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Host Immune Evasion by the *Pseudomonas Aeruginosa* Virulence Factor LecB

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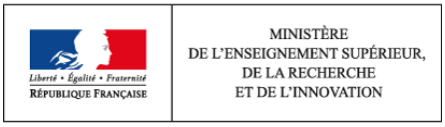

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Résumé

Introduction

Pseudomonas aeruginosa est une bactérie Gram-négative très répandue qui est naturellement présente dans le sol et l'eau (Green et al., 1974; Schroth et al., 2018). Elle fait partie de la microflore cutanée des animaux et des humains mais provoque rarement des infections chez les organismes sains (Cogen et al., 2008). Dans les hôpitaux, cependant, elle pose un risque sérieux pour la santé et fait partie des agents pathogènes les plus problématiques qui affectent généralement les personnes dont le système immunitaire est affaibli (Andonova and Urumova, 2013). Ainsi, les infections chez les patients atteints de mucoviscidose, de cancer et de brûlures sont très courantes et peuvent engendrer des effets allant d'infections de la peau, des oreilles, des voies urinaires et des poumons jusqu'aux bactériémie et septicémie des brûlures et blessures (Gellatly and Hancock, 2013). Ces infections se caractérisent par une morbidité et une mortalité élevées (Chatterjee et al., 2016) dues à la remarquable adaptabilité, variabilité, au développement de la résistance ainsi qu'à la formation de biofilms du pathogène (Andonova and Urumova, 2013).

En raison de sa résistance à de nombreux antibiotiques, le traitement des infections causées par *Pseudomonas aeruginosa* est difficile et nécessite de nouvelles stratégies thérapeutiques. L'une de ces approches thérapeutiques alternatives aux antibiotiques est le développement de substances qui agissent sur les facteurs de virulence de cette bactérie (Chatterjee et al., 2016; Grishin et al., 2015). Les facteurs de virulence sont constitués de molécules cellulaires ou extracellulaires qui facilitent l'invasion bactérienne et son évasion de la défense de l'hôte permettant sa dissémination (Johnson, 2018; Sharma et al., 2017). Comme le ciblage de ces facteurs n'affecte pas directement la viabilité bactérienne mais seulement leur capacité à infecter les humains, la résistance à ces composés antivirulents devrait se développer plus lentement que celle aux agents antimicrobiens et représente ainsi un outil prometteur (Grishin et al., 2015).

L'un de ces facteurs de virulence de *Pseudomonas aeruginosa* est la lectine LecB, une petite protéine soluble de 47 kDa qui se fixe au fucose (Imberty et al., 2004) et qui a été initialement isolée de l'isolat clinique PAO1 par Gilbao-Farber et *al*. dans les années 1970 comme protéine capable d'agglomérer les érythrocytes humains et animaux (Gilboa-Garber, 1972). *In vitro*, LecB s'est révélée impliquée dans la fixation du pathogène aux cellules humaines et animales (Boukerb et al., 2014; Chemani et al., 2009; Noskova et al., 2015;

Wentworth et al., 1991) ainsi que dans la formation de biofilm (Johansson et al., 2008; Tielker et al., 2005). Cependant, son rôle dans l'infection *in vivo* est encore incertain en particulier son impact sur la réponse immunitaire cutanée.

La peau est l'un des principaux sites d'infection et les complications des infections cutanées par *P. aeruginosa* entraînent le plus souvent une bactériémie. Les pathogènes ou antigènes envahissant la peau sont normalement efficacement capturés par les cellules dendritiques qui migrent ensuite hors de la peau à travers les vaisseaux lymphatiques vers les ganglions lymphatiques drainant la peau (Worbs et al., 2016). Ils peuvent également être transportés directement vers les ganglions lymphatiques drainant vers la lymphe. Au sein du ganglion lymphatique les antigènes et les pathogènes sont capturés par les macrophages et les cellules dendritiques résidents et traités pour leur présentation aux lymphocytes naïfs afin de déclencher une réponse immunitaire protectrice spécifique du pathogène (Carrasco and Batista, 2007; Junt et al., 2007; Worbs et al., 2016). Cependant, *P. aeruginosa* produit une série de facteurs de virulence qui aident à éluder le système de défense de l'hôte, lui permettant de persister dans les tissus et de se disséminer dans tout l'organisme (Gellatly and Hancock, 2013; Johnson, 2018). Etant donné son rôle dans l'adhésion cellulaire (Boukerb et al., 2014; Chemani et al., 2009; Wentworth et al., 1991) et la formation de biofilms (Johansson et al., 2008; Tielker et al., 2005), LecB peut également jouer un rôle important dans l'évasion de la réponse immunitaire de l'hôte. Par conséquent, une meilleure compréhension de l'interaction entre cette lectine et les cellules immunitaire cutanées et ganglionnaires favoriserait probablement le développement de nouvelles approches préventives et/ou thérapeutiques chez les patients.

Résultats

Afin d'étudier l'impact de la lectine LecB de *Pseudomonas aeruginosa* sur les ganglions lymphatiques drainant la peau (sdLN), nous avons d'abord cherché à identifier les cellules cibles de LecB dans ces ganglions lymphatiques et ensuite à étudier son impact sur ces cellules.

Afin d'identifier les cellules cibles potentielles de LecB dans les ganglions lymphatiques drainant la peau, nous avons injecté LecB purifiée, marquée par fluorescence par voie intradermique dans le pavillon de l'oreille et par voie sous-cutanée dans la patte arrière de souris C57BL/6 pour cibler respectivement les sdLN auriculaires et poplités. Les cellules

cibles de LecB dans ces ganglions lymphatiques ont été analysées par immunofluorescence et cytométrie en flux. Nous avons constaté que LecB se liait à toutes les cellules dendritiques résidantes dans les ganglions lymphatiques et dérivées de la peau ainsi qu'à tous les types de macrophages ganglionnaires, avec une plus grande affinité pour les macrophages sous-capsulaires et médullaires associés aux sinus. Cependant, nous avons trouvé que LecB se liait particulièrement aux cellules endothéliales lymphatiques et sanguines, dont les veinules à endothélium épais.

Sachant que les veinules à endothélium épais sont médiatrices de l'entrée des lymphocytes du sang dans le ganglion lymphatique, nous avons étudié si la liaison de LecB à ces cellules endothéliales affecte l'entrée des lymphocytes. Vingt-quatre heures après l'administration intradermique ou sous-cutanée de LecB, le nombre de cellules totales dans les ganglions lymphatiques drainant a triplé. Cette augmentation du nombre de cellules est principalement attribuable à une augmentation des lymphocytes B et T et, dans une moindre mesure, à une augmentation des monocytes inflammatoires et des neutrophiles. La mesure de l'entrée des cellules après injection intraveineuse de lymphocytes B et T traçables a toutefois montré que cette augmentation n'était pas due à une infiltration accélérée des lymphocytes.

Comme LecB se fixe également aux cellules endothéliales lymphatiques et que ces cellules régulent la sortie des lymphocytes des ganglions lymphatiques, nous avons analysé si une sortie limitée des lymphocytes par LecB pouvait expliquer l'augmentation des lymphocytes dans les ganglions lymphatiques après injection de LecB.

Nous avons mesuré le nombre de cellules après blocage de l'entrée des lymphocytes du sang dans le ganglion lymphatique par l'injection d'un anticorps anti-CD62L dans des souris C57BL/6 avant l'injection de LecB ou de PBS. L'inhibition de la sortie par LecB maintiendrait le nombre de lymphocytes, contrairement à une diminution du nombre de lymphocytes dans les ganglions lymphatiques drainant au PBS. Le nombre de lymphocytes B et T dans les ganglions lymphatiques drainant au PBS a été significativement réduit après le blocage de l'entrée par rapport au contrôle non traité et cette diminution a été récupérée par LecB, indiquant une inhibition de la sortie des lymphocytes par LecB.

Afin d'élucider le mécanisme par lequel LecB inhibe la sortie des lymphocytes du ganglion lymphatique, nous avons analysé les molécules d'adhésion des cellules endothéliales lymphatiques impliquées dans la migration des lymphocytes dans le ganglion lymphatique par immunofluorescence et cytométrie en flux. Vingt-quatre heures après l'injection sous-cutanée de LecB dans la patte arrière de souris C57BL/6, nous avons constaté une diminution significative de l'intensité médiane de fluorescence de la protéine de surface cellulaire

« murine chloride channel calcium-activated 1 » (mCLCA1) sur les cellules endothéliales lymphatiques des ganglions lymphatiques poplités, alors que l'intensité médiane de fluorescence de la molécule d'adhésion « vascular cell adhesion protein 1 » (VCAM-1) est considérablement accrue. Étant donné que mCLCA1 sur les cellules endothéliales lymphatiques se lie fortement à l'intégrine « lymphocyte function-associated antigen 1 » (LFA-1) sur les lymphocytes B et T, permettant ainsi leur adhésion à l'endothélium lymphatique (Furuya et al., 2010), un déclin de cette protéine de surface pourrait expliquer la sortie limitée des lymphocytes du ganglion lymphatique par LecB.

De plus, nous avons montré *in vitro* par microscopie confocale que l'exposition de cellules endothéliales primaires humaines à LecB modifie considérablement l'organisation de l'actine, ce qui suggère une fonction de barrière endothéliale renforcée qui pourrait également expliquer l'inhibition de la sortie des lymphocytes du ganglion.

Conclusion

En conclusion, nous avons montré que la lectine LecB de *Pseudomonas aeruginosa*, lorsqu'elle est injectée dans la peau, se draine dans les ganglions lymphatiques par la lymphe, où elle se fixe aux cellules endothéliales lymphatiques et sanguines ainsi qu'aux macrophages associés aux sinus. LecB a inhibé la sortie des lymphocytes B et T hors du ganglion lymphatique, ce qui a entraîné une accumulation de lymphocytes dans le ganglion lymphatique. Cette inhibition de la sortie pourrait être due au déclin de la protéine mCLCA1 sur les cellules endothéliales lymphatiques observé avec LecB; mCLCA1 étant impliqué dans l'adhésion des lymphocytes à ces cellules. La pertinence physiologique de LecB sur la sortie des lymphocytes des ganglions lymphatiques reste à élucider dans le contexte d'une réponse immunitaire chez des souris immunisées. Il est possible que l'inhibition de la sortie par LecB affecte la sortie des plasmocytes des ganglions lymphatiques vers la moelle osseuse et limite ainsi la production à long terme d'anticorps. Nous allons donc immuniser les souris par injection sous-cutanée d'ovalbumine avec un adjuvant en l'absence et en présence de LecB. Nous déterminons le titre d'anticorps anti-ovalbumine dans le sérum par un dosage immuno-enzymatique (ELISA) et le nombre de plasmocytes qui ont quitté les ganglions lymphatiques vers la moelle osseuse.

Nous nous attendons à ce que ces résultats donnent un aperçu important sur la compréhension actuelle des mécanismes régissant l'évasion immunitaire de *Pseudomonas aeruginosa*. Ceci

pourrait ainsi expliquer la persistance et la récurrence des infections en séquestrant des plasmocytes protecteurs produisant des anticorps dans le ganglion lymphatique. Par conséquent, les approches thérapeutiques contre LecB pourraient fournir un nouveau moyen contre *Pseudomonas aeruginosa* et éventuellement ouvrir la voie au développement de vaccins.

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List of Abbreviations

A

ACAID: anterior chamber associated immune deviation

ACKR4: atypical chemokine receptor 4

ADP: adenosine diphosphate

AJ: adherens junction

APC: antigen-presenting cell

B

BCR: B-cell receptor

BEC: blood endothelial cell

C

cAMP: cyclic adenosine monophosphate

CCL: chemokine C-C motif ligand

CCR: C-C chemokine receptor

CF: cystic fibrosis

Con A: concanavalin A

CR: cysteine-rich

CRD: carbohydrate-recognition domain

CTL: C-type lectin

D

DC: dendritic cell

dDC: dermal dendritic cell

E

E. coli: *Escherichia coli*

EGF: epidermal growth factor

ENA-78: epithelial neutrophil-activating protein-78

EPS: extracellular polymeric substances

ER: endoplasmic reticulum

ESAM: endothelial cell-selective adhesion molecule

F

FDC: follicular dendritic cell

FRC: fibroblastic reticular cell

G

GlyCAM-1: glycosylation-dependent cell adhesion molecule-1

GRK2: guanine nucleotide-binding protein-coupled receptor kinase-2

GTP: guanosine triphosphate

H

HA: hyaluronic acid

HEV: high endothelial venule

HIV: human immunodeficiency virus

HSPG: heparin sulfate proteoglycan

HUVEC: human umbilical vein endothelial cell

I

ICAM: intercellular adhesion molecule

IFN-I: type I interferon

IFN-γ: interferon-gamma

IGF-1R: insulin like growth factor-1 receptor

IL-22: interleukin 22

ILP: invadosome-like protrosions
IPTG: isopropyl-β-D-thiogalactoside

J

JAM: junctional adhesion molecule

L

LBRC: lateral border recycling compartment
LC: Langerhans cell
Le^a: Lewis a
LEC: lymphatic endothelial cell
LFA-1: lymphocyte f unction-associated antigen 1
LN: lymph node
LPAM: lymphocyte Peyer patch adhesion molecule
LPS: lipopolysaccharide
LYVE-1: lymphatic vessel endothelial hyaluronan receptor 1

M

MAdCAM-1: mucosal addressin cell adhesion molecule 1
MARCO: macrophage receptor with collagenous structure
MBP: mannose-binding protein
MCM: medullary sinus macrophage
MCMV: murine cytomegalovirus
MCP-1: monocyte chemotactic protein-1
MHC-II: major histocompatibility complex class II
MLC: myosin light chain
MLCK: myosin light chain kinase
MLCP: myosin light chain phosphatase
MR: mannose receptor
MSM: medullary sinus macrophage

N

NADPH: nicotinamide adenine dinucleotide phosphate
NF-κB: nuclear factor-kappa B
NHK: normal human keratinocyte
NK T cell: natural killer T cell
NOD: nucleotide-binding oligomerization domain-containing protein
NPG: p-nitrophenyl-α-D-galactoside

P

PECAM-1: platelet endothelial cell adhesion molecule 1
PHA: phytoheamagglutinin
PLVAP: plasmalemma vesicle-associated protein
PNAd: peripheral-node addressin
PP1c: protein phosphatase 1c
PRR: pattern recognition receptor
PWM: pokeweed mitogen
Pyk2: proline-rich tyrosine kinase 2

R

RANTES: regulated on activation, normal T cell expressed and secreted
ROCK: Rho kinase
ROS: reactive oxygen species

S

S1P: sphingosine-1-phosphate

S1PR1: sphingosine-1-phosphate receptor
SCS: subcapsular sinus
Siglec: sialic acid-binding immunoglobulin-like lectin
SphK: sphingosine kinase
SSM: subcapsular sinus macrophage

T

T2SS: type II secretion system
T3SS: type III secretion system
TEM: transendothelial migration
TJ: tight junction
TLR: Toll-like receptor
TM: transmembrane
TNF-α: tumor necrosis factor alpha
T_{reg}: regulatory T cell

V

VCAM: vascular cell adhesion molecule
VE-cadherin: vascular endothelial cadherin
VEGF: vascular endothelial cell growth factor
VE-PTP: vascular endothelial-phosphotyrosine phosphatase
VLA-4: very late antigen-4
VSV: vesicular stomatitis virus

W

WGA: wheat germ agglutinin
WHO: World Health Organization

Z

ZO: zonula occludens

INTRODUCTION

Introduction

Pseudomonas aeruginosa is a widespread, Gram-negative bacterium that is ubiquitously present in nature (Spiers et al., 2000). As an opportunistic pathogen, it rarely provokes infections in healthy organisms but poses a serious health risk in hospitals, where it typically affects people with a weakened immune system or limited epithelial barrier function (Andonova and Urumova, 2013; Gales et al., 2001). As such, infections in cystic fibrosis, cancer and burn wound patients are very common and range from skin, ear, urinary tract and lung infections to bacteraemia and wound/burn sepsis (Gellatly and Hancock, 2013). These infections demonstrate a high morbidity and mortality (Chatterjee et al., 2016) due to the adaptability, development of antimicrobial resistance and biofilm formation of the pathogen (Andonova and Urumova, 2013). Due to its resistance to many antibiotics, therapy of *P. aeruginosa* infections is challenging and calls for novel treatment strategies. One of these alternative treatment approaches to antibiotics is the design of substances that act on the virulence factors of this pathogen (Chatterjee et al., 2016; Grishin et al., 2015). Virulence factors consist of cell-associated or extracellular molecules that facilitate the bacterial invasion into the host and evasion from host defense, enabling dissemination (Johnson, 2018). As targeting these factors does not directly affect bacterial viability, but only impacts their ability to infect humans, resistance to these compounds is expected to develop more slowly than to antimicrobial agents and represents a promising tool (Grishin et al., 2015).

One of these virulence factors of *P. aeruginosa* is the lectin LecB, a small, soluble, fucose-binding protein (Gilboa-Garber et al., 2000). *In vitro*, LecB has been shown to be involved in the attachment of the pathogen to human and animal cells (Boukerb et al., 2014; Chemani et al., 2009; Nosková et al., 2015; Wentworth et al., 1991) as well as in biofilm formation (Johansson et al., 2008; Tielker et al., 2005). Its role in *in vivo* infection, however, is still uncertain, especially its impact on the cutaneous immune response. The skin is one of the main infection sites, and complications of *P. aeruginosa* skin infections most commonly result in bacteraemia. Skin-invading pathogens or antigens are normally efficiently captured by dendritic cells (DCs), which then migrate out of the skin through lymphatic vessels into skin-draining lymph nodes (LNs) (Worbs et al., 2016), or are directly transported to the draining LNs via the lymph. Within the LN, antigens and pathogens are captured by resident macrophages and DCs and processed for their presentation to naïve lymphocytes to mount a protective pathogen-specific immune response (Carrasco and Batista, 2007; Junt et al., 2007;

Worbs et al., 2016). *P. aeruginosa*, however, produces an array of virulence factors that help to elude the host defense system enabling it to persist in the tissue and to disseminate throughout the body (Gellatly and Hancock, 2013; Johnson, 2018). Given its role in cell adhesion (Boukerb et al., 2014; Chemani et al., 2009; Wentworth et al., 1991) and biofilm formation (Johansson et al., 2008; Tielker et al., 2005), LecB may also play an important role in the evasion of the host immune response. Hence, a better understanding of the interaction between this lectin and the cutaneous and lymph node immune cells could be useful for the development of novel preventative and/or therapeutic approaches in infected patients.

1 Pseudomonas aeruginosa

Pseudomonas aeruginosa is a common Gram-negative bacterium that naturally occurs in soil and water (Green et al., 1974; Schroth et al., 2018). Due to its ability to survive under a variety of environmental conditions (e.g., at temperatures ranging from 4 to 42°C) (LaBauve and Wargo, 2012) and to use a wide range of environmental compounds as nutrient source (Spiers et al., 2000), this bacterium is ubiquitously present and has been found amongst other in hospital waste water, respiratory equipment, solutions, medicines, disinfectants, sinks, mops, food mixture and vegetables (Schwartz et al., 2015). This versatility probably arises from its large genome (5.2 – 7.1 Mbp) that is rich in regulatory genes, allowing the bacterium to colonize diverse ecological niches (Spiers et al., 2000). *P. aeruginosa* is also a member of the human natural microflora and can be found on the skin and in the mouth but rarely provokes infections in healthy organisms (Cogen et al., 2008). In hospitals, however, it poses a serious health risk and is one of the most problematic hospital-acquired pathogens due to its ubiquitous nature (Andonova and Urumova, 2013). As an opportunistic pathogen, it typically affects immunocompromised people or people with breached epithelia resulting from intravenous lines, urinary catheters, or endotracheal tubes (Gales et al., 2001). Consequently, infections in patients suffering from AIDS, neutropenia, cystic fibrosis, cancer and burn wounds are very common and range from skin, ear, urinary tract and lung infections to bacteraemia and wound/burn sepsis (Gellatly and Hancock, 2013). These infections are marked by a high morbidity and mortality (Chatterjee et al., 2016), arising from the remarkable adaptability, variability, development of resistance as well as biofilm formation of the pathogen (Andonova and Urumova, 2013).

