

183. Cousin N, Bartel S, Scholl J, Tacconi C, Egger A, Thorhallsdottir G, Neri D, Dieterich LC, Detmar M. Antibody-Mediated Delivery of VEGF-C Promotes Long-

Lasting Lymphatic Expansion That Reduces Recurrent Inflammation. Cells. 2022;12(1):1-15.

184. Schwager S, Renner S, Hemmerle T, Karaman S, Proulx ST, Fetz R, Golding-Ochsenbein AM, Probst P, Halin C, Neri D, Detmar M. Antibody-mediated delivery of VEGF-C potently reduces chronic skin inflammation. JCI Insight. 2018;3(23):1-18.

185. Dyring-Andersen B, Skov L, Jensen P. Chronic lymphoedema caused by recurrent infections in a patient with allergic hand eczema. Dermatol Reports. 2011;3(1):22.

186. Hampton HR, Bailey J, Tomura M, Brink R, Chtanova T. Microbe-dependent lymphatic migration of neutrophils modulates lymphocyte proliferation in lymph nodes. Nat Commun. 2015;6(7139):1-11.

187. Abadie V, Badell E, Douillard P, Ensergueix D, Leenen PJ, Tanguy M, Fiette L, Saeland S, Gicquel B, Winter N. Neutrophils rapidly migrate via lymphatics after Mycobacterium bovis BCG intradermal vaccination and shuttle live bacilli to the draining lymph nodes. Blood. 2005;106(5):1843-50.

188. Rigby DA, Ferguson DJ, Johnson LA, Jackson DG. Neutrophils rapidly transit inflamed lymphatic vessel endothelium via integrin-dependent proteolysis and lipoxin-induced junctional retraction. J Leukoc Biol. 2015;98(6):897-912.

189. Teijeira A, Halin C. Breaching their way through: Neutrophils destroy intercellular junctions to transmigrate rapidly across lymphatic endothelium. J Leukoc Biol. 2015;98(6):880-2.

190. Sakaguchi S, Wing K, Miyara M. Regulatory T cells - a brief history and perspective. Eur J Immunol. 2007;37(S1):116-23.

191. Ring S, Schafer SC, Mahnke K, Lehr HA, Enk AH. CD4+ CD25+ regulatory T cells suppress contact hypersensitivity reactions by blocking influx of effector T cells into inflamed tissue. Eur J Immunol. 2006;36(11):2981-92.

192. Himmel ME, Crome SQ, Ivison S, Piccirillo C, Steiner TS, Levings MK. Human CD4+ FOXP3+ regulatory T cells produce CXCL8 and recruit neutrophils. Eur J Immunol. 2011;41(2):306-12.

193. Goral A, Sledz M, Manda-Handzlik A, Cieloch A, Wojciechowska A, Lachota M, Mroczek A, Demkow U, Zagozdzon R, Matusik K, Wachowska M, Muchowicz A. Regulatory T cells contribute to the immunosuppressive phenotype of neutrophils in a mouse model of chronic lymphocytic leukemia. Exp Hematol Oncol. 2023;12(1):1-16.

194. Ou Q, Power R, Griffin MD. Revisiting regulatory T cells as modulators of innate immune response and inflammatory diseases. Front Immunol. 2023;14(2):1-16.

195. Rudensky AY, Campbell DJ. In vivo sites and cellular mechanisms of T reg cell-mediated suppression. J Exp Med. 2006;203(3):489-92.

196. Piao W, Xiong Y, Li L, Saxena V, Smith KD, Hippen KL, Paluskievicz C, Willsonshirkey M, Blazar BR, Abdi R, Bromberg JS. Regulatory T Cells Condition Lymphatic Endothelia for Enhanced Transendothelial Migration. Cell Rep. 2020;30(4):1052-62.