In the acute phase of infection, *P. aeruginosa* synthesizes a potent array of cell-associated and extracellular virulence factors **(Figure 1)** that are critical for the initial colonization of the pathogen (Smith et al., 2006). They not only promote the adhesion of the pathogen to the host cell but also enable the pathogen to evade and modulate host defense systems for tissue invasion and dissemination (Johnson, 2018). A single polar flagellum and several shorter type IV pili found at a cell pole confer motility and function as main adhesins, allowing the pathogen to bind to host epithelial cells (Feldman et al., 1998; Persat et al., 2015). In a murine *P. aeruginosa* burn infection model, both flagellum and pili have been shown to enhance the virulence of the pathogen by increasing its colonization and dissemination throughout the host organism (Sato et al., 1988). *P. aeruginosa* possess five protein secretion systems, of which the type II (T2SS) and type III (T3SS) secrete the majority of the known toxins (Jyot et

al., 2011). Upon cell contact, the T3SS is activated (Bleves et al., 2010). The T3SS consists of a needle complex on the bacterial surface that injects toxic effector proteins from the bacterial cytosol directly into the host cells (Hauser, 2009). The four secreted effector proteins ExoS, ExoT, ExoU and ExoY mimic and thereby subvert the activity of host proteins (Bleves et al., 2010). ExoS and ExoT both function as guanosine triphosphate (GTP)ase-activating proteins and adenosine diphosphate (ADP)-ribosyltransferases (Hauser, 2009). As such, they have been shown to disrupt the host cell actin cytoskeleton, which is associated with decreased phagocytosis and reduced cell-cell adherence, which facilitate the penetration of the pathogen and promote its persistence and dissemination (Frithz-Lindsten et al., 1997; Garrity-Ryan et al., 2000). ExoU is a potent phospholipase A_2, which is highly cytotoxic and has been shown to disrupt cellular membranes and to induce rapid necrotic cell death of host cells (Finck-Barbancon et al., 1997; Hauser et al., 1998; Sato and Frank, 2004). ExoY has been characterized as an adenylyl cyclase, which increases the intracellular cyclic adenosine monophosphate (cAMP) concentration in mammalian cells (Hauser, 2009). The resulting effects include increased endothelial permeability (Sayner et al., 2004), disruption of the actin cytoskeleton and inhibition of bacterial internalization (Cowell et al., 2005). The T2SS secretes exotoxin A, which functions as ADP-ribosyltransferase and inhibits eukaryotic protein biosynthesis, thereby inducing apoptosis of host cells (Michalska and Wolf, 2015). Moreover, the T2SS secretes several proteases with broad substrate specificity and that often act synergistically in the degradation of connective tissues and important host immune effectors. The elastases LasA and LasB as well as protease IV, for instance, have been shown to degrade elastin (Cowell et al., 2003; Kessler et al., 1997; Wilderman et al., 2001) and collagen (Heck et al., 1986). Components of the immune system targeted and degraded by the secreted proteases include immunoglobulin (Döring et al., 1981; Heck et al., 1990), cytokines/chemokines (e.g., tumor necrosis factor alpha (TNF-α), interferon-gamma (IFN-γ) (Parmely et al., 1990), interleukin 22 (IL-22) (Bradshaw et al., 2018), chemokine C-C motif ligand 5 (CCL5/RANTES), monocyte chemotactic protein-1 (MCP-1), and epithelial neutrophil-activating protein-78 (ENA-78) (Leidal et al., 2003)) as well as complement (Hong and Ghebrehiwet, 1992; Schultz and Miller, 1974).

Other virulence factors contributing to the pathogenesis of *P. aeruginosa* include siderophores (e.g., pyoverdin, iron-chelating molecules) (Peek et al., 2012), pyocyanin, alginate, hemolysins, and the lectins LecA and LecB (Chatterjee et al., 2016).

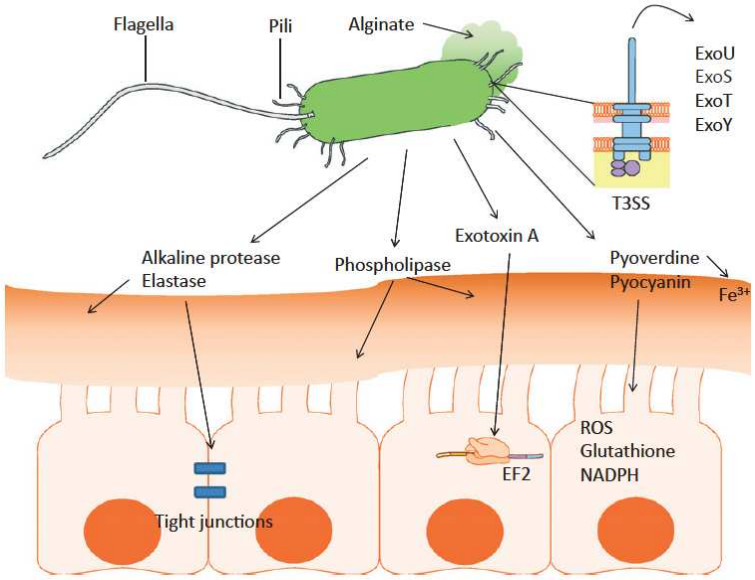


Figure 1. Virulence factors produced by *Pseudomonas aeruginosa*

The adhesins flagella and type IV pili mediate de binding of the bacteria to the host, which activates the T3SS for injection of toxic effector proteins directly into the host cell. Several proteases degrade connective tissues and host immune effectors, which facilitates the invasion and the dissemination of the pathogen throughout the body. Secreted phospholipase can disrupt host cell membranes, whereas exotoxin A induces cell apoptosis by inhibiting host cell protein biosynthesis. Pyoverdine chelates iron ions and pyocyanin interferes with host cell electron transport pathways and redox cycling. Reprinted from (Gellatly and Hancock, 2013) with permission.

Besides the intrinsic host immune evasion strategies mentioned above, which impede the clearance of the pathogen by the immune system, the high antibiotic resistance in *P. aeruginosa* complicates the treatment of infections (Pang et al., 2019). Consistent with this, the World Health Organization (WHO) classed *P. aeruginosa* in 2017 among the three most critical multidrug-resistant bacteria, for which there is an urgent need for the development of new antibiotics for the treatment of infections (Tacconelli et al., 2017). *P. aeruginosa* disposes several mechanisms to counteract the action of antibiotics, which can be classified into intrinsic, acquired and adaptive resistance (Pang et al., 2019). The intrinsic resistance is based on the pathogen low outer membrane permeability that restricts the access of antibiotics into the bacterial cells (Angus et al., 1982; Yoshimura and Nikaido, 1982). Indeed, the membrane permeability of *P. aeruginosa* has been shown to be 12- to 100-fold lower than that of *Escherichia coli* (Bellido et al., 1992; Yoshimura and Nikaido, 1982). In addition, *P. aeruginosa* possesses efflux pumps that actively export antibiotics out of the cell (Pan et al.,

2016), and it produces antibiotic-inactivating enzymes, such as β-lactamases and aminoglycoside-modifying enzymes, which degrade or modify antibiotics (Pang et al., 2019). The acquired resistance can result from horizontal transfer of resistance genes or mutational changes, which contributes to the development of multi-drug-resistance strains (Breidenstein et al., 2011). In addition, multiple triggers, including antibiotics or environmental stimuli such as biocides, polyamines, pH, anaerobiosis, cations and carbon sources or biofilm formation can induce adaptive antibiotic resistance by modulating the expression of genes encoding efflux pumps, cell envelope components or enzymes (Breidenstein et al., 2011).

Biofilms consist of organized bacterial communities that grow on a surface embedded in a matrix of extracellular polymeric substances (EPS) composed of polysaccharides (alginate, Pel and Psl), proteins, and DNA synthesized by these bacteria (Rasamiravaka and Labtani, 2015). The EPS matrix provides a physical and chemical barrier shielding the bacteria from unfavorable environmental conditions, host immune responses, mechanical forces, and antimicrobial agents (Hall-Stoodley and Stoodley, 2009; Lieleg et al., 2011). Hence, biofilm-associated infections are often untreatable and develop into a chronic state (Bjarnsholt, 2013). *P. aeruginosa* isolates from chronic infections have been shown to differ phenotypically from isolates of acute infections. In contrast to acute infection, many virulence factors are downregulated in the chronic state, including the T3SS, flagella and pili, which renders the isolates less immunostimulatory and less cytotoxic, whereas the bacteria acquire a mucoid phenotype, characterized by the overproduction of the EPS alginate (Hogardt and Heesemann, 2010).

The bacterial virulence factors and biofilm formation are regulated by quorum sensing (Breidenstein et al., 2011). Quorum sensing represents a cell-cell communication system in single-celled organisms that is based on diffusible, low-molecular-weight signal molecules (Lee and Zhang, 2015). When a critical cell population density is reached, these signal molecules accumulate and diffuse through the bacterial cell envelope, where they activate transcriptional regulators resulting in a coordinated regulation of gene expression (Smith and Iglewski, 2003).

2 Lectins

2.1 Lectins in general

The term “lectin” was originally introduced to describe a heterogeneous class of plant seed-derived proteins that were able to agglutinate erythrocytes in a blood group-specific manner (Boyd and Shapleigh, 1954). Since the agglutinating property of these proteins has been found to be based on their specific binding to saccharides on the surface of erythrocytes, Goldstein et *al*. proposed a new definition of a lectin as “a sugar-binding protein of nonimmune origin that agglutinates cells or precipitates glycoconjugates” (Goldstein et al., 1980). This definition, however, implies that the lectin molecule contains at least two carbohydrate-binding sites, which allows the crosslinking of adjacent cells with complementary cell surface glycoconjugates, and therefore excludes monovalent carbohydrate-binding proteins (Barondes, 1988). Consequently, the definition of lectins was broadened by Barondes and now classifies any “carbohydrate-binding protein other than an enzyme or an antibody” (Barondes, 1988).

Lectins are ubiquitously present and occur in all types of organisms, including animals, plants, bacteria, fungi and viruses **(Figure 2)** (Vasta et al., 2011). Although they share many biological properties, they form a heterogeneous group of proteins that vary in size, composition and structure (Sharon and Lis, 2004). Accordingly, diverse molecular weights have been observed among lectins, ranging from 26 kDa for the wheat germ agglutinin (WGA) to 400 kDa for the horseshoe crab lectin (Sharon and Lis, 1972). Moreover, lectins exist in both soluble and cell-associated form (Goldstein et al., 1980). Their diversity can be best exemplified by two types of animal lectins. Based on their carbohydrate-recognition domain (CRD), which mediates the carbohydrate-binding, animal lectins have been classified in structurally related families (Sharon and Lis, 2004). While the most widely occurring C-type lectin (CTL) family comprises mainly large, asymmetric transmembrane glycoproteins with one or more CRDs that require Ca^{2+} for their activity, the family of galectins represents a group of small, soluble, non-glycosylated proteins that are found both intracellularly and extracellularly and function independently of Ca^{2+} (Ghazarian et al., 2011; Gorelik et al., 2001; Sharon and Lis, 2004).

The binding of a lectin with a specific carbohydrate relies on hydrophobic (van der Waals) interactions and hydrogen bonds, whereas the specificity is achieved by specific hydrogen bonds and metal coordination bonds to key hydroxyl groups that differ in different sugars (Drickamer, 1997). Binding to these key hydroxyl groups orientates the sugar in the binding

site, which additionally excludes sterically unwanted recognition and thereby contributes further to the selective binding (Drickamer, 1997).

Consistent with their ubiquitous presence, endogenous lectins are involved in a wide range of biological processes, including cell adhesion, cell proliferation and programmed cell death, glycoprotein trafficking, immune system function and inflammation (Gorelik et al., 2001), some of which will be mentioned below.

Animal lectins have many different functions, but primarily act as recognition molecules within the immune system (Kilpatrick, 2002). The mannose-binding protein (MBP), a member of the collectins, belonging to the C-type lectin family, is an example of a pattern recognition receptor (PRR) of the innate immune system (De Schutter and Van Damme, 2015a). Synthesized by the liver and secreted into the serum (Uemura et al., 2002), this lectin binds to a wide range of carbohydrates, including N-acetyl-D-glucosamine, mannose, N-acetyl-mannosamine, fucose and glucose (De Schutter and Van Damme, 2015a). This broad recognition spectrum allows the interaction with many bacteria, viruses, yeasts, fungi and protozoa for complement activation and lysis of the pathogen (De Schutter and Van Damme, 2015b). Similar to the MBP, the C-type lectin mannose-specific receptor on the surface of macrophages and dendritic cells recognizes and phagocytoses many bacteria, yeasts, parasites and mycobacteria (Stahl and Ezekowitz, 1998).

The family of selectins plays an important role in the recruitment of leukocytes from the blood into inflamed tissue and in the constitutive homing of lymphocytes into lymph nodes (Ley, 2003). These C-type lectins are present on the surface of leukocytes (L-selectins), endothelial cells (E- and P-selectins) and platelets (P-selectins) (De Schutter and Van Damme, 2015a). By binding to fucosylated and sialylated glycoprotein ligands on the endothelial and leukocyte cell surface, respectively, leukocytes are tethered to the vascular wall, which initiates their adhesion cascade for subsequent extravasation from the blood into the inflamed tissue or into the LN (Ley, 2003).

Beside its functions in the immune system, lectins are also involved in intracellular glycoprotein trafficking (Sharon and Lis, 2004). Calnexin, a membrane-bound lectin of the endoplasmic reticulum (ER) and its soluble homolog calreticulin function as molecular chaperones that ensure the proper folding and assembly of newly synthesized glycoproteins for their secretion to the cell surface (Halperin et al., 2014; Sharon and Lis, 2004). Moreover, the mannose-6-phosphate receptors, the only members of the P-type lectin family, are transmembrane glycoproteins that function as sorting receptors for lysosomal enzymes. By

binding to mannose 6-phosphate residues of newly synthesized hydrolases in the Golgi apparatus, they direct these enzymes to the lysosomal compartment (Gary-Bobo et al., 2007). Some plant lectins have been described to have mitogenic properties toward mammalian cells (Sharon and Lis, 2004). Consistent with this, the phytoheamagglutinin (PHA) isolated from the red kidney bean (*Phaseolus vulgaris*) and concanavalin A (Con A) extracted from jack bean (*Canavalia ensiformis*), have been shown to stimulate the proliferation of T lymphocytes by binding to and activation of the T cell receptor (Gorelik et al., 2001). The pokeweed mitogen (PWM), the lectin from *Phytolacca Americana,* has been described to induce proliferation in both T and B lymphocytes (Gorelik et al., 2001). Moreover, several plant lectins, including ricin from the seeds of the castor oil plant (*Ricinus communis*), abrin, found in the seeds of the rosary pea (*Abrus precatorius*), Con A, PHA, and WGA, have been demonstrated to be cytotoxic to various normal and malignant cells by inducing apoptosis (Gorelik et al., 2001).

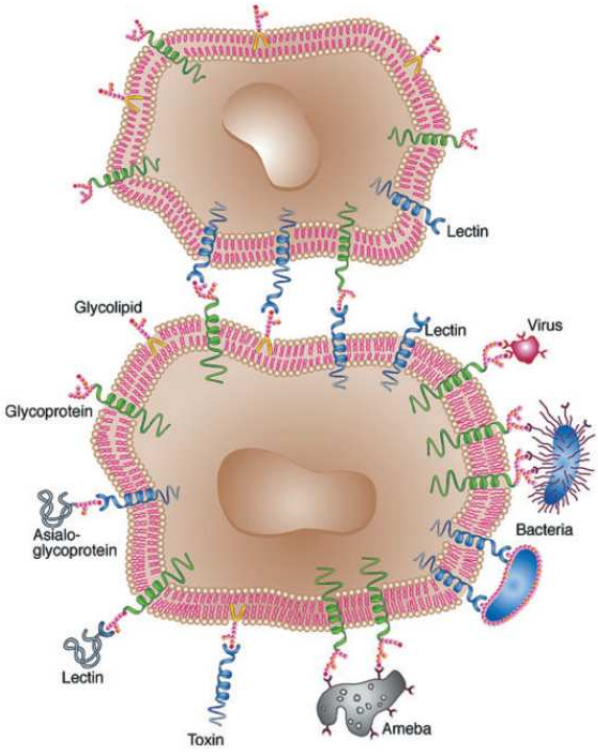


Figure 2. Cell surface lectin – carbohydrate interactions

Lectins recognize and bind to cell surface carbohydrates, glycoproteins or glycolipids and mediate the adhesion of different kind of cells (e.g. animal, plant and bacterial cells) and viruses to other cells. Reprinted from (Sharon and Lis, 2004) with permission.

Bacterial lectins are found in almost all bacteria and are present in secreted form, on the cell surface, in the periplasmic space, intracellularly or on cell fimbriae/ pili or on flagella (Gilboa-Garber et al., 2008). They interact with glycoproteins and glycolipid receptors on host cells, and thereby facilitate the attachment of bacteria to host cells, which precedes bacterial colonization and infection (Gilboa-Garber et al., 2008). Accordingly, bacterial lectins are often called adhesins and most bacteria produce multiple adhesins with different carbohydrate specificities (Ohlsen et al., 2009). The urophathogenic *E. coli* strain 536, for instance, disposes several types of fimbrial adhesins, including mannose-specific type-1 fimbriae, galabiose-specific P fimbriae, and sialyl-galactoside-binding S-fimbriae (Hacker et al., 1990). The carbohydrate specificity of the lectins determines not only the species specificity but also leads to tissue tropism of the pathogen (Ohlsen et al., 2009). Consistent with this, the *E. coli* strain K99 can cause lethal diarrhea in piglets, calves and lambs but not in human. This is due to the specific binding of its fimbrial lectin to glycolipids and glycoproteins containing N-glycolylsialyl residues, which are found in the intestine of the susceptible animals, but not in normal human tissue (Kyogashima et al., 1989).
Although the binding of adhesins to their receptors is generally weak, they often cluster on the membrane, which results in high avidity suited for host invasion (Esko and Sharon, 2009).

2.2 Lectins of Pseudomonas aeruginosa

Pseudomonas aeruginosa produces two soluble lectins, LecA and LecB (formerly named PA-IL and PA-IIL), whose genes *lecA* and *lecB* are unique in the *Pseudomonas* genus (Gilboa-Garber, 1982). They have been initially isolated in the 1970s from the clinical isolate PAO1 by Gilbao-Garber et *al*. as proteins capable of agglutinating human and animal erythrocytes (Gilboa-Garber, 1972). LecA and LecB both occur as a homotetramer (51 and 47 kDa, respectively) **(Figure 3)**, consisting of four subunits of 121 and 114 amino acids, respectively (Imberty et al., 2004). Each subunit of LecA contains one Ca^{2+}-ion (Cioci et al., 2003), whereas each LecB subunit contains two Ca^{2+}-ions, which mediate the binding to carbohydrate ligands (Loris et al., 2003; Mitchell et al., 2002). Among the monosaccharides, LecA exhibits a strict binding specificity for D-galactose, with the exception of N-acetyl-D-galactosamine, which can bind, albeit with a lower affinity than galactose (Cioci et al., 2003). In addition, it binds to multiple glycoproteins and glycosphingolipids, including

glycoconjugates containing terminal non-substituted α-Gal1-4Gal disaccharide (i.e., the human P_1 and P^k blood group antigens) and αGal1-3-Gal disaccharide, the xeno-antigen present on non-human tissues (Cioci et al., 2003; Imberty et al., 2004; Lanne et al., 1994). With a dissociation constant in the micromolar range, LecB disposes among lectin-monosaccharide interactions an unusually high affinity for L-fucose and fucose-containing oligosaccharides, and binds also to mannose and other saccharides with lower affinity (Mitchell et al., 2005). This strong interaction arises from the two Ca^{2+} ions that are, in contrast to other calcium-containing lectins, both involved in the sugar binding (Mitchell et al., 2002). Although the sequence of LecB varies among different *P. aeruginosa* strains, the glycan binding preferences are similar and differ only in their affinities (Sommer et al., 2016). So far, the Lewis a (Le^a) antigen, a fucosylated oligosaccharide present on red blood cells, platelets and lymphocytes and diverse human tissue and in soluble form in the salvia, milk and other body fluids (Velliquette and Westhoff, 2019), has been identified as the highest affinity natural ligand for this lectin with a K_D value of 200 nM (Perret et al., 2005).

Like most microbial and plant lectins, both LecA and LecB are relatively resistant to high temperature (up to 70°C), extreme pH, proteolysis and other denaturing treatments (Imberty et al., 2004). In addition, they are, as mentioned before, able to agglutinate human and animal erythrocytes (Gilboa-Garber, 1972). This hemagglutinating capacity has been shown to rely on LecA binding to B, P^k and P_1 blood group antigens, whereas LecB binds to H, A and B blood group antigens on the surface of erythrocytes (Gilboa-Garber et al., 1994b). Interestingly, the temperature profiles in their hemagglutinating activity have been shown to be opposing, with LecA exhibiting the highest activity at 4°C and LecB at 42°C (Gilboa-Garber and Sudakevitz, 1999).

The expression of the LecA and LecB gene is regulated with other virulence factors of *Pseudosomas aeruginosa* and controlled by the quorum sensing system *rhl (Winzer et al., 2000)*. The expression of *lecA* has been shown to be additionally regulated by the stationary-phase sigma factor RpoS (Winzer et al., 2000). Accordingly, both lectins have been demonstrated to be synthesized at high cell densities, i.e., during the transition to the stationary phase. During the exponential stage of growth, however, neither of them has been detectable in whole-cell protein extracts of the *P. aeruginosa* PAO1 strain (Winzer et al., 2000). Interestingly, several molecules produced by the host organism under stress conditions have been shown to increase the synthesis of LecA, including IFN-γ (Wu et al., 2005), adenosine (Patel et al., 2007), norepinephrine (Alverdy et al., 2000) and k-opioids (Zaborin et al., 2012).

Concerning the subcellular localization of the lectins, controversial results have been reported. LecA and LecB have been shown to be abundantly present in the cytoplasm of stationary phase bacteria and in small amounts on the cytoplasmic and outer membranes and in the periplasmic space (Glick and Garber, 1983). Rosenau and colleagues, in contrast, found LecB mainly in the periplasmic space (Bartels et al., 2011), whereas Jaeger and colleagues reported LecB to reside in the outer membrane of *Pseudomonas aeruginosa* (Tielker et al., 2005). In biofilm cells, LecB has been demonstrated to be exposed on the surface, from which it can be released by the addition of L-fucose (Funken et al., 2012). On the outer membrane surface, LecB is most likely bound to carbohydrate ligands (Tielker et al., 2005). The major outer membrane porin OprF has been suggested as an interaction partner of LecB (Funken et al., 2012). Consistent with this, in an OprF-deficient *P. aeruginosa* mutant, LecB was no longer present in the outer membrane but instead in the culture supernatant (Funken et al., 2012). Although LecB resides in the outer membrane, it lacks any of the secretion signals known so far, which makes a translocation via the type I pathway or via the Sec or Tat pathway unlikely (Tielker et al., 2005). A transient N-glycosylation of LecB, however, has been suggested to be necessary for its proper transport to the cell surface (Bartels et al., 2011).

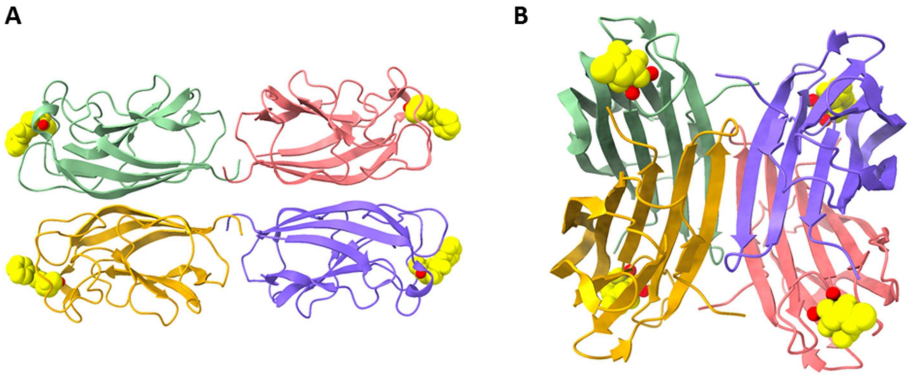


Figure 3. Crystal structure of *P. aeruginosa* lectins LecA and LecB bound to their ligands

(A) Cartoon representation of LecA bound to galactose (PDB: 5MIH) (Wagner et al., 2017).

(B) Cartoon representation of LecB bound to fucose (PDB: 1OXC) (Loris et al., 2003).

Individual monomers are represented in different colors. Calcium ions are shown as red spheres, and lectin-bound galactose and fucose are shown as yellow spheres.

2.3 Functions of LecA and LecB

LecA and LecB have been shown to be involved in the adhesion of *P. aeruginosa* to epithelial cells and to impact epithelial cell biology. In addition, both lectins play a role in biofilm formation.

2.3.1 Adhesion

The ability of LecA and LecB to bind to human and animal glycoproteins (Gilboa-Garber et al., 1994a; Kirkeby et al., 2007) together with LecB location on the outer cell membrane (Tielker et al., 2005) suggest that these lectins are involved in the adhesion of *P. aeruginosa* to human tissue **(Figure 4A)**. Consistent with this, binding to the human lung epithelial cell line A549 has been shown to be significantly decreased in a *P. aeruginosa lecA* and *lecB* mutant compared to the PAO-1 wild-type strain (Chemani et al., 2009) and to be inhibited by lectin ligands in a dose-dependent manner (Boukerb et al., 2014), supporting a role for LecA and LecB in bacterial adhesion. In line with these results, *P. aeruginosa* cell lysates have been shown to increase the amount of adhered bacteria to cultured rabbit corneal epithelial cells, which could partially be reversed by addition of the LecA- and LecB-specific sugars D-galactose and D-mannose, respectively (Wentworth et al., 1991). The authors concluded that lectins released from the cytoplasm of the lysed bacteria promoted the bacterial adhesion to the epithelial cells. Similarly, binding of *P. aeruginos*a to the immortalized human airway epithelial cells NuLi from healthy donor and CuFi from cystic fibrosis patient could be inhibited by anti-LecB antibodies (Noskova et al., 2015). Cystic fibrosis (CF) is characterized by decreased terminal sialylation and increased terminal fucosylation of mucins and other epithelial glycoproteins in the lung (Scanlin and Glick, 1999). Consequently, the epithelium in CF offers preferential ligands for LecB and may promote the adhesion of the pathogen in the lung. Consistent with this hypothesis, bacterial adhesion to CF epithelial cells has been shown to be increased compared to normal epithelium (Noskova et al., 2015). In contrast with the previous finding, the binding ability of a *P. aeruginosa* LecB mutant to human tracheobronchial mucin was not impaired compared to the wild-type (Sonawane et al., 2006). Moreover, LecA and LecB have been shown to be involved in the binding of *P. aeruginosa* to fibronectin, a glycoprotein of the extracellular matrix, as addition of the saccharides sialic acid, N-acetyl-glucosamine and N-acetylgalactosamine, galatose and fucose could inhibit the binding (Rebiere-Huet et al., 2004).

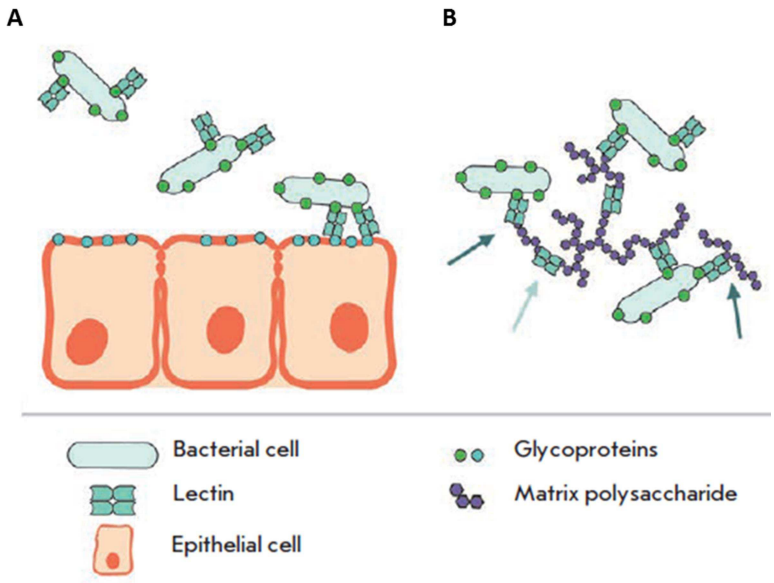


Figure 4. Proposed functions of the *P. aeruginosa* lectins LecA and LecB

(A) Lectin binding to host glycoproteins promotes the adhesion of *P. aeruginosa* to epithelial cells

(B) Lectin binding to biofilm matrix polysaccharides may stabilize the biofilm matrix and enhance the retention of bacterial cells within the growing biofilm (Passos da Silva et al., 2019). Reprinted modified from (Grishin et al., 2015).

2.3.2 Biofilm

In addition to cell adhesion, LecA and LecB have been reported to play an important role in biofilm formation. LecA and LecB-deficient *P. aeruginosa* mutants have been shown to form only poorly developed biofilms with a decreased thickness and surface coverage compared to the biofilms formed by the bacterial wild-type (Diggle et al., 2006; Tielker et al., 2005). Moreover, biofilm growth of a wild-type *P. aeruginosa* strain was inhibited in the presence of isopropyl-β-D-thiogalactoside (IPTG) or p-nitrophenyl-α-D-galactoside (NPG) and mature wild-type biofilms could be dispersed by the addition of IPTG, which suggests that LecA contributes to biofilm formation (Diggle et al., 2006). The exact role of LecA and LecB in biofilm formation, however, remains unclear. LecB has been described to bind to the surface of *P. aeruginosa* biofilms, which could be inhibited by preincubation of LecB with L-fucose, showing that LecB ligands are exposed on the surface of *P. aeruginosa* biofilms (Tielker et al., 2005). Indeed, LecB has been recently identified to function as an important structural protein in the biofilm matrix of *P. aeruginosa*. By binding to the key exopolysaccharide Psl, LecB has been suggested to stabilize the biofilm matrix leading to increased retention of cells and exopolysaccharides within the growing biofilm **(Figure 4B)** (Passos da Silva et al.,

2019). Furthermore, LecB has been shown to be required for proper type IV pili assembly, which is required for biofilm formation (Sonawane et al., 2006).

Due to its role in biofilm formation, LecB represents an attractive target for antimicrobial therapy and led to the development of glycomimetics that disrupt LecB-sugar interactions. The application of multivalent fucosyl-peptide dendrimers has been reported to successfully prevent biofilm formation and to disrupt already formed biofilms (Johansson et al., 2008).

2.3.3 Epithelial cells

The epithelium forms a protective layer of cells that covers the outer surfaces of the body such as the skin, and lines the internal surfaces of organs (O'Brien et al., 2002). Consequently, for an infection to occur, *P. aeruginosa* must adhere to epithelial cells for the subsequent colonization and eventual dissemination throughout the body. Therefore, several studies focused on the direct effect of LecB on epithelial cells.

Both lectins have been found to contribute to the cytotoxicity of *Pseudomonas aeruginosa* to human lung epithelial cells *in vitro*. Accordingly, the cytotoxicity of the PAO-1 wild-type strain to the human epithelial cell line A549 was higher than the cytotoxicity of the *lecA* and *lecB* mutant and could also be significantly reduced by addition of the LecA- and LecB-specific inhibitors α-methyl-D-galactoside and α-methyl-D-fucoside, respectively (Chemani et al., 2009). Moreover, LecA and LecB have been demonstrated to bind to cilia of human nasal mucosa epithelial and to inhibit their beating frequency, which could be prevented by pre- and co-incubation with D-galactose and L-fucose, respectively (Adam et al., 1997; Gustke et al., 2012; Mewe et al., 2006). Since beating epithelial cell cilia are important for clearing mucus and foreign particles (e.g., bacteria) from the airways (Chilvers et al., 2003), both lectins likely contribute to the pathogenicity of *P. aeruginosa* in respiratory tract infections. Moreover, LecA has been reported to negatively impact the intestinal epithelium by reducing the transepithelial electrical resistance of cultured human colon epithelial cell line Caco-2 monolayers (Laughlin et al., 2000). In addition, the permeability for mannitol, a paracellular permeability probe, was increased by LecA, which could be prevented by coincubation with N-acetyl-D-galactosamine (Laughlin et al., 2000). As the protein levels of the tight junctional proteins Zonula occludens-1 (ZO-1) and occludin were also reduced in these cells after LecA treatment, the increased permeability likely resulted from disrupted tight junctions (Laughlin et al., 2000).

In the human lung epithelial cell line H1299, LecB has been described to attenuate cell migration and proliferation and to activate NF-κB signaling (Cott et al., 2016). Concomitantly, LecB caused β-catenin degradation, which may be involved in the former events and may contribute to *P. aeruginosa*-induced delay of tissue repair (Cott et al., 2016). Moreover, LecB was shown to associate with the insulin like growth factor-1 receptor (IGF-1R) in cultured normal human keratinocytes (NHKs), leading to its internalization, albeit without receptor activation (Landi et al., 2019). LecB also blocked the cell cycle and induced cell death in these keratinocytes, suggesting that LecB may contribute to the persistence of chronic *P. aeruginosa* wound infections (Landi et al., 2019).

2.3.4 *In vivo* studies

In vivo, LecA and LecB have been shown to be linked to the pathogenicity of *P. aeruginosa*. In a *P. aeruginosa*-induced acute lung injury mouse model, lung permeability, bacterial burden and dissemination were reduced in a *lecA* and *lecB* mutant compared to the wild-type strain (Chemani et al., 2009). Moreover, the administration of LecA and LecB inhibitors reduced *P. aeruginosa-induced* lung injury and mortality in these mice (Chemani et al., 2009). A clinical trial conducted in adult, chronic CF patients, has shown that inhalation with a fucose/galactose solution during 21 days could reduce the bacterial count, TNF-α expression as well as neutrophil numbers in the sputum of the patients, whereas the lung function remained unchanged (Hauber et al., 2008). It should be taken in account, however, that this study has been conducted in a small number of patients without any control group that received a lectin-unspecific sugar. In another phase II study, treatment of *P. aeruginosa* induced otitis externa diffusa with a D-galactose, D-mannose, N-acetylneuraminic acid solution has been reported to be as successful as a local antibiotic application (Steuer et al., 1993), pointing to an important role of these lectins in the pathogenesis of *P. aeruginosa.* Similar results have been demonstrated in a case report of a patient suffering from a *P. aeruginosa*-induced respiratory tract infection who could be successfully treated with a solution containing LecA- and LecB-specific sugars even if conventional antibiotic treatment failed (von Bismarck et al., 2001).

Even though these results clearly show that both lectins play an important role in binding of *P. aeruginosa* to human cells and in biofilm formation, the precise underlying mechanism and its role in *in vivo* infection have still not been fully elucidated. Since LecB is also

required for the normal assembly of type IV pili in *P. aeruginosa* and secretion of other proteins (Sonawane et al., 2006), its implication in infection can be either direct by promoting the bacterial binding to carbohydrate ligands on the host cell surface or indirect by affecting other bacterial adhesins (Grishin et al., 2015).

3 The Lymphatic System

The lymphatic vascular system plays a crucial role in body function by maintaining fluid balance, absorbing dietary fats in the intestine and transporting them back into the blood, and by carrying antigens and immune cells from the periphery to draining LNs (Liao and von der Weid, 2015). It consists of a network of vessels present throughout the body, which is usually particularly dense in superficial tissues, such as the mucous membranes of the gastro-intestinal and respiratory tracts and the skin (Skobe and Detmar, 2000). The brain and spinal cord, in contrast, are devoid of lymphatic vessels (Moore and Bertram, 2018). Small initial lymphatic vessels collect excess fluid and proteins from the interstitial space and merge into larger collecting lymphatic vessels that transport lymph into the draining LN. From there lymph enters efferent lymphatic vessels to drain into downstream LNs and to return to the blood circulation via the subclavian veins **(Figure 5)** (Liao and von der Weid, 2015).

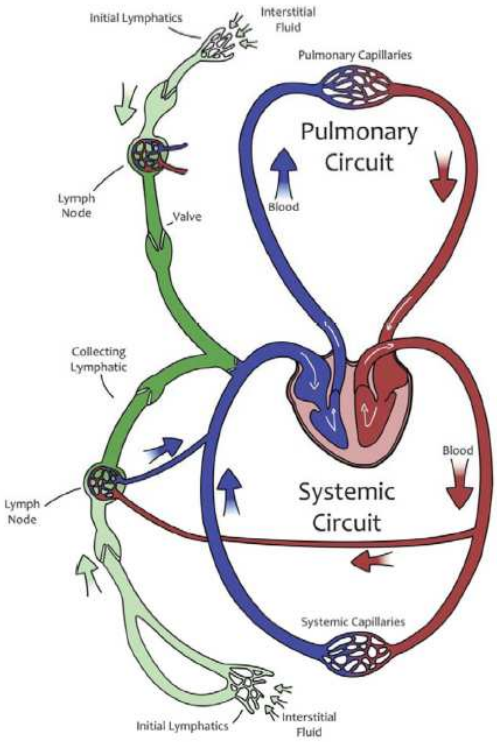


Figure 5. Schematic of the lymphatic vascular system and the blood circulation

Small initial lymphatics throughout the body collect excess fluid and proteins from the interstitial space and merge into larger collecting lymphatics, which transport the lymph into the draining lymph node. Lymph exiting the lymph node can either drain into downstream lymph nodes or return to the blood circulation via the subclavian vein. Reprinted from (Moore and Bertram, 2018) with permission.

3.1 Lymphatic transport of antigens and cells to lymph nodes

3.1.1 Antigen entry into initial lymphatic vessels

Small initial lymphatic vessels (lymphatic capillaries) collect excess fluid and proteins that have extravasated from the blood vessels and accumulate within the interstitium **(Figure 6A)** (Skobe and Detmar, 2000). The endothelium of initial lymphatic vessels is composed of intertwined oak leaf-shaped cells that form overlapping flaps at their junctions **(Figure 6B, C)**. These flaps act as primary valves assuring unidirectional flow of fluid into the lymphatic vessels **(Figure 6D)** (Baluk et al., 2007). Discontinuous, button-like junctions (buttons) at the sides of flaps join adjacent endothelial cells and are composed of the adherens junction protein vascular endothelial (VE)-cadherin and tight junction proteins, including occludin, claudin-5, zonula occludens (ZO)-1, endothelial cell-selective adhesion molecule (ESAM), and junctional adhesion molecule-A (JAM-A) (Baluk et al., 2007). The platelet endothelial

cell adhesion molecule (PECAM-1/CD31) and the lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) are distributed in a complementary manner to VE-cadherin at the tips of the flaps (Baluk et al., 2007). The buttons have been suggested to serve as anchors along the sides of flaps, permitting easy entry of fluid and other content from peripheral tissues through the flaps without the need of disassembly and reformation of endothelial junctions (Baluk et al., 2007). Hence, initial lymphatic vessels provide highly permeable portals, through which infectious pathogens such as bacteria and viruses could directly enter (Liao and von der Weid, 2015).