197. de Haij S, Jansen JH, Boross P, Beurskens FJ, Bakema JE, Bos DL, Martens A, Verbeek JS, Parren PW, van de Winkel JG, Leusen JH. In vivo cytotoxicity of type I CD20 antibodies critically depends on Fc receptor ITAM signaling. Cancer Res. 2010;70(8):3209-17.

198. Boross P, van Montfoort N, Stapels DA, van der Poel CE, Bertens C, Meeldijk J, Jansen JH, Verbeek JS, Ossendorp F, Wubbolts R, Leusen JH. FcRgamma-chain ITAM signaling is critically required for cross-presentation of soluble antibody-antigen complexes by dendritic cells. J Immunol. 2014;193(11):5506-14.

199. Overdijk MB, Jansen JH, Nederend M, Lammerts van Bueren JJ, Groen RW, Parren PW, Leusen JH, Boross P. The Therapeutic CD38 Monoclonal Antibody Daratumumab Induces Programmed Cell Death via Fcgamma Receptor-Mediated Cross-Linking. J Immunol. 2016;197(3):807-13.

200. Lehmann CHK, Baranska A, Heidkamp GF, Heger L, Neubert K, Luhr JJ, Hoffmann A, Reimer KC, Bruckner C, Beck S, Seeling M, Kiessling M, Soulat D, Krug AB, Ravetch JV, Leusen JHW, Nimmerjahn F, Dudziak D. DC subset-specific induction of T cell responses upon antigen uptake via Fcgamma receptors in vivo. J Exp Med. 2017;214(5):1509-28.

201. Monach PA, Nigrovic PA, Chen M, Hock H, Lee DM, Benoist C, Mathis D. Neutrophils in a mouse model of autoantibody-mediated arthritis: critical producers of Fc receptor gamma, the receptor for C5a, and lymphocyte function-associated antigen 1. Arthritis Rheum. 2010;62(3):753-64.

202. Kovacs M, Nemeth T, Jakus Z, Sitaru C, Simon E, Futosi K, Botz B, Helyes Z, Lowell CA, Mocsai A. The Src family kinases Hck, Fgr, and Lyn are critical for the generation of the in vivo inflammatory environment without a direct role in leukocyte recruitment. J Exp Med. 2014;211(10):1993-2011.

203. Nemeth T, Futosi K, Sitaru C, Ruland J, Mocsai A. Neutrophil-specific deletion of the CARD9 gene expression regulator suppresses autoantibody-induced inflammation in vivo. Nat Commun. 2016;7(1):1-13.

204. Furman D, Campisi J, Verdin E, Carrera-Bastos P, Targ S, Franceschi C, Ferrucci L, Gilroy DW, Fasano A, Miller GW, Miller AH, Mantovani A, Weyand CM, Barzilai N, Goronzy JJ, Rando TA, Effros RB, Lucia A, Kleinstreuer N, Slavich GM. Chronic inflammation in the etiology of disease across the life span. Nat Med. 2019;25(12):1822-32.

9. Bibliography of the candidate's publications

9.1 Publications included in the dissertation

I. **Aradi P.**, Kovács G., Kemecsei É., Molnár K., Sági S.M., Horváth Z., Mehrara B.J., Kataru R.P., Jakus Z. (2024) *Lymphatics-dependent modulation of the sensitization and elicitation phases of contact hypersensitivity*. J Invest Dermatol, 26:S0022-202X(24)00261-6; IF: 5.7

II. Németh T., Futosi K., Szabó M., **Aradi P.**, Saito T., Mócsai A., Jakus Z. (2019) *Importance of Fc Receptor γ-Chain ITAM Tyrosines in Neutrophil Activation and in vivo Autoimmune Arthritis*. Front Immunol, 10(1):252; IF: 5.085