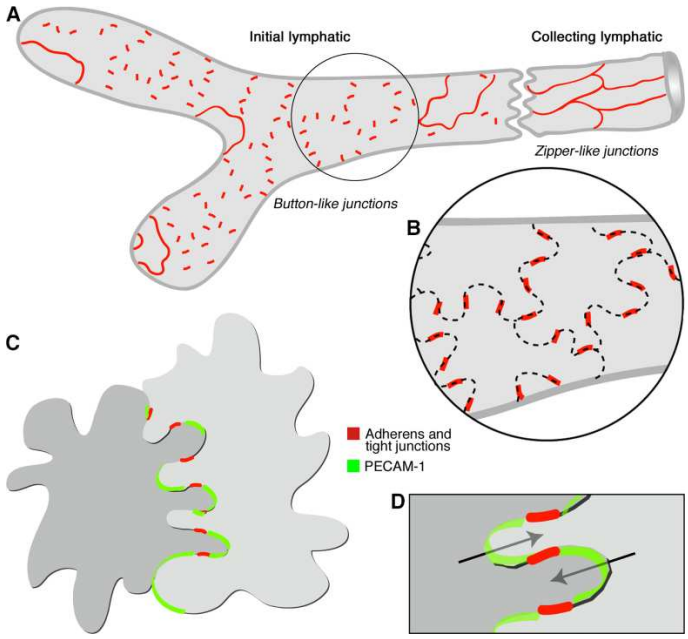


Figure 6. Button-like junctions (buttons) in initial lymphactics.

(A) Schema displaying discontinuous, button-like junctions in endothelial cells of initial lymphatic vessels and continuous zipper-like junctions in collecting lymphatic vessels.

(B) Enlarged view of oak leaf-shaped endothelial cells (dashed line) of initial lymphatics with buttons (red) along the sides of the overlapping flaps.

(C) Schema of two intertwined oak leaf-shaped endothelial cells forming overlapping flaps. Adherens and tight junction proteins (red) form anchors along the sides of flaps and PECAM-1 (CD31, green) is distributed in a complementary manner at the tips of the flaps.

(D) Enlarged view of flaps formed by intertwined initial lymphatics endothelial cells. Adherens and tight junction proteins forming the buttons, allow unidirectional fluid entry without repetitive disruption and reformation of junctions. Reprinted from (Baluk et al., 2007).

3.1.2 Cell entry into initial lymphatic vessels

In addition to antigens, lymphatic vessels transport immune cells, mainly recirculating memory T cells and antigen-loaded DCs, from the periphery to draining LNs **(Figure 7)** (Jackson, 2019). In the steady-state, only small numbers of immature DCs and effector memory T cells migrate from peripheral tissues to draining LNs. During inflammation, however, the number of migrating DCs increases significantly, which carry endocytosed antigens for immune priming in the draining LNs (Jackson, 2019). In both conditions, the migration is mainly driven by chemotaxis (Saeki et al., 1999). The most important regulator of DC migration is the C-C chemokine receptor 7 (CCR7) on DCs (Förster et al., 1999). Accordingly, CCR7-deficient DCs have been shown to fail to migrate from the periphery to draining LNs upon inflammatory stimulation (Clatworthy et al., 2014; Förster et al., 1999; Tal et al., 2011) as well as under steady-state conditions from the skin (Ohl et al., 2004). CCR7 has two ligands, the chemokine C-C motif ligand 19 (CCL19) and CCL21 (Weber et al., 2013). While CCL19 has been shown to be dispensable for the migration of DCs into lymphatic vessels, CCL21 has been demonstrated to be essential for this process under both homeostatic and inflammatory conditions (Britschgi et al., 2010; Weber et al., 2013). Indeed, the lymphatic endothelium has been reported to secrete endogenous CCL21 that is immobilized either on the cell surface or the interstitial matrix, creating a gradient, which directs CCR7-bearing DCs toward the lymphatic vessels (Weber et al., 2013). $CCL21^+$ puncta close to the $LYVE\text{-}1^+$ flaps of initial lymphatic vessel endothelial cells have been shown to mediate the docking of DCs on the endothelium for subsequent transmigration (Tal et al., 2011).

The transmigration of the lymphatic endothelium requires under inflammatory conditions the interaction of β_2-integrins and their counterreceptors intercellular adhesion molecule-1 (ICAM-1) (Johnson et al., 2006; Nitschke et al., 2012; Xu et al., 2001) and vascular cell adhesion molecule-1 (VCAM-1) (Johnson et al., 2006). In the resting state, however, integrins are dispensable for DC transmigration, as DCs from mice lacking all integrins were still able to migrate to LNs by means of chemokine-directed amoeboid motion, when transferred into a non-inflamed dermis (Lammermann et al., 2008). Consistent with this, under inflammatory conditions cytokines, such as TNF-α and TNF-β, increase the expression of integrin ligands, such as ICAM-1 and VCAM-1, on lymphatic endothelial cells (Johnson et al., 2006). Moreover, the lymphatic endothelium increases the synthesis and release of

chemokines, including CCL5, CCL2 (MCP-1) and CCL20, which attracts DCs, lymphocytes, monocytes and neutrophils (Johnson et al., 2006).

In addition to integrin signaling, Jackson and colleagues showed that interactions between LYVE-1 expressed within the flaps of the lymphatic endothelium and its ligand hyaluronic acid (HA) on the DC plasma membrane are involved in the adhesion to and transmigration of DCs across the lymphatic endothelium (Johnson et al., 2017).

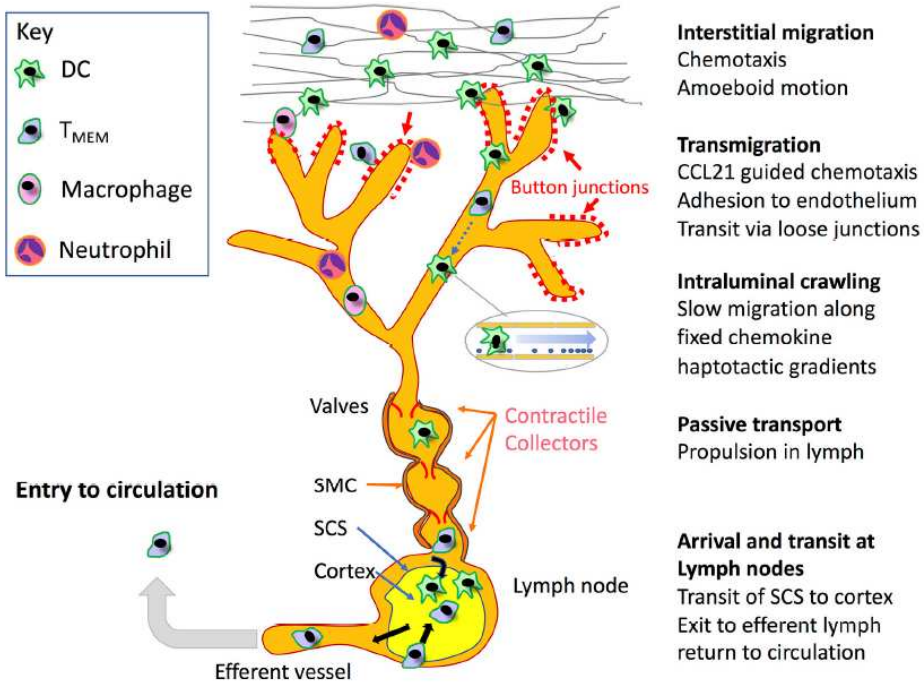


Figure 7. Leukocyte migration through the lymphatic system

Upon inflammation, leukocytes (e.g., memory T cells and antigen-carrying DCs) are mobilized from peripheral tissues to migrate toward initial lymphatic vessels via chemokine-directed amoeboid motion for access to draining LNs. Immobilized CCL21 on the surface of initial lymphatic vessels mediate the docking of leukocytes on the endothelium for subsequent integrin-dependent transmigration through discontinuous, button-like endothelial junctions. Within the vessels, leukocytes crawl along a CCL21 gradient on the endothelial surface and enter larger collecting lymphatics. Collecting lymphatics are covered by smooth muscle cells and contain one-way valves, which transport the cells with the lymph by intrinsic pumping to the draining LN. The migrating cells enter the LN through the subcapsular sinus and transmigrate to the underlying cortex to prime naïve lymphocytes in the case of DCs or travel through the medullary sinuses to enter the cortex in the case of memory T cells. While DCs remain within the LN, where they die, memory T cells exit the LN via efferent vessels to re-enter the blood circulation. Reprinted from (Jackson, 2019).

Effector T cells, rather than naïve T cells, migrate as DCs in a CCR7-dependent manner from tissues into draining LNs via the lymph under both homeostatic and inflammatory conditions (Bromley et al., 2005; Brown et al., 2010; Debes et al., 2005). Just as for DCs, the transmigration of T cells depends on integrin-mediated adhesion by the interaction of the β_2 integrin lymphocyte function-associated antigen 1 (LFA-1) and its ligand ICAM-1 (Teijeira et al., 2017). The lipid sphingosine-1-phosphate (S1P), which is known to regulate T cell exit from LNs, has also been shown to mediate the migration of T cells via afferent lymphatics. Indeed, increased S1P levels in peripheral tissues or agonizing of S1P receptors retained T cell within the tissue and inhibited their migration to draining LNs (Ledgerwood et al., 2007). Moreover, the interaction between the macrophage mannose receptor (MR) on lymphatic endothelial cells and CD44 on the plasma membrane of T cells has been described to increase the adhesion of T cells to the lymphatics, thereby fostering T cell migration across the lymphatic endothelium (Marttila-Ichihara et al., 2008; Salmi et al., 2013).

3.1.3 Antigen and cell transport through afferent lymphatic vessels

Initial lymphatic vessels congregate to larger collecting lymphatics, which direct lymph to the draining LN **(Figure 7)** (Moore and Bertram, 2018). Collecting lymphatics are characterized by endothelial cells that are, like blood endothelial cells, interconnected by continuous, zipper-like junctions (zippers) **(Figure 6A)** (Baluk et al., 2007). These zippers are composed of VE-cadherin and tight junction proteins, including occludin, claudin-5, ZO-1, ESAM, and JAM-A, which strongly reduce their permeability to peripheral fluid (Baluk et al., 2007). Moreover, unlike initial lymphatics, collecting vessels loose LYVE-1 expression and possess a continuous basement membrane and luminal one-way valves that prevent back flow (Liao and von der Weid, 2015). They are covered by smooth muscle cells that contract periodically, which together with the valve action, transport lymph by intrinsic pumping to the draining LN, as observed in the chambers of the heart (Moore and Bertram, 2018). Although these morphological characteristics underline the role of collecting lymphatics in transporting lymph and cells rather than collecting material, it is still uncertain whether antigens and cells can directly enter from peripheral tissue (Liao and von der Weid, 2015).

4 The Lymph Node

Afferent lymphatic vessels, which transport extracellular fluid from a particular peripheral tissue, ultimately converge into draining LNs **(Figure 8)** (Junt et al., 2008). As sites of induction of protective adaptive immune responses against invading pathogens, LNs constitute important organs of the immune system (Junt et al., 2008). Here, antigen-sampling cells of the innate immune system capture pathogens arriving with the lymph to reduce their spread and dissemination into the blood stream (Junt et al., 2008). Moreover, LNs assure the encounter of antigen-presenting cells (APCs) with rare antigen-specific lymphocytes to induce an efficient antigen-specific immune responses, and provide a niche for the survival and differentiation of lymphocytes (Junt et al., 2008). Hence, LNs ensure the generation of high-affinity antibodies that neutralize extracellular pathogens, the induction of cytokine-secreting effector T helper cells and activation of cytotoxic T lymphocytes that lyse infected cells (Junt et al., 2008). To fulfill these tasks, LNs display a highly organized structure with distinct B and T lymphocyte compartments, which are supported by networks of non-hematopoietic endothelial and mesenchymal stromal cells (Mueller and Germain, 2009). These stromal cells comprise amongst others lymphatic endothelial cells (LECs), blood endothelial cells (BECs), follicular dendritic cells (FDCs) and fibroblastic reticular cells (FRCs) (Mueller and Germain, 2009). LNs can be divided into three main compartments: the cortex, separated from the capsule by the subcapsular sinus (SCS), which forms the B lymphocyte area with primary follicles; the adjacent paracortex, in which T lymphocytes are concentrated; and the medulla, a labyrinth of lymph-draining sinuses that are separated by medullary cords, which contain many plasma cells, some macrophages and memory T lymphocytes **(Figure 8)** (von Andrian and Mempel, 2003). Lymph carrying antigens and antigen-presenting cells such as DCs from peripheral tissues enters the LNs through several afferent lymphatics that are lined by LECs, into the SCS (Girard et al., 2012). From there, lymph is channeled into the cortex via cortical sinuses, and collected in the medullary sinuses to exit the LN via efferent lymphatics (von Andrian and Mempel, 2003). All LN sinuses are lined by LYVE-1^+ LECs, which express chemokines and adhesion molecules to facilitate the migration or egression of incoming or exiting DCs, B and T lymphocytes (Cohen et al., 2014). Moreover, $CD169^+$ macrophages and LN-resident DCs are associated with the lymphatic sinuses and efficiently sample antigens from the sinus lumen (Carrasco and Batista, 2007; Gerner et al., 2015; Phan et al., 2009; Phan et al., 2007). Blood-borne cells, such as naïve B and T lymphocytes, mainly enter the LN via specialized post-capillary

venules, the high endothelial venules (HEVs) **(Figure 8A)** (Warnock et al., 1998). The BECs of the HEVs display many sialomucins (also called peripheral-node addressins (PNAds)) on their cell surface, which are important for the entry of lymphocytes into the LN (Miyasaka et al., 2016).

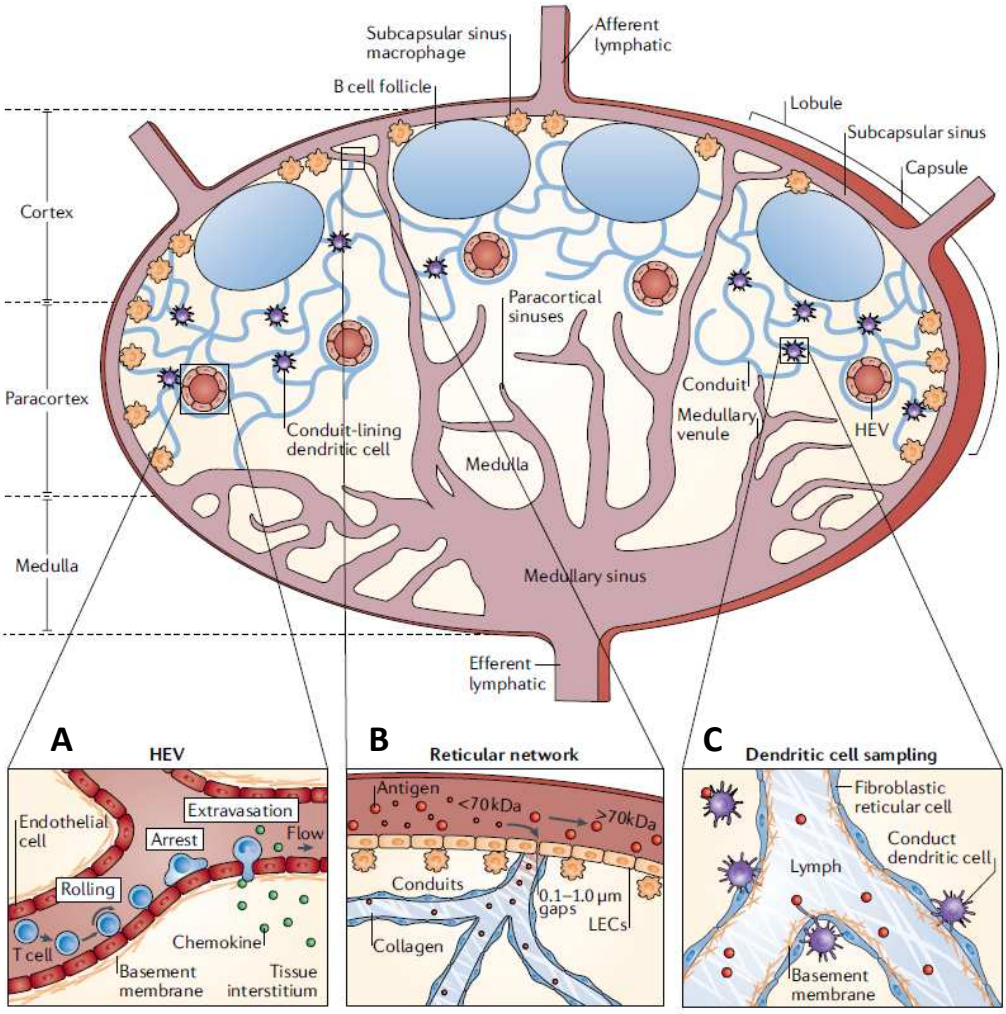


Figure 8. Lymph node structure and cells

A cross-section of a lymph node (LN) shows the different compartments within the LN. The cortex contains B cell follicles, which are located just underneath the subcapsular sinus. The paracortex harbors the T cell zone, in which dendritic cells prime T cells, while the medulla contains medullary sinuses.

(A) Blood-borne naïve lymphocytes enter the LN through high endothelial venules in the paracortex by means of an adhesion cascade, which is initiated by rolling of lymphocytes along the HEV wall and followed by firm arrest and subsequent extravasation.

(B) Antigens that enter the LN with the lymph in the subcapsular sinus, gain dependent on their size either access to the fibroblastic reticular cell (FRC)-formed conduits for rapid transport to the HEVs lumen or drain through the LN via cortical and medullary sinuses.

(C) Dendritic cells that are associated with the FRC-conduits sample quickly antigens from the conduit lumen for early T cell activation. Reprinted modified from (Schudel et al., 2019) with permission.

The two stromal cell subsets, FDCs in the cortex and FRCs in the paracortex, both form dense and complex three-dimensional networks that define the B cell and T cell compartment, respectively (Bajénoff et al., 2006). These networks serve as scaffolds for the migration of lymphocytes and dendritic cells and facilitate their encounter and interaction for the induction of an immune response (Bajénoff et al., 2006). Moreover, the FRC network provide a conduit system for low molecular weight substances (e.g., chemokines, antigens), which are rapidly transported from the SCS to the paracortical HEVs for the modulation of leukocyte recruitment (chemokines) or rapid induction of an immune response (antigens) **(Figure 8B, C)** (Gretz et al., 2000; Sixt et al., 2005; Stein et al., 2000).

4.1 Cell-independent antigen transport to the lymph node

The fate of lymph-borne antigens arriving via the afferent lymphatics into the LN SCS, largely depends on their size **(Figure 8B)** (Gretz et al., 2000; Sixt et al., 2005). Large particulate antigens (molecular weight >70 kDa or radius >4 nm), such as bacteria and viral particles, cannot enter the LN cortex and drain from the SCS through the LN via cortical and medullary sinuses (Gretz et al., 2000; Sixt et al., 2005). $CD169^{+}$ sinus-associated macrophages (Carrasco and Batista, 2007; Junt et al., 2007; Phan et al., 2007) and dendritic cells (Sixt et al., 2005) efficiently sample antigens from the lymphatic vessel lumen to induce early B and T cell activation even before the arrival of the peripheral tissue-derived antigen-loaded DCs (Gerner et al., 2015).

Small lymph-borne antigens with a molecular radius less than ~4 nm or a molecular weight less than 70 kDa, in contrast, have been shown to quickly drain from the SCS through the LN cortex and into the lumen of HEVs in the T cell zone (Gretz et al., 2000; Sixt et al., 2005). Antigens drain within the inner tubular space of FRC-formed conduits that are continuous with the fibers in the SCS floor and extend from the SCS to the paracortical HEVs (Gretz et al., 2000; Sixt et al., 2005). The glycoprotein PLVAP (plasmalemma vesicle-associated protein), expressed by sinusoidal LECs, has been shown to form sieve-like diaphragms in transendothelial channels in LECs that connect the sinus with the conduits (Rantakari et al., 2015). PLVAP thereby functions as “gatekeeper” for these conduits, controlling the size-

selective entry of lymph-borne antigens at the SCS floor to the conduits (Rantakari et al., 2015). These conduits contain a core of collagen bundles, covered by a microfibrillar layer, which in turn is ensheated by a basement membrane and an outer layer of FRCs (Sixt et al., 2005). They not only quickly deliver antigens to naïve lymphocytes entering the LN via the HEVs, but also transport chemokines from peripheral inflamed tissues to HEVs to modulate leukocyte recruitment (Gretz et al., 2000; Stein et al., 2000). Consistent with this, von Andrian and colleagues showed that the inflammatory MCP-1 (CCL2) released from inflamed skin has been transported via the lymph to the HEV lumen in draining LNs. There, MCP-1 recruited blood-borne monocytes via activation of integrin-dependent arrest of rolling monocytes (Palframan et al., 2001).

A similar albeit less dense conduit system has also been described by Carroll and colleagues in the LN B cell zone, which rapidly drains small soluble antigens from the SCS to the underlying B cell follicles (Roozendaal et al., 2009). Unlike the conduits in the T cell zone, they are not ensheated by FRCs but by FDCs (Bajénoff and Germain, 2009). These conduits contain gaps, exposing them to B cells, which has been suggested to accelerate the encounter of antigens with their cognate B cells (Roozendaal et al., 2009). Alternatively, FDCs ensheating the conduits may capture antigens from the conduits for delivery to and activation of follicular B cells (Bajénoff and Germain, 2009). The transport of antigens via this follicular conduit system probably also explains the free diffusion of small antigens via gaps from the SCS floor into the follicles described by Jenkins and colleagues (Pape et al., 2007).

The regulated transport of lymph-borne antigens within the LN ensures that the vast majority of antigens entering with the lymph is captured and filtered out to elicit a protective immune response and to prevent the dissemination of lymph-borne pathogens into the blood circulation (Chang and Turley, 2015).

4.2 Cell entry into the lymph node via afferent lymphatic vessels

DCs draining via the collecting lymphatic vessels arrive into the LN SCS, just beneath the outer LN capsule. Their migration across the sinus floor into the LN parenchyma is, as their migration into initial lymphatic vessels, mediated by CCR7/CCL21-dependent chemotaxis (Jackson, 2019). The SCS ceiling lining lymphatic endothelial cells express CCRL1 (ACKR4), the atypical chemokine receptor for CCL19 and CCL21 (Ulvmar et al., 2014). CCRL1 has been shown to scavenge the endogenous CCR7 ligands from the SCS ceiling, thereby creating a chemokine gradient, which directs the migration of DCs through the SCS

floor **(Figure 9)** (Ulvmar et al., 2014). Interestingly, Förster and colleagues showed that transmigration of DC across the SCS alters the SCS floor LECs and $CD169^+$ SCS macrophages locally, which permits the subsequent transmigration of naïve T cells (Braun et al., 2011). When these T cells are intralymphatically injected in the absence of DCs, however, they have been shown to enter the LN parenchyma via the medullary sinuses (Braun et al., 2011). Once inside the LN parenchyma, both DCs and T cells move along the FRC scaffold to the T cell zone (Acton et al., 2012; Bajénoff et al., 2006). This translocation relies on CCR7 signaling (Braun et al., 2011), which is mediated by CCL19 and CCL21 expressed by FRCs (Luther et al., 2000; Schumann et al., 2010). Moreover, FRCs, like LECs, highly express the transmembrane glycoprotein podoplanin (gp38) (Farr et al., 1992; Schacht et al., 2003). It has been shown that the interaction of podoplanin on FRCs with its C-type lectin receptor CLEC-2 on DCs reduces the actomyosin contractility of FRCs and promotes actin polymerization in DCs, which permits their spread and migration along the reticular cell scaffold (Acton et al., 2012; de Winde et al., 2018).

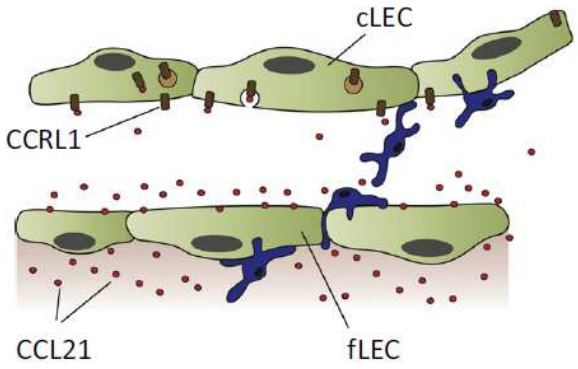


Figure 9. Subcapsular sinus lining LECs mediate cell entry across floor LECs

The migration of immune cells across the SCS floor into the LN parenchyma relies on CCR7/CCL21-dependent chemotaxis. The atypical chemokine receptor CCRL1 on the surface of the SCS ceiling-lining LECs scavenges the endogenous CCR7 ligand CCL21 from the SCS ceiling to create a chemokine gradient, which directs the migration of the cells through the SCS floor (Ulvmar et al., 2014). Reprinted from (Chang and Turley, 2015) with permission.

4.3 Cell entry into the lymph node from the blood

Under physiological circumstances, naïve (mature, non-activated) lymphocytes recirculate continuously between the peripheral blood and LNs in search of their cognate antigen (Takeda et al., 2017). They enter the LNs via specialized post-capillary venules, the high endothelial venules (HEVs), which are located in the LN paracortex (Warnock et al., 1998). After scanning the LN for a cognate antigen, lymphocytes exit the LN via the efferent lymphatics (Takeda et al., 2017). These processes of entry and exit of lymphocytes are tightly balanced to maintain cell numbers within the LN in the non-inflamed state (Mionnet et al., 2011). Indeed, HEV endothelial cells have been shown to regulate lymphocyte numbers within the LN by keeping incoming lymphocytes within pockets **(Figure 10)** on their abluminal side and releasing them into the LN parenchyma in proportion to the rate of lymphocyte egress from the LN (Mionnet et al., 2011).

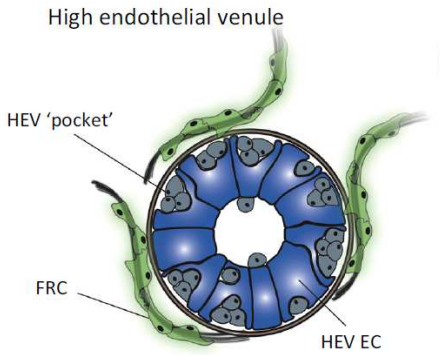


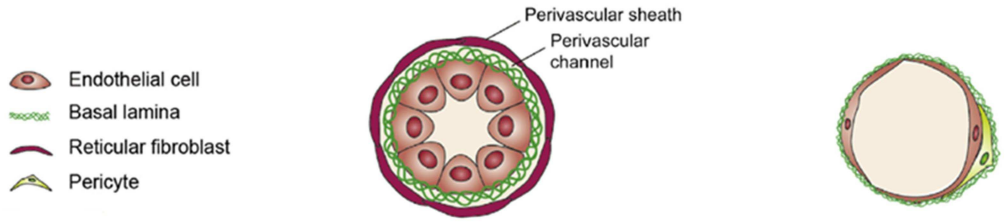
Figure 10. High endothelial venules pockets

HEV endothelial cells keep naïve lymphocytes that enter the LN from the blood within pockets on their abluminal side to regulate lymphocyte numbers within the LN. Depending on the rate of lymphocyte egress, retained lymphocytes are released into the LN parenchyma (Mionnet et al., 2011). Reprinted from (Chang and Turley, 2015) with permission.

4.3.1 High endothelial venules

HEVs are morphologically distinct from other blood vessels **(Figure 11)** (Miyasaka et al., 2016). Unlike normal blood vessels with a flat endothelium, HEVs are lined by high cuboidal endothelial cells (Warnock et al., 1998). Moreover, they present a thickened apical glycocalyx, a thickened basal lamina, and a perivascular sheath that is built by FRCs (Poosarla et al., 2015). The channel formed between the basal lamina and the surrounding sheath of FRCs is called perivascular channel (Poosarla et al., 2015). While they share

common adhesion molecules such as CD31 and ICAM-2 with normal venules, they specifically express large amounts of highly glycosylated and sulphated forms of sialomucins, including CD34, podocalyxin, endoglycan, glycosylation-dependent cell-adhesion molecule-1 (GlyCAM-1), endomucin and nepmucin (Miyasaka et al., 2016). These sialomucins are also called peripheral-node addressins (PNAds) and share a unique sugar structure on O-glycans, the 6-sulfo sialyl Lewis X (Le^x) (Takeda et al., 2017).



		HEV	Normal venules
Morphology	Endothelium	Tall and plump	Flat
	Basal lamina	Thick	Thin
	Perivascular sheath	Concentrically-arranged FRCs	Thin layers of pericytes or smooth muscle cells
Gene expression	HEV sialomucins	++	- ~ ±
	Chemokines	++	- ~ ±
	ICAM-1	++	- ~ ±
	ICAM-2	+	+
	CD31	+	+
	VE-cadherin	+	+

Figure 11. Comparison of morphology and gene expression of HEVs and normale venules.
HEV are morphologically distinct from normal venules with their high cuboidal endothelial cells, thick basal lamina and perivascular sheath formed by fibroblastic reticular cells. They also specifically express high amounts of sialomucins, chemokines and adhesion molecules, which mediate the entry of naïve lymphocytes from the blood into the LN. Reprinted modified from (Miyasaka et al., 2016).

4.3.2 Adhesion cascade

The entry of lymphocytes into the LN via HEVs is controlled by an adhesion cascade, initiated by lymphocyte tethering/rolling along the HEV wall, followed by firm arrest of leukocytes to the HEV wall and completed by transendothelial migration **(Figure 12)** (Miyasaka et al., 2016).

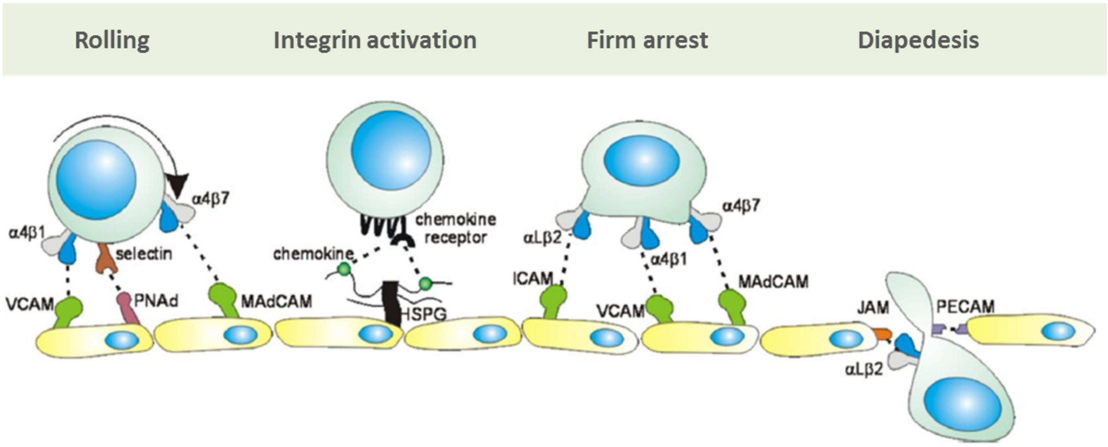


Figure 12. Adhesion cascade of lymphocytes transmigrating across high endothelial venules (HEVs)
In HEVs, binding of peripheral node addressins (PNAds) to L-selectin (CD62L) on lymphocytes mediates rolling of the latter on the HEV surface. HEV endothelial cells produce chemokines that are presented on the cell surface by heparin sulfate proteoglycans (HSPGs), which bind to chemokine receptors and activate the integrins αLβ2 (lymphocyte function-associated antigen-1, LFA-1), α4β1 (very late antigen-4, VLA-4), and α4β7 (lymphocyte Peyer patch adhesion molecule, LPAM). Interaction of these integrins with their ligands ICAM-1 (intercellular adhesion molecule 1), VCAM-1 (vascular cell adhesion molecule 1), and MAdCAM-1 (mucosal addressin cell adhesion molecule 1) leads to firm arrest of lymphocytes to the endothelium and subsequent diapedesis, which involves the junction adhesion molecules (JAMs) and PECAM-1 (CD31). Reprinted modified from (T Pals et al., 2007).

4.3.2.1 Tethering/ Rolling

The initial tethering of lymphocytes on the HEV wall relies on the weak and transient interaction of PNAds with CD62L (L-selectin), a cell adhesion molecule that is expressed on the lymphocyte surface (Miyasaka et al., 2016). CD62L specifically recognizes the 6-sulfo sialyl Le^x on the PNAds, which causes the lymphocytes to slow their movement and to roll along the HEV wall (Miyasaka et al., 2016).

4.3.2.2 Firm arrest/ adhesion

Chemokines presented on the luminal surface of HEV endothelial cells then trigger integrin activation on lymphocytes **(Table 1)** (Miyasaka et al., 2016). The rapid activation of the cell-surface β_2-integrin lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18, $\alpha_L\beta_2$) causes its binding to the adhesion molecules intracellular adhesion molecule-1 (ICAM-1) and

ICAM-2 on the HEV endothelial surface (Takeda et al., 2017). Moreover, the chemokine-activated α4 integrins (the alpha unit of the very late antigen-4 (VLA-4)) can bind vascular cell adhesion molecule-1 (VCAM-1) and these interactions finally result in shear-resistant firm arrest and adhesion of lymphocytes to the HEV wall (Takeda et al., 2017).

The chemokines CCL21 and CCL19, which interact with their receptor CCR7 on lymphocytes, have been shown to be essential for lymphocyte migration from the blood into LNs (Baekkevold et al., 2001; Gunn et al., 1999; Stein et al., 2000). While the lymphoid tissue-specific isoform CCL21-ser is constitutively synthesized by HEV endothelial cells (Gunn et al., 1998) and FRCs, CCL19 is produced only by FRCs, and translocated to the luminal surface of HEVs (Baekkevold et al., 2001). The crucial role of these chemokines in lymphocyte entry via HEVs has been demonstrated in *plt/plt* mice that are deficient for CCL19 (Luther et al., 2000; Nakano and Gunn, 2001) and the CCL21-ser isoform (Nakano and Gunn, 2001; Stein et al., 2000; Vassileva et al., 1999). These mice display greatly reduced T cell numbers within the LNs due to abrogated firm arrest of lymphocytes on the HEV wall and thus defective migration into the LNs (Nakano et al., 1998; Nakano et al., 1997). Exogenous CCL19 and CCL21 could both separately restore T cell homing in *plt/plt* mice (Baekkevold et al., 2001; Stein et al., 2000). Moreover, mice lacking CCR7 present defects in lymphocyte homing to LNs (Förster et al., 1999), indicating that the interaction of both CCL19 and CCL21 with their counterreceptor CCR7 is required for the firm adhesion of lymphocyte to HEVs and their subsequent migration into the LN.

Furthermore, the chemokine CXCL12 and its receptor CXCR4 have been found to be involved in B and T cell migration across HEVs (Okada et al., 2002). Like CCL19, CXCL12 is not produced by HEVs, but by FRCs that surround HEVs, and displayed on the HEV lumen, whereas its receptor CXCR4 is found on the lymphocyte surface (Okada et al., 2002). Altough *plt/plt* mice showed normal B cell numbers in LNs (Nakano et al., 1997), the LN homing of transferred CCR7-deficient B cells in wild-type mice was reduced (Förster et al., 1999), indicating that B cell migration relies not solely on CCR7-signaling. Consistent with this, Cyster and colleagues have identified both CCR7 and CXCR4 as main chemokine receptors mediating B cell entry into LNs (Okada et al., 2002). Accordingly, injected CXCR4-deficient B cells have been shown to migrate to LNs only at a low level in *plt/plt* mice (lacking the CCR7 ligands) but normally in wild-type mice (Okada et al., 2002). In line with these findings, CCL12 desensitization in wild-type mice did not affect LN homing of B cells contrary to CCL19 desensitization, which resulted in a decreased B cell accumulation in LNs (Okada et al., 2002). These results indicate that CCR7-signaling in B cell migration is

only partially redundant with signaling of CXCR4, whereas the contribution of CXCR4 seems to be fully redundant with the contribution of CCR7 (Okada et al., 2002). Similarly, CXCR4 signaling has also been shown to play a minor role T cell homing to LNs, but appears to be fully redundant with signaling of CCR7 (Okada et al., 2002). The differences in T and B cell homing have been suggested to result from their differential responsiveness to CCL19 and CCL21, but similar responsiveness to CXCL12 (Okada et al., 2002). Indeed, T cells show a ~10-fold higher surface level of CCR7 than B cells, and are also more responsive to CCL19 and CCL21 in *in vitro* chemotaxis assays (Okada et al., 2002).

In the same study, CXCR5, highly expressed on naïve B cells, and its ligand CXCL13, have been shown to not be involved in B cell homing to LNs contrary to its contribution to B cell migration to Peyer's patches. Accordingly, while CXCL13 has been found in Peyer's patch HEVs, it was absent in LN HEVs and LN homing was not affected in CXCR5-deficient B cells (Okada et al., 2002). In contrast, Miyasaka and colleagues have shown contradictory results since they could detect CXCL13 in LN HEVs and have observed reduced B cell migration to mesenteric LNs in CXCL13-deficient mice compared to wild-type mice (Ebisuno et al., 2003). In addition, CXCL13 has been reported to stimulate several integrins in B cells, such as LFA-1 and α4 integrin, *in vitro*, thereby inducing the firm arrest of B cells (Kanemitsu et al., 2005). These results indicate that LN HEVs display CXCL13 on their luminal surface and that the interaction of the chemokine with its receptor CXCR5 on naïve B cells crucially supports their firm arrest on the HEV wall.

Table 1. Proteins and molecules involved in the adhesion and transmigration of B and T lymphocytes across HEVs (Miyasaka et al., 2016).

Cell type	Rolling	Integrin activation	Adhesion	Intraluminal crawling	Transmigration
T cells	CD62L – PNAd	CCR7 – CCL19, CCL21 CXCR4 – CXCL12	LFA-1 – ICAM-1, ICAM-2	LFA-1 – ICAM-1	LFA-1 / ICAM-1? JAM
B cells	CD62L – PNAd	CCR7 – CCL19, CCL21 CXCR4 – CXCL12 CXCR5 – CXCL13	LFA-1 – ICAM-1, ICAM-2	LFA-1 – ICAM-1	LFA-1 / ICAM-1? JAM

4.3.2.3 Intraluminal crawling and transmigration

Once firmly adhered to the HEV wall, lymphocytes crawl along the vessel lumen (intraluminal crawling) in search for a permissive transmigration site, which is mainly dependent on the constitutively, highly expressed endothelial ICAM-1 (Boscacci et al., 2010). Transendothelial migration (TEM), the migration across the endothelium, is a process of sequential steps involving different molecules on the surface of the endothelium and within the cytoplasm (Muller, 2011). Although adhesion molecules localized at endothelial cell junctions, such as PECAM-1 (CD31), CD99, VE-cadherin or members of the JAM family, have been described *in vitro* and/or *in vivo* to be involved in the TEM process, the precise underlying molecular mechanisms are still not defined (Petri and Bixel, 2006).

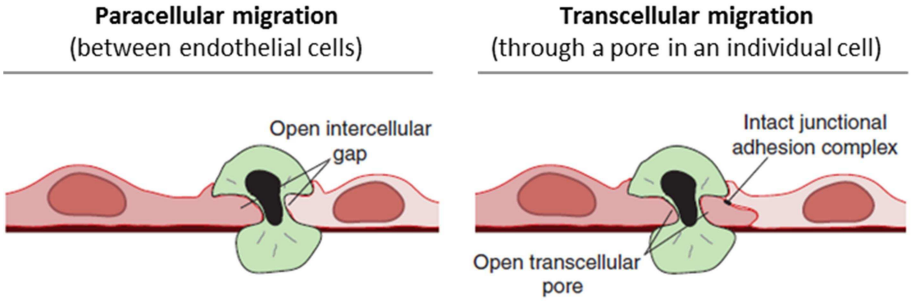


Figure 13. Modes of transendothelial migration

Immune cells can migrate across a vascular epithelium either between two adjacent endothelial cells (paracellular migration) or through a pore in an individual endothelial cell (transcellular migration). Reprinted modified from (Carman, 2009) with permission.

TEM occurs *in vivo* either between two adjacent endothelial cells at interendothelial cell junctions (paracellular migration) or directly through individual endothelial cells (transcellular migration) **(Figure 13)** (Anderson and Anderson, 1976; Marchesi et al., 1964; Schoefl, 1972; Wenk et al., 1974). The mode of migration appears to depend on multiple factors, such as the tightness of the endothelial junctions, the type and localization of the endothelium within the vascular system and the signaling of the leukocyte and/or endothelium (Filippi, 2016). Leukocytes, including lymphcoytes, monocytes and basophils, have been described to form both *in vitro* and *in vivo* highly dynamic invadosome-like protrusions (ILPs), with which they probe the local surface resistance of the endothelium to find a site of less resistance, permissive to transmigration (Carman et al., 2007; Martinelli et

al., 2014). Accordingly, in endothelia with tight intercellular junctions as at the blood-brain barrier, TEM takes predominantly place by the transcellular route, thereby leaving the tight junctions intact (Faustmann and Dermietzel, 1985; Lossinsky et al., 1989; Wolburg et al., 2005). Moreover, leukocytes appear to have different preferential transmigration routes. In an *in vitro* flow assay, human peripheral blood lymphocytes have been shown to transmigrate transcellularly across TNF-α-activated human umbilical vein endothelial cells (HUVECs), whereas neutrophils migrated through interendothelial cell junctions (Nieminen et al., 2006). Nevertheless, the exact molecular mechanisms controlling the route of migration need still to be defined.

4.3.2.3.1 Paracellular migration

TEM via the paracellular route requires the disassembly of interendothelial cell junctions to form a paracellular gap, through which leukocytes can migrate (Carman, 2009).
Endothelial permeability relies not only on the integrity of intercellular junctions, but also on actomyosin-based cell contractility (Wojciak-Stothard and Ridley, 2002). Hence, paracellular TEM results from multiple cell adhesion molecule-triggered signals that induce the disassembly of the intercellular junctions and the induction of actomyosin-based contractility within the endothelium, which finally results in the loosening of the cell junctions (Muller, 2011).

4.3.2.3.1.1 Endothelial cell junctions

Endothelial cells are interconnected by adherens junctions (AJs) and tight junctions (TJs) **(Figure 14A)**, composed of transmembrane proteins that mediate intercellular adhesion via homophilic interactions (Dejana, 2004). All junctional adhesion proteins are associated with the actin cytoskeleton, which not only stabilizes the junction but also permits their dynamic regulation of junction opening and closing (Dejana, 2004). In contrast to epithelial cells, in which TJs concentrate toward the apical site of the cell interface, endothelial TJs are found intermingled with AJs along the cell-cell contact (Dejana, 2004). TJs are composed of claudins, occludins, members of the JAM family, and ESAM **(Figure 14A)** (Dejana, 2004). Members of the Zonula occludens (ZO) family of proteins, including ZO-1-3, connect them to the actin cytoskeleton (Garcia-Ponce et al., 2015). The main AJ protein is the endothelial

cell-specific VE-cadherin (Lampugnani et al., 1992). With its extracellular domain, VE-cadherin binds other cadherins in a homophilic and calcium-dependent manner, thereby forming mechanically stable cell adhesion complexes between two adjacent cells (Geyer et al., 1999). Its cytoplasmic tail is linked to the AJ proteins p120, β-catenin and plakoglobulin **(Figure 14B)**. α-catenin binds to β-catenin and plakoglobulin and connects the cadherin complex to the actin cytoskeleton (Dejana et al., 2008). Under resting conditions, the vascular endothelial-phosphotyrosine phosphatase (VE-PTP) is bound to VE-cadherin and maintains the cadherin complex in a non-phosphorylated state that is critical for the barrier integrity (Reglero-Real et al., 2016). Consequently, signals that promote the phosphorylation of VE-cadherin, such as kinases, destabilize the endothelial junctions and facilitate vascular leakage and leukocyte migration (Wessel et al., 2014).

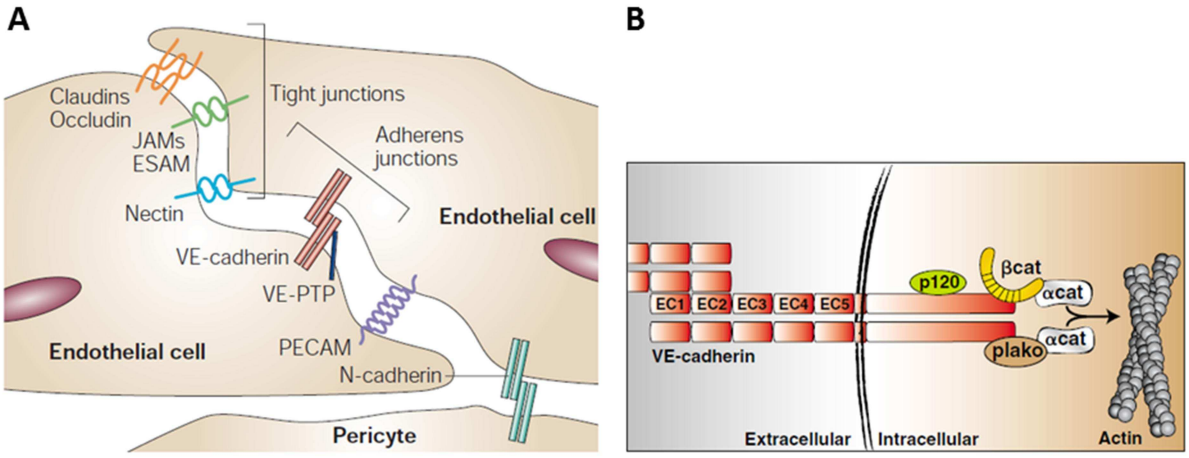


Figure 14. Endothelial cell junctions

(A) Endothelial cell junctions contain tight junctions, composed of claudins, occludins, junctional adhesion molecules (JAMs), and endothelial cell selective adhesion molecule (ESAM). Adherens junctions are formed by VE-cadherin, which is associated with vascular endothelial protein tyrosine phosphatase (VE-PTP) through its extracellular domain. Nectin contributes to the organization of tight and adherens junctions. Additional adhesion proteins such as PECAM-1 (CD31) cluster outside the junctions. Homophilic interactions between the extracellular domains of these proteins mediate the intercellular adhesion. N-cadherin is excluded from VE-cadherin^{+} junctions and possibly mediates the contact between endothelial cells and pericytes. Reprinted from (Dejana, 2004) with permission.

(B) VE-cadherin binds other cadherins through its extracellular domains (EC1-5) to promote strong adhesion between two adjacent endothelial cells. Its cytoplasmic tail is bound to p120, β-catenin (βcat) and plakoglobin (plako), with the two latters being bound to α-catenin (αcat). α-catenin connects the protein complex with the actin cytoskeleton. Reprinted from (Dejana et al., 2008) with permission.

4.3.2.3.1.2 Actin cytoskeleton

Endothelial cell junctions are connected to the actin cytoskeleton, which is required for their stabilization, while allowing their dynamically regulated opening and closure (Dejana, 2004). Hence, remodeling and the contractility of the actin cytoskeleton are important mechanisms that regulate the endothelial barrier and vascular permeability (Garcia-Ponce et al., 2015). Endothelial cells contain different forms of F-actin (Garcia-Ponce et al., 2015). Circumferential cortical actin rings are thought to stabilize the interendothelial junctions and barrier function (Garcia-Ponce et al., 2015). Under conditions of increased permeability and junctional instability, e.g., during inflammation, cortical actin rings are less prominent and reassemble into contractile cytoplasmic stress fibers (Aghajanian et al., 2008). Accordingly, under physiological conditions, large arterioles of the rat mesentery showed a prominent circumferential actin ring, capillaries displayed diffuse actin staining, and postcapillary venules, a main site of leukocyte extravasation, had a thin cortical actin ring with few short central fibers (Thurston and Baldwin, 1994). Under inflammatory conditions, the endothelial cytoskeleton needs to be dynamically remodeled to allow the transmigration of leukocytes (Garcia-Ponce et al., 2015). Contractility of the cytoskeleton results from the interaction of actin filaments with the myosin motor proteins, which are regulated by phosphorylation of their light chain (myosin light chain, MLC) (Garcia-Ponce et al., 2015). The two kinases MLC kinase (MLCK) and Rho kinase (ROCK) are responsible for the phosphorylation, while dephosphorylation is mediated by the MLC phosphatase (MLCP) (Garcia-Ponce et al., 2015). These enzymes are in turn controlled by two GTPases, Rho and Rac, which act antagonistically: while Rho increases the actomyosin contractility, thereby promoting the breakdown of intercellular junctions, Rac sustains junction integrity (Wojciak-Stothard and Ridley, 2002).

4.3.2.3.1.3 Mechanism of paracellular transmigration

Upon firm adhesion of leukocytes to the vessel wall through LFA-1 – ICAM-1 interaction, ICAM-1 has been shown to cluster densely at the leukocyte-endothelial cell interface and surrounding the cells during transmigration (Shaw et al., 2004). This clustering leads to and is maintained by the tyrosine kinase Src-dependent phosphorylation of cortactin, an F-actin binding protein that associates ICAM-1 with the endothelial cytoskeleton **(Figure 15)** (Yang et al., 2006a; Yang et al., 2006b). In addition, Src and Proline-rich Tyrosine Kinase 2 (Pyk2)

have been shown in HUVECs to phosphorylate VE-cadherin on the p120- and β-catenin-binding sites, tyrosine residues 658 and 731, respectively. This phosphorylation led to uncoupling of p120 and β-catenin from the cytoplasmic tail of VE-cadherin and thus disassembly of the intercellular junctions (Potter et al., 2005). Consistent with these findings, Greenwood and colleagues have demonstrated that phosphorylation of VE-cadherin enhanced transendothelial migration of lymphocyte across brain microvascular cells (Turowski et al., 2008)

Clustering of VCAM-1 on the endothelium surface upon cell adhesion activates the GTPase Rac1 that stimulates vascular NADPH (nicotinamide adenine dinucleotide phosphate) oxidase for the generation of reactive oxygen species (ROS) (van Buul et al., 2005). Rac-induced ROS generation has been shown to activate the kinase Pyk2, which in turn phosphorylated β-catenin, thereby destabilizing AJs (van Buul et al., 2005). In addition, ROS has been described to strongly inactivate protein phosphatases by oxidation of a critical cysteine residue in the catalytic site (Kim et al., 2003; Tai et al., 2002). Given that the phosphatase VE-PTP plays a crucial role in maintaining endothelial cell junctional integrity by maintaining VE-cadherin in a non-phosphorylated state (Nawroth et al., 2002), increased ROS levels may negatively affect VE-PTP and contribute to the disruption of the AJs. Deem and Cook-Mills have additionally shown that VCAM-induced ROS release activates endothelial cell-associated matrix metalloproteases (MMPs), which were needed for the efficient migration of lymphocytes across a LN-derived endothelial cell line (Deem and Cook-Mills, 2004). The authors suggested that these MMPs may degrade endothelial cell junction proteins, thereby promoting the migration of lymphocytes.

Both ICAM-1 and VCAM-1 clustering have been shown to increase the concentration of cytosolic free Ca^{2+} in the endothelial cells (Cook-Mills et al., 2004; Couraud et al., 2000), which activates the myosin light-chain kinase (MLCK) and causes the actin-myosin fibers to contract (Hixenbaugh et al., 1997). The activity of MLCK has been described to be reinforced by the inactivation of protein phosphatase 1 (PP1). PP1 inactivation results from its phosphorylation by Rho kinase (ROCK), which is activated by the ICAM-1-activated RhoA GTPase (Muller, 2014). This contraction has been suggested to contribute to the opening of interendothelial cellular junctions, thereby promoting TEM (Hixenbaugh et al., 1997). The net result of these events is the contraction of the endothelial cell body against weakened interendothelial cell junctions, which allows the transcellular migration of leukocytes (Muller, 2011).

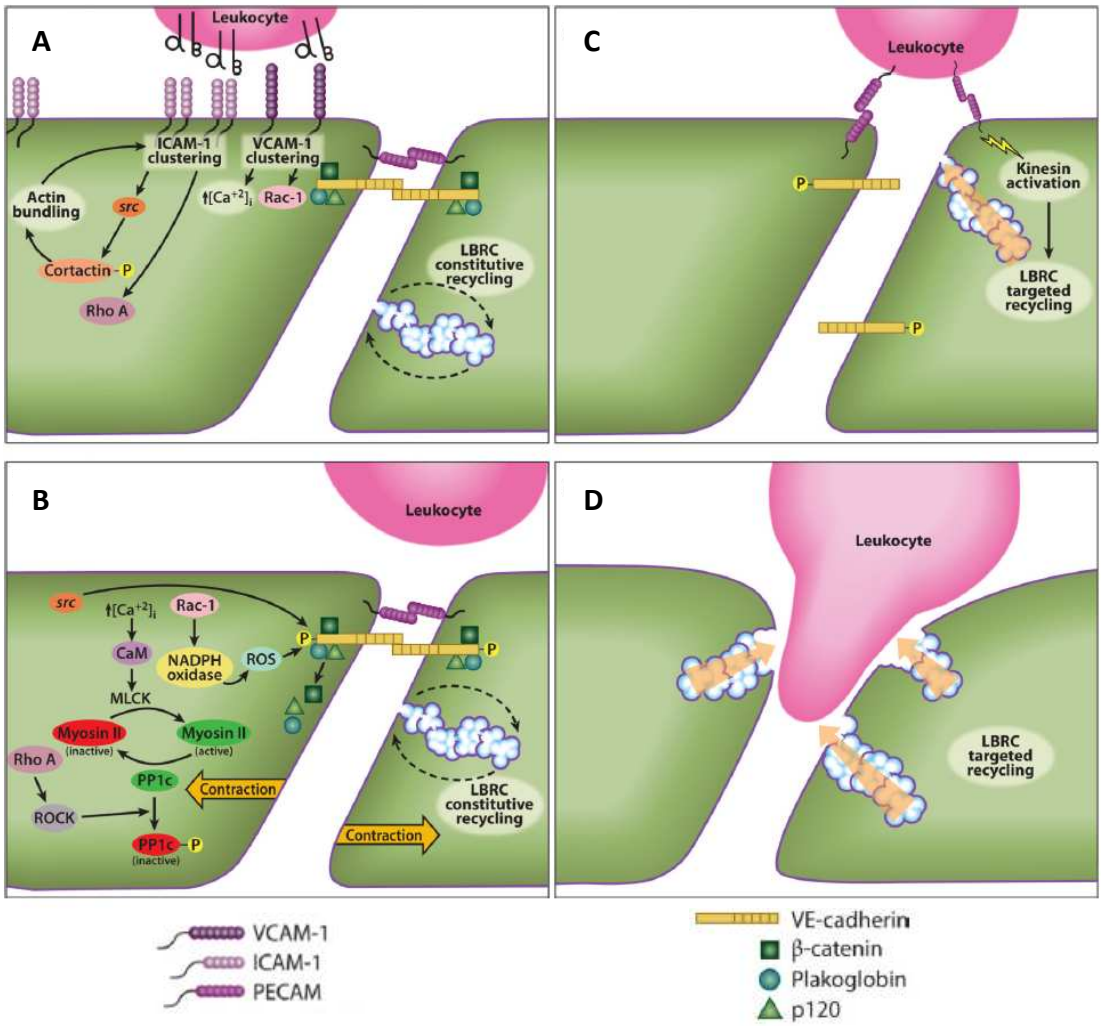


Figure 15. Molecular mechanisms of transcellular migration of leukocytes across an endothelium

(A) Binding of lymphocyte integrins to intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) on the endothelium leads to clustering of ICAM-1 and VCAM-1, which activates tyrosine kinase Src, GTPases RhoA and Rac-1 and increases cytosolic Ca^{2+}. Src-dependent phosphorylation of the F-actin binding protein cortactin maintains ICAM-1 clustering.

(B) These signals lead to the activation of myosin light-chain kinase (MLCK) and inactivation of protein phosphatase 1c (PP1c), which causes the contraction of the actin-myosin fibers. Phosphorylation of vascular endothelial cadherin (VE-cadherin) results in the disassembly of the endothelial intercellular junctions.

(C) Homophilic platelet/endothelial cell adhesion molecule 1 (PECAM-1) interactions between leukocytes and endothelial cells activate kinesin molecular motors in the endothelial cell and directs the trafficking of the lateral border recycling compartment (LBRC) to the vicinity of the migrating leukocyte.

(D) Targeted recycling of junctional proteins within the LBRC displaces components of the junctions laterally and thereby provides surface area and unligated molecules for the transmigrating leukocyte.

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For the passage of the leukocytes along the junctional border, targeted intracellular recycling of endothelial cell junctional proteins is required (Mamdouh et al., 2008). The lateral border recycling compartment (LBRC) is an endothelial cell-specific internalization organelle, found in close proximity of endothelial cell junctional lateral borders (Mamdouh et al., 2008). The LBRC contains the key junctional proteins PECAM-1, JAM-A and CD99, but not VE-cadherin (Feng et al., 2015; Mamdouh et al., 2009) and recycles them constitutively along the endothelial border. During TEM, homophilic PECAM-1 interactions between the transmigrating leukocytes and the endothelial cells direct the trafficking of the LBRC membrane at the cell border at the site of leukocyte transmigration (Mamdouh et al., 2008). Targeted recycling of junctional proteins within the LBRC displaces components of the junctions laterally, thereby providing not only additional surface area but also unligated molecules for the leukocytes, on which to migrate (Muller, 2011). VE-cadherin, in contrast, is not internalized into the LBRC (Mamdouh et al., 2009) but appears to be pushed out of the way by transmigrating leukocytes (Shaw et al., 2001). While lymphocytes and activated lymphoblasts transmigrate in a PECAM-1-independent manner, the targeted recycling of membrane from the LBRC has been shown to be required (Mamdouh et al., 2008).

4.3.2.3.2 Transcellular migration

TEM via the transcellular route involves the fusion of the leukocyte and endothelial cell membranes to form a transcellular pore, which allows the leukocytes to move through the cell (Muller, 2014). This mode of transmigration leaves the tight junctions intact, which may be important for specially tight endothelia such as the one at the blood-brain barrier (Muller, 2014). The molecules and mechanisms that mediate transcellular migration are similar to those controlling paracellular migration (Filippi, 2016). As described above, during their lateral migration over the endothelium, leukocytes constantly protrude and retract invadosome-like protrusions (ILPs) into the endothelium surface, thereby forming cell surface invaginations in the endothelial cells (Carman et al., 2007; Martinelli et al., 2014). This protrusive behavior has been shown to be required for efficient transcellular diapedesis (Carman et al., 2007) and has been suggested to be used to probe the endothelial cell surface for sites of low endothelial resistance for subsequent transcellular pore formation (Carman, 2009). As for the paracellular migration, the LBRC is also required for the transcellular route

with the exception that it itself forms the transcellular pore, allowing the passage of leukocytes (Mamdouh et al., 2009).

For the subsequent transcellular migration, the membranes of the leukocyte and endothelial cells must fuse and the required energy has been proposed to be possibly supplied by ILPs (Carman, 2009). Muller and colleagues showed that leukocytes are surrounded by membrane recruited from the LBRC that contained PECAM-1, JAM-A, and CD99 (Mamdouh et al., 2009). Both paracellular and transcellular migration of myeloid cells have been demonstrated to rely on PECAM-1 and CD99. Hence, the LBRC membranes form a transcellular channel of membranes lined with the required adhesive molecules for the interaction with the cells as they pass the endothelial cell (Mamdouh et al., 2009). The signals triggering the recruitment of the LBRC to the apical cell surface, however, are still not defined.

4.4 Migration to the T cell and B cell zone

Once naïve lymphocytes have entered the LN via the HEVs, they may either be nested within HEV pockets until being released or directly enter the underlying perivascular channel (Mionnet et al., 2011). They exit the channels via flaps formed by the membranes of two adjacent FRCs to reach the LN parenchyma (Bajénoff et al., 2006). Within the parenchyma, T and B cells move along the FRC fibers to home to the T cell zone and B cell follicles, respectively **(Figure 16A)** (Bajénoff et al., 2006). FDCs in the B cell follicles produce the chemokine CXCL13, which attracts the incoming CXCR5-bearing B cells toward the follicles **(Figure 16B)** (Ansel et al., 2000; Förster et al., 1996). FRCs in the paracortex secrete the chemokines CCL19 and CCL21, which interact with the chemokine receptor CCR7 on both B and T cells **(Figure 16C)** (Bajénoff et al., 2006). Within their respective compartment, B and T cells move along the FDC and FRC network, respectively, in search of cognate antigen (Bajénoff et al., 2006). T cells interact with the conduit-associated DCs that sample antigens from the conduit lumen, whereas B cells encounter antigen-capturing FDCs or $CD169^{+}$ macrophages for their activation (Mueller and Germain, 2009).

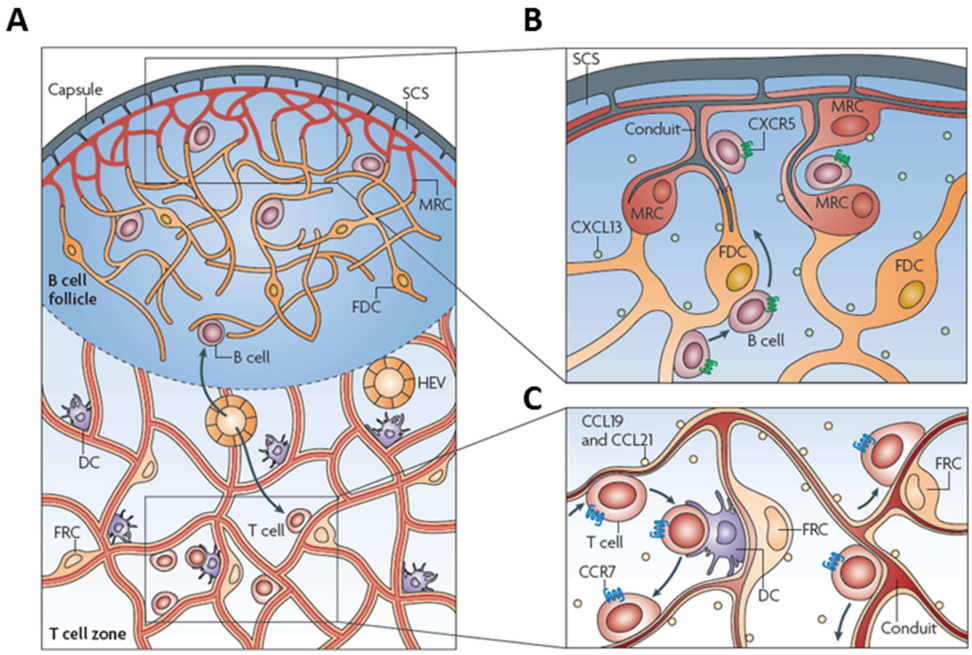

Figure 16. T and B cell migration within the lymph node

(A) B and T cells enter the LN through high endothelial venules in the T cell zone and move along the fibroblastic reticular cell (FRC) network to home to the B cell follicle and T cell zone, respectively.

(B) B cells express the CXC-chemokine receptor 5 (CXCR5) and are attracted by CXCL13-producing follicular dendritic cells in the B cell follicles. Within the follicles, B cells migrate along the FDC network in search of cognate antigen.

(C) In the T cell zone, C-C chemokine receptor 7 (CCR7)-bearing T cells move along the FRC network, which is rich in CC-chemokine ligand 19 (CCL19) and CCL21. They interact with conduit-associated dendritic cells, which capture antigens from the conduit lumen and present them to T cells (Mueller and Germain, 2009).

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4.5 Lymphocyte exit from the LN

Naïve B and T cells that have probed the LN for antigens without having encountered their cognate antigen, are ultimately returned to the circulation (Girard et al., 2012). They exit the LN via the efferent vessels that reconnect either directly or via other intervening LNs to the subclavian vein (Jackson, 2019). Lymphocyte egress depends on sphingolipid sphingosine-1-phosphate (S1P) and its receptor sphingosine-1-phosphate receptor (S1PR1), expressed on lymphocytes. Differential concentrations of S1P in the blood, the LN and the lymph as well as cyclical ligand-induced modulation of S1PR1 on lymphocytes are required for lymphocyte recirculation **(Figure 17)** (Rosen and Goetzl, 2005).

S1P is a pleiotropic autocrine and paracrine signaling lipid (Rosen and Goetzl, 2005). As a metabolite of the normal turnover of the plasma membrane sphingolipid ceramide, S1P is generated in most cells (Rivera et al., 2008b). Ceramide is cleaved by ceramidases to sphingosine (Aoki et al., 2016). The two sphingosine kinases (SphKs) SphK1 and SphK2 catalyze the subsequent phosphorylation of sphingosine to form S1P (Aoki et al., 2016). S1P levels in most tissues, including the LN, are very low since the produced S1P is either dephosphorylated by S1P phosphatases or irreversibly degraded by S1P lyase (Rivera et al., 2008b). In blood and lymph, however, S1P levels have been found to be high (Pham et al., 2010). In the blood, S1P is abundantly synthesized by hematopoietic cells, with erythrocytes being the major contributors (Schwab and Cyster, 2007). LECs are the main producers of S1P in the lymph and the activity of endothelial Sphk1 and Sphk2 for the generation of S1P has been shown to be crucial for lymphocyte egress (Pham et al., 2010). Accordingly, in mice deficient of Sphk1 and Sphk2 in LECs, the egress of both B and T cells from the LN is blocked (Pham et al., 2010).

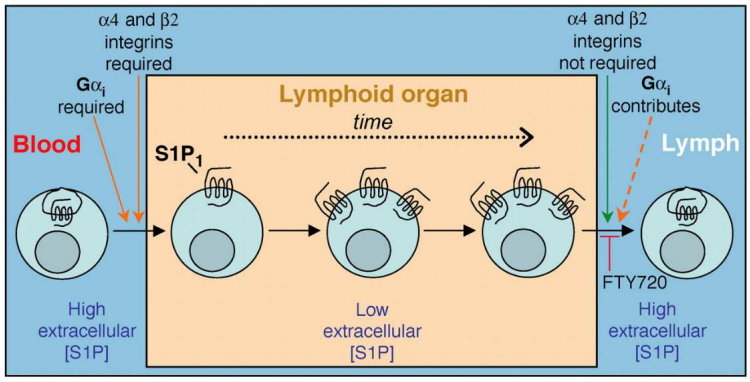

Figure 17. Lymphocyte egress depends on sphingolipid sphingosine-1-phosphate (S1P) and its receptor sphingosine-1-phosphate receptor (S1PR1)

Blood is rich in extracellular S1P, which leads to down-modulation of S1PR1 on lymphocytes in the blood. Upon migration into the lymph node, in which S1P concentrations are low due to S1P lyase-mediated degradation, lymphocytes start to reacquire S1PR1 on their cell surface and become responsive to S1P, which directs them to the S1P-rich cortical sinuses for egress. The drug FTY720 (Fingolimod) can suppress the egress of lymphocytes by causing S1PR1 internalization and degradation. Reprinted from (Lo et al., 2005).

S1PR1, expressed on the lymphocyte surface, is essential for lymphocyte egress by sensing S1P concentration gradients established between blood/lymph and LNs (Rivera et al., 2008a). Upon ligand binding, the receptor is quickly desensitized by phosphorylation of its cytoplasmic tail by the heterotrimeric guanine nucleotide-binding protein-coupled receptor kinase-2 (GRK2) and by receptor internalization (Willinger et al., 2014). Hence, the abundance of S1PR1 on the cell surface is low on naïve lymphocytes entering the LN from the blood (high S1P levels), which likely prevents their immediate egress through cortical sinuses in close proximity to HEVs (Lo et al., 2005). Within the LN (low S1P level), S1PR1 is reacquired on the cell surface and responsive to S1P and directs lymphocytes to the cortical sinuses for transmigration (Lo et al., 2005). Within the lymph, S1PR1 is again down-modulated (Lo et al., 2005).

4.6 Recruitment and retention of lymphocytes during immunity

While under homeostatic conditions lymphocyte numbers within the LNs are kept fairly constant, they dramatically increase upon inflammatory stimulation (Chang and Turley, 2015). This increase results from the enhanced recruitment of naïve lymphocyte from the blood and from their concomitant retention within the LN by reduced egress (Chang and Turley, 2015). Innate signals, such as LPS or CpG, which bind to the Toll-like receptors (TLRs) 4 and 9, respectively, have been shown to cause an increase in the diameter of the arterial vessels feeding the LN, thereby increasing the blood flow through the LN and thus the delivery of naïve lymphocytes (Soderberg et al., 2005). The enhanced cellular input is concomitantly sustained by an expansion of the vasculature within the LN. Increased expression of vascular endothelial cell growth factor (VEGF) leads to rapid proliferation of lymphatic and blood endothelial cell, including HEVs, which increase in numbers (Kumar et al., 2012). In addition to the enhanced cellular input, lymphocyte egress is transiently shut down during inflammation (Shiow et al., 2006). Egress depends on the expression of S1PR1 on lymphocytes and its ligand S1P (Aoki et al., 2016). Inflammatory cytokines, such as IFN-α/β or TNF-α, have been shown to mediate lymphocyte sequestration (Shiow et al., 2006). They induce the expression of the transmembrane protein CD69 on the surface of lymphocytes upon activation (Shiow et al., 2006). CD69 has been demonstrated to form a complex with S1PR1, which results in a down-modulation of the receptor (Shiow et al., 2006). Work of Cyster and colleagues has confirmed this interaction and has suggested that

CD69 induces a conformation of the receptor similar to the one of the ligand-bound state, leading to S1PR1 internalization and degradation (Bankovich et al., 2010). This down-modulation of S1PR1 decreases in turn the responsiveness of lymphocytes to S1P produced by lymphatic endothelial cells, thereby inhibiting their egress (Bankovich et al., 2010).

5 Antigen-Capturing Cells within the Lymph Node

Lymph-borne antigens and pathogens that are transported via lymphatics to the draining lymph nodes are captured by $CD169^+$ macrophages that line the subcapsular sinus floor, acting as efficient filter to prevent the spread of pathogens into the deeper LN parenchyma and their systemic dissemination (Junt et al., 2007). These macrophages are part of the first line of defense and induce rapid B cell responses by transferring the captured antigens to the underlying B cell follicles (Louie and Liao, 2019). Complementary to this, LN-resident dendritic cells that are in close proximity to the lymphatic sinuses efficiently sample lymph-borne antigens from the lymphatic sinus lumen and induce early T cell activation even before the arrival of the peripheral tissue-resident dendritic cells migrating to the draining LNs (Gerner et al., 2015).

5.1 Lymph node macrophages

Based on their distinct location within the LN, three main macrophages can be distinguished: the subcapsular sinus macrophages (SSMs), the medullary sinus macrophages (MSMs), and the medullary cord macrophages (MCMs), all of which exhibit differences in their function and expression of surface markers **(Figure 18)** (Gray and Cyster, 2012). All LN macrophages subsets have been found to be $CD11b^+CD11c^{lo}$, which distinguishes them from the $CD11b^+CD11c^{hi}$ classical dendritic cells (Junt et al., 2007; Phan et al., 2009).

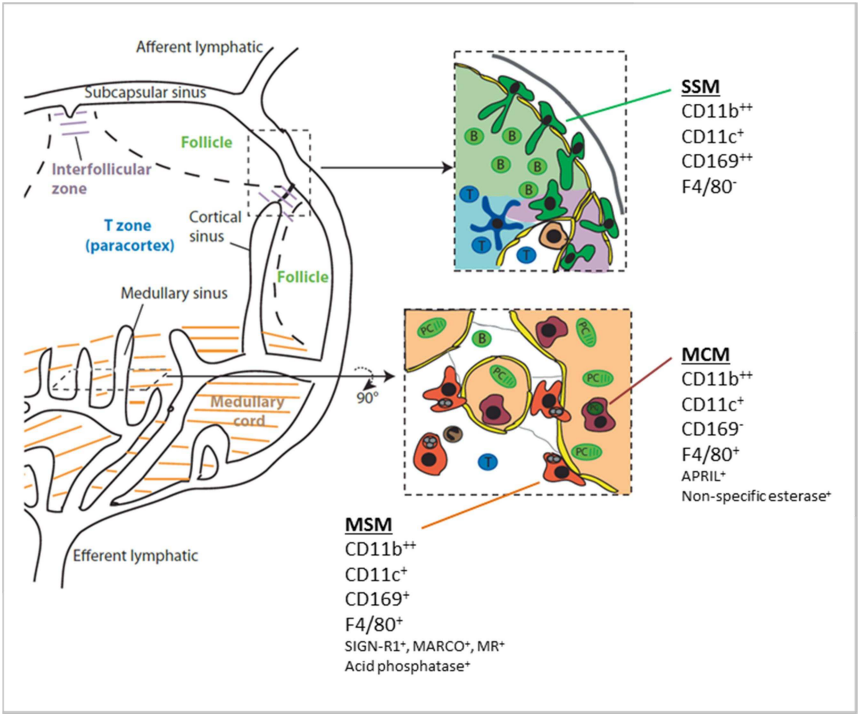

Figure 18. Schema of macrophage subsets within the LN and their phenotypic markers

The structure of a LN is schematically depicted on the left and the regions, where LN macrophages reside, are displayed enlarged in the boxes on the right. The CD169^{hi}F4/80^{-} SSMs line the subcapsular sinus floor with their heads protruding into the sinus lumen to capture lymph-borne antigens, and their tails extending into the underlying B cell follicles (green). The CD169^{-}F4/80^{+} MCMs reside within the medullary cords between the medullary sinuses and are involved in the clearance of apoptotic plasma cells. The highly phagocytic CD169^{+}F4/80^{+} MSMs are attached to the medullary sinus walls and to reticular fibers within the lumen. They dipose many pathogen-recognizing receptors and bind any lymph-borne antigen within the medulla.

B, B cell; T, T cell; PC, plasma cell; SSM, subcapsular sinus macrophages; MCM, medullary cord macrophages; MSM, medullary sinus macrophages. Reprinted modified from (Gray and Cyster, 2012) with permission.

5.1.1 Subcapsular sinus macrophages

Subcapsular sinus macrophages (SSMs) highly express CD169 (Siglec-1), but are negative for the mouse macrophage marker F4/80 (Junt et al., 2007; Phan et al., 2009). CD169, formerly termed sialoadhesin, belongs to the sialic acid-binding immunoglobulin-like lectin (Siglec) family, a group of sialic acid-binding receptors expressed on specific subsets of tissue macrophages (Taylor et al., 2005). Although not primarily involved in endocytosis, CD169 appears to be present on the plasma membrane of macrophage cellular processes, where it recognizes sialoconjugates on host and microbial ligands (Gordon et al., 2014). The

best characterized ligands contain terminal α-2,3 sialylated residues, found amongst others on heavily O-glycosylated molecules, such as the mucin CD43 (Gordon et al., 2014), widely expressed on hematopoietic cells (Mody et al., 2007), and the epithelial glycoprotein Mucin-1 (Gordon et al., 2014). Moreover, CD169 can be bound in a sialic acid-independent manner by the cysteine-rich domain of the mannose receptor (MR), expressed on medullary sinus macrophages (Gordon et al., 2014). When released in soluble form, MR may promote the capture of mannose-rich antigens by SSMs (Gray and Cyster, 2012). CD169 has also been shown to interact with the macrophage galactose-type N-acetylgalactosamine-specific lectin-1 (MGL1). Kumamoto and colleagues have reported that during the sensitization phase of contact hypersensitivity, dermal migratory $MGL1^+$ macrophages concentrated in the subcapsular sinus of draining lymph nodes by the interactions with N-glycans on SSM CD169 (Kumamoto et al., 2004).

Located underneath the subcapsular sinus, just above the underlying B cell follicles, SSMs form a first line of defense against lymph-borne pathogens entering the LN via the afferent lymphatic vessels. With their “heads” protruding into the subcapsular sinus lumen, SSMs capture mainly large, lymph-borne particles (> 70 kDa) that cannot diffuse freely through the sinus floor or gain access to the conduits (Gretz et al., 2000; Roozendaal et al., 2009; Sixt et al., 2005). They act as efficient filter to prevent the spread of pathogens into the deeper LN parenchyma and their systemic dissemination (Junt et al., 2007). Unlike dendritic cells, SSMs have been shown to be mostly non-migratory, maintaining their transcellular position with their “tails” being stable and likely attached to the extracellular matrix (Carrasco and Batista, 2007; Phan et al., 2007). SSMs have been shown to be poorly endocytotic and to synthesize only small amounts of lysosomal proteins, explaining their poor degradative activity (Phan et al., 2007). Captured antigens by their heads are instead retained on the surface and translocated to their “tails” that extend into the underlying B cell follicles **(Figure 19)** (Phan et al., 2007). SSMs have been shown to capture different viruses including vesicular stomatitis virus (VSV), adenovirus, vaccinia virus (Junt et al., 2007) and murine cytomegalovirus (MCMV) (Farrell et al., 2015) and also bacterial pathogens such as *P. aeruginosa* (Kastenmüller et al., 2012).

Cognate antigens presented by SSMs, can be directly recognized by migrating follicular B lymphocytes by their B-cell receptor (BCR) and become activated (Phan et al., 2007). It has been suggested that the adhesion between SSMs and B cells may be facilitated either directly by the means of the BCR or indirectly involving integrins. Indeed, engagement of the BCR has been reported to increase the expression of the lymphocyte function-associated antigen 1

(LFA-1) and/ or α4-integrins and thus the adhesiveness of the B cells to the respective ligands, ICAM-1 and VCAM-1 (Dang and Rock, 1991; Junt et al., 2007), both expressed on SSMs (Junt et al., 2007). Immune complexes in contrast have been shown to be collected from SSMs by non-cognate B lymphocytes through the complement receptor 1 and 2. They were then transported into the follicles for delivery to follicular dendritic cells, which probably relies on their high immune complex-binding capacity (Phan et al., 2007).

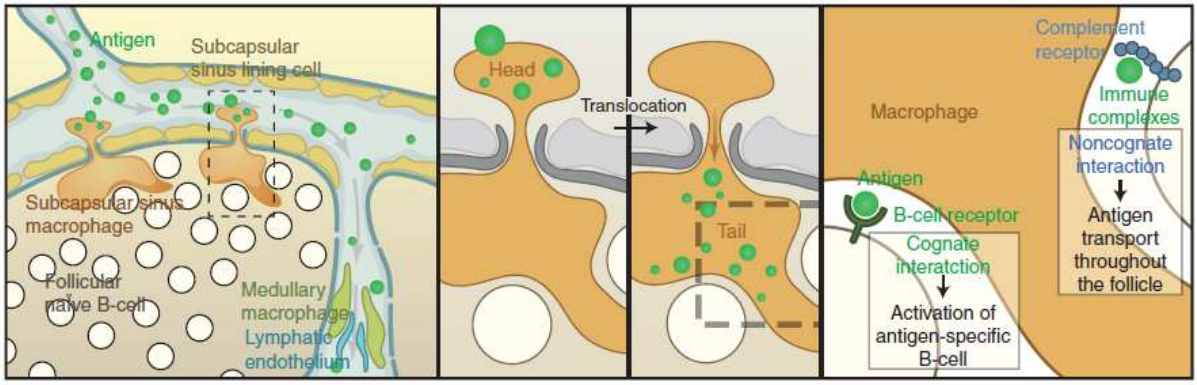

Figure 19. Subcapsular sinus macrophages capture lymph-borne antigens and pathogens

Lymph-borne antigens and pathogens that are transported via lymphatics to the draining lymph nodes are sampled by $CD169^{+}$ macrophages that line the subcapsular sinus (SCS) floor, acting as efficient filter to prevent the spread of pathogens into the deeper LN parenchyma and their systemic dissemination (Junt et al., 2007). These macrophages are part of the first line of defense and induce rapid B cell responses. With their heads protruding into the SCS lumen, they capture antigens and translocate them to their "tails" that extend into the underlying B cell follicles (Phan et al., 2007). Cognate antigens can be directly recognized by antigen-specific B cells by their B cell receptor for activation, whereas immune complexes can be collected from SSMs by non-cognate B lymphocytes through the complement receptors for delivery to follicular dendritic cells (Phan et al., 2007). Reprinted from (Martinez-Pomares and Gordon, 2007) with permission.

In addition to their function in antigen capture and presentation, SSMs play an important role in antiviral defense (Farrell et al., 2015; Iannacone et al., 2010; Junt et al., 2007; Phan et al., 2007). SSMs have been shown to be permissive for viral replication, thereby preventing viral systemic spread. Subcutaneous infection with the neurotropic virus VSV has been demonstrated to lead to viral replication exclusively within SSMs, which in turn produce high levels of type I interferon (IFN-I) and recruit IFN-I-producing plasmacytoid dendritic cells (Iannacone et al., 2010). The strong IFN-I response has been shown to protect intranodal nerves from infection, thereby preventing the invasion of the central nervous system by VSV (Iannacone et al., 2010). Indeed, genome-wide mRNA profile analyses revealed higher

expression of the pattern recognition receptor RIG-I sensing intracellular VSV, in SSMs compared to MSMs, suggesting a preferential response of SSMs to intracellular viruses (Kuka and Iannacone, 2014). Their permissivity to viral replication and subsequent IFN-I production has been shown to rely on B cell-derived lymphotoxin (LT) α1β2. In mice deficient for LTα1β2 in B cells, SSMs displayed a MSM-like phenotype, unable to replicate VSV and to prevent VSV neuroinvasion (Moseman et al., 2012). LTα1β2 may dampen the macrophage responsiveness to autocrine IFN-I. Indeed, $CD169^+$ metallophilic macrophages, the splenic counterparts of SSMs, have been reported to synthesize Usp18. This protein inhibited IFN-I signaling, which resulted in reduced IFN-I responsiveness of macrophages and thus enabled them to replicate VSV (Honke et al., 2011). In the LN, tonic exposure to LTα1β2 on follicular B cells could play a similar role in attenuating the responsiveness of SSMs to autocrine IFN-I (Kuka and Iannacone, 2014).

SSMs also contribute to the host defense against bacterial infections. Kastenmüller et *al*. have shown that upon bacterial infection, SSMs sense the pathogen by the means of NLR-based inflammasomes and generate active IL-1β and IL-18. IL-1β promotes neutrophil recruitment to eliminate extracellular bacteria, whereas IL-18 actives innate lymphoid cells including NK cells, γδ T cells, NK T cells, and innate-like $CD8^+$ T cells to produce IFN-γ, which in turn enhances the phagocytic activity of the macrophages toward intracellular bacteria (Kastenmüller et al., 2012).

5.1.2 Medullary sinus macrophages

Medullary sinus macrophages (MSMs) adhere to the medullary sinus walls and reticular fibers in the lumen (Gray and Cyster, 2012). As SSMs, they express CD169, albeit to a lesser extent compared to SSMs (Iannacone et al., 2010; Junt et al., 2007). In addition, MSM express F4/80 (Gray and Cyster, 2012). F4/80 (also termed epidermal growth factor (EGF)-like module-containing mucin-like hormone receptor-like 1, EMR1) belongs to a small leukocyte family of EGF-TM7 seven transmembrane G protein-coupled receptor (GPCR)-like adhesion receptors (Lin et al., 2005) and serves as a mouse macrophage cell membrane marker (Gray and Cyster, 2012). Although its exact role is still not known, it has been shown to be crucially implicated in the process of anterior chamber associated immune deviation (ACAID) model of ocular peripheral tolerance. Stein-Streilein and colleagues have reported that $F4/80^+$ cells migrate from the anterior chamber of the eye to the marginal sinus of the

spleen. There they interact with natural killer (NK) T cells, which was essential for the generation of $CD8^+$ regulatory T cells to induce systemic tolerance (Lin et al., 2005). Moreover, MSMs express the macrophage receptor with collagenous structure (MARCO), a class A scavenger receptor involved in sensing and clearing pathogens (Arredouani, 2014). This phagocytic receptor recognizes different isolated microbial components as well as intact Gram-positive and Gram-negative organisms (Mukhopadhyay et al., 2011). Additionally, it has been demonstrated that MARCO cooperates with intracellular pathogen sensors by internalizing ligands for recognition by the cytosolic Toll-like receptors (TLR)-3, nucleotide-binding oligomerization domain-containing protein 2 (NOD2) and the inflammasome component NALP3, thereby shaping the immune response (Mukhopadhyay et al., 2011). The type II C-type lectin SIGN-R1 (CD209), the murine homolog of human DC-SIGN, is also expressed by MSMs (Gray and Cyster, 2012). SIGN-R1 functions as pathogen recognition receptor and binds specific polysaccharide antigens such as dextrans and mannan present on the surface of encapsulated bacteria, which are rapidly internalized for lysosomal degradation (Geijtenbeek et al., 2002). MSMs also express the C-type lectin mannose receptor (MR, CD206) (Gordon et al., 2014). The MR binds a broad range of microorganisms, including bacteria (e.g., *Mycobacterium tuberculosis, Streptococcus pneumonia, Yersinia pestis*), fungi (e.g., *Candida albicans, Pneumocystis carinii, Cryptococcus neoformans*), virus (e.g., HIV, influenza virus, dengue virus), and parasites (e.g., *Leishmania*), and is highly endocytic and phagocytic (Azad et al., 2014). Its extracellular regions are composed of an N-terminal cysteine-rich (CR) domain that binds glycoproteins with terminal GlcNAc residues (e.g., CD169 on SSMs), a fibronectin II domain that binds to collagen, and eight carbohydrate recognition domains that bind mannose and fucose with high affinity (Azad et al., 2014).

Consistent with their broad expression of receptors, MSMs are poorly selective and bind to any lymph-borne particulate such as bacteria, nanoparticles and apoptotic cells (Kuka and Iannacone, 2014). Like SSMs, MSMs also capture viruses such as VSV, but in contrast to SSMs, MSMs are resistant to viral replication and appear to not produce pro-inflammatory cytokines (Iannacone et al., 2010). MSMs have been shown to express higher levels of TLR4 and TLR13 than SSMs, which both recognize VSV (Moseman et al., 2012), suggestive for detection and elimination of extracellular viruses (Kuka and Iannacone, 2014). Indeed, MSMs contain high levels of endosomal degradative enzymes, and rapidly clear lymph-borne pathogens (Phan et al., 2009) as well as apoptotic polymorphonuclear cells by phagocytosis (Gray and Cyster, 2012).

5.1.3 Medullary cord macrophages

In addition to MSMs, Cyster and colleagues have described another medullary macrophages subset, the CD169$^-$F4/80$^+$ medullary cord macrophages (MCMs) (Gray and Cyster, 2012). MCMs possess, contrary to MSMs, a low acid phosphatase and high non-specific esterase activity. Moreover, MCMs are less phagocytic than MSMs but specifically clear apoptotic plasma cells (Gray and Cyster, 2012). They also provide trophic factors such as APRIL that promote the survival of plasma blasts and plasma cells (Gray and Cyster, 2012).

5.2 Lymph Node Dendritic Cells

Dendritic cells are potent antigen-presenting cells that are crucial for the priming of naïve T cells and for the maintenance of peripheral tolerance (Worbs et al., 2016). They efficiently sample exogenous or self-antigens in peripheral tissues, such as the skin, and migrate to draining LNs, where they present antigens directly to naïve T cells or transfer them to LN-resident DCs (Tomura et al., 2014). While under steady-state conditions, DCs are largely tolerogenic, leading to dampened T cell responses, in a proinflammatory environment, DCs undergo a maturation process, which provides them the necessary motility to migrate to the skin-draining LNs and competence to prime naïve T cells (Iberg et al., 2017). This maturation program includes the upregulation of the CCR7, major histocompatibility complex class II (MHC-II) and co-stimulatory molecules, such as CD80, CD86 and CD40, and imparts the capacity to release cytokines, which promote the differentiation of naïve antigen-specific T cell into effector cells, and which activate other immune cells (Probst et al., 2003; Spörri and Reis e Sousa, 2005).

Murine skin-draining LNs contain conventional MHC-II^{int} LN-resident DCs and MHC-II^{hi} skin-derived migratory DCs (Gerner et al., 2012). Based on their anatomical location in the resting skin and their cell surface expression of the C-type lectin Langerin (CD207) and the integrin CD103, skin DCs can be further subdivided in three main subsets: the Langerin$^+$CD103$^-$ Langerhans cells (LCs) reside in the epidermis, while in the dermis, the Langerin$^+$CD103$^+$ dermal DCs (dDCs) and the Langerin$^-$CD103$^-$ dDCs can be found **(Figure 20)** (Igyártó et al., 2011). The different DC subsets have been shown to exhibit specific, non-redundant functions, which is also reflected in their distinct spatial distribution within the LN. LN-resident DCs are mainly located deep in the T cell zone and have been shown to sample small protein antigens from the LN conduits (Gretz et al., 2000; Sixt et al., 2005). In addition,

a specialized population of LN-resident DCs has been reported to reside within the lymphatic sinus floor of the interfollicular zones, cortico-medullary junctions and medullary regions of the LN (Gerner et al., 2015). These DCs have been shown to be able to capture lymph-borne microbial and vaccination-derived particulates (e.g., *Escherichia coli*, *Staphylococcus aureus* bioparticles and microspheres of different sizes) from the sinus lumen and to induce early T cell activation and generation of effector T cell responses much sooner than and independently of migratory DCs (Gerner et al., 2015). The skin-derived Langerin$^-$CD103$^-$ dDCs are mainly found in the interfollicular and outer paracortical regions within the LN (Gerner et al., 2012) and specifically prime CD4$^+$ T cells by capturing and processing extracellular antigens for MHC-II presentation (del Rio et al., 2007; Kim and Braciale, 2009). LCs and CD103$^+$ dDCs, in contrast, accumulate deeper in the T cell zone (Gerner et al., 2012). CD103$^+$ dDCs have been shown to most efficiently cross-present extracellular antigen by MHC-I molecules to CD8$^+$ T cells among all skin-derived DCs (Bedoui et al., 2009; del Rio et al., 2007; Kim and Braciale, 2009). Accordingly, mice deficient for CD103$^+$ dDCs show an impaired priming of CD8$^+$ T cells (Murphy et al., 2013). The role of LCs is less clear but recent evidence suggests that they might contribute to peripheral tolerance (West and Bennett, 2018). Indeed, LCs have been shown to mediate deletional tolerance in CD4$^+$ (Igyarto et al., 2009; Shklovskaya et al., 2011) and CD8$^+$ T cells (Flacher et al., 2014; Gomez de Agüero et al., 2012) upon migration to skin-draining LNs, and to activate regulatory T (T_{reg}) cells (Gomez de Agüero et al., 2012; Kitashima et al., 2018). In epicutaneous infections with the pathogenic *Candida albicans* or *Staphylococcus aureus* in mice, however, LCs have been reported to be necessary and sufficient for the generation of protective T helper 17 cells (Igyártó et al., 2011). In addition to their different functions, skin-derived DCs also show distinct migration patterns toward and different lifespans within the LN. While under steady-state conditions, migrated Langerin$^-$ dDCs had the shortest lifespan within the LN with ~2 days, CD103$^+$ dDCs stayed 4.5 days and LCs 4 days (Tomura et al., 2014). Upon inflammatory stimulation of the skin by a chemical stressor or mechanical injury, Langerin$^-$ dDCs have been shown to be the first skin-derived DC subset that migrated to the draining LN after one day, whereas CD103$^+$ dDCs and LCs could be detected only on day three and four (Tomura et al., 2014).

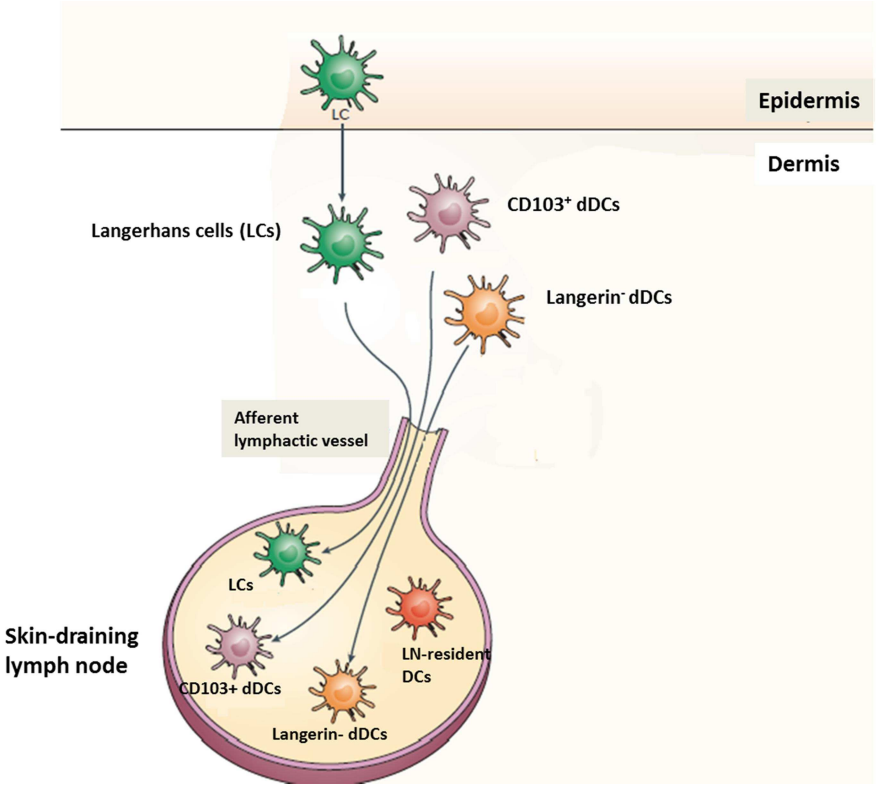

Figure 20. Dendritic cell subsets within the skin-draining lymph node

Skin-draining LNs typically contain MHC-II^{int} resident DCs and three subsets of MHC-II^{hi} migratory skin-derived DCs: the Langerin^{+}CD103^{-} Langerhans cells (LCs) that reside in the epidermis, and the dermal Langerin^{+}CD103^{+} DCs (CD103^{+} dDCs) and Langerin^{-}CD103^{-} dDCs (Langerin^{-} dDCs) (Gerner et al., 2012; Igyártó et al., 2011). Reprinted modified from (Malissen et al., 2014) with permission.

Thesis Objective

Pseudomonas aeruginosa produces a wide array of virulence factors and exhibit a high intrinsic resistance to many antibiotics, which leads to persistent infections associated with high morbidity and mortality. The severity and outcome of these infections depend on the appropriate response of the host and the bacterial virulence factors that subvert host responses. The *P. aeruginosa* lectin LecB has been shown *in vitro* to be involved in the adhesion of the bacterium to host epithelial cells and to play a role in biofilm formation. However, its role in the pathophysiology of *P. aeruginosa* infections and/or evasion of the host immune response is not completely understood. Therefore, the aim of my thesis was to study the function of the *P. aeruginosa* lectin LecB *in vivo* by analyzing the impact of cutaneously administered LecB on the immune cells of skin-draining lymph nodes. Firstly, I identified the target cells of LecB within the lymph node and secondly, I analyzed its impact on these cells. A better understanding of the interactions between this lectin and the cutaneous and lymph node immune cells is crucial to an overall comprehension of the pathophysiology and would likely foster the development of novel preventative and/or therapeutic strategies to treat *Pseudomonas aeruginosa* infections.

MATERIAL AND METHODS

Material and Methods

Table 2. Antibodies used in experiments. The host and target species, the fluorochrome, supplier and reference number are listed.

Antibodies (clone)	Supplier	Reference
Rat anti-Mouse **I-A/I-E** AF700 (M5/114.15.2)	BioLegend	107622
Armenian Hamster anti-Mouse **CD11c** PE-Cy7 (HL3)	BD Pharmingen	558079
Rat anti-Mouse **CD103** PE (M290)	BD Pharmingen	557495
Rat anti-Mouse **Langerin** AF647 (929F3.01)	Novus Biologicals	DDX0362A647-100
Rat anti-Mouse **CD11b** PerCP Cy5.5 (M1/70)	BioLegend	101228
Rat anti-Mouse **CD169** PE (3D6.112)	BioLegend	142404
Rat anti-Mouse **CD169** AF594 (3D6.112)	Biolegend	142416
Rat anti-Mouse **F4/80** APC (BM8)	BioLegend	123116
Rat anti-Mouse **CD45R/B220** FITC (RA3-6B2)	BD Pharmingen	553088
Armenian Hamster anti-Mouse **CD3ε** PE (145-2C11)	BD Pharmingen	553064
Armenian Hamster anti-Mouse **CD3ε** APC (145-2C11)	BD Pharmingen	553066
Mouse anti-Mouse **CD45.1** APC-eF780 (A20)	eBioscience	47-0453-80
Mouse anti-Mouse **CD45.2** APC (104)	BD Pharmingen	558702

Rat anti-Mouse **CD45** APC (30-F11)	BD Pharmingen	559864
Rat anti-Mouse **CD45** APC/Cy7 (30-F11)	BioLegend	103116
Rat anti-Mouse **TER-119** APC-eF780 (TER-119)	eBioscience	47-5921
Rat anti-Mouse **CD31** PE (390)	BioLegend	102407
Rat anti-Mouse **CD31** biotin (390)	eBioscience	13-0311-82
Syrian Hamster anti-Mouse **gp38** Podoplanin PE/Cy7 (8.1.1)	BioLegend	127412
Rat anti-Mouse **PNAd** biotin (MECA-79)	Biolegend	120804
Rat anti-Mouse **MAdCAM-1** biotin (MECA-367)	eBioscience	13-5997-85
Rat anti-Mouse **MAdCAM-1** PE (MECA-367)	Santa Cruz Biotechnology	sc-19604 PE
Rat anti-Mouse **CD106/VCAM-1** APC (429 (MVCAM.A))	BioLegend	105718
Rat anti-Mouse **CD54/ICAM-1** APC (YN1/1.7.4)	BioLegend	116120
Syrian Hamster anti-Mouse **CLCA1** (10.1.1)	Andy Farr, University of Washington, Seattle, US	Furuya et *al.* 2010
Rat anti-Mouse **Ly-6G** APC (1A8-Ly6G)	eBioscience	17-9668-82
Rat anti-Mouse **Ly-6C** PE (AL-21)	BD Pharmingen	560592
*Invivo*MAb Rat anti-Mouse **L-Selectin/CD62L** (Mel-14)	Bio X Cell	BE0021

Rabbit anti-Human **CD144/VE