9.2 Publications not included in the dissertation

III. Szőke D., Kovács G., Kemecsei É., Bálint L., Szoták-Ajtay K., **Aradi P.**, Styevkóné Dinnyés A., Mui B.L., Tam Y.K., Madden T.D., Karikó K., Kataru R.P., Hope M.J., Weissman D., Mehrara B.J., Pardi N, Jakus Z. (2021) *Nucleoside-modified VEGFC mRNA induces organ-specific lymphatic growth and reverses experimental lymphedema*. Nat Commun, 12(1):3460; IF: 17.694

IV. Bálint L., Ocskay Z., Deák B.A., **Aradi P.**, Jakus Z. (2020) Lymph Flow Induces the *Postnatal Formation of Mature and Functional Meningeal Lymphatic Vessels*. Front Immunol, 10(1):3043; IF: 7.561

V. Csete D., Simon E., Alatshan A., **Aradi P.**, Dobó-Nagy C., Jakus Z., Benkő S., Győri D.S., Mócsai A. (2019) *Hematopoietic or Osteoclast-Specific Deletion of Syk Leads to Increased Bone Mass in Experimental Mice*. Front Immunol, 10(1):937; IF: 5.085

VI. Reed H.O., Wang L., Sonett J., Chen M., Yang J., Li L., **Aradi P.**, Jakus Z., D'Armiento J., Hancock W.W., Kahn M.L. (2019) *Lymphatic impairment leads to pulmonary tertiary lymphoid organ formation and alveolar damage*. J Clin Invest, 129(6):2514-2526; IF: 11.864

VII. Szarka E., **Aradi P.**, Huber K., Pozsgay J., Végh L., Magyar A., Gyulai G., Nagy G., Rojkovich B., Kiss É., Hudecz F., Sármay G. (2018) *Affinity Purification and Comparative Biosensor Analysis of Citrulline-Peptide-Specific Antibodies in Rheumatoid Arthritis*. Int J Mol Sci, 19(1):326; IF: 4.183

10. Acknowledgements

First and foremost, I extend my sincere gratitude to my supervisor, Dr. Zoltán Jakus, whose guidance and support have been invaluable in navigating my research journey. I am deeply thankful for his insightful ideas and invaluable advice throughout my entire PhD career.

I also wish to express my gratitude to Prof. Dr. László Hunyady, the former head of the Physiology Department, and to Attila Mócsai, the current head of the Physiology Department, for their support and encouragement. Additionally, I am grateful to Prof. Dr. Erzsébet Ligeti and Prof. Dr. Péter Enyedi, the chairpersons of the Molecular Medicine Program of the PhD School, for their attention and guidance.

Special thank goes to all members of the Lymphatic Physiology Research Group of the Hungarian Academy of Sciences. I am particularly grateful to Éva Kemecsei and Dr. Zalán Horváth for their assistance during experiments, Dr. Gábor Kovács for his expertise in mouse foot injections, Kitti Szoták-Ajtay for her guidance in basic histological experiments, and László Bálint for his unwavering support and assistance throughout my PhD journey. I also want to acknowledge Eszter Marinkás-Puszta and Valéria Németh for their outstanding contributions to laboratory work, as well as all others who have brightened my days during the past few years.

I extend my thanks to Janka Zsófia Csepregi and Anita Orosz from the Mócsai laboratory for their guidance and assistance with the contact hypersensitivity mouse model.

This work was supported by the National Research, Development and Innovation Office (K139165, TKP2021-EGA-29, TKP2021-EGA-24, NVKP_16-1-2016-0039), the European Union and the Hungarian Government (VEKOP-2.3.2-16-2016-00002, VEKOP-2.3.3-15-2016-00006, EFOP-3.6.3-VEKOP-16-2017-00009). Z.J. is a recipient of the János Bolyai Research Scholarship of the Hungarian Academy of Sciences (BO/00898/22) and the New National Excellence Program of the Ministry for Culture and Innovation from the source of the National Research, Development and Innovation Fund (UNKP-23-5-SE-10).

Lastly, I am deeply grateful to my family and friends for their unwavering support and encouragement throughout this journey. They have been my pillars of strength during challenging times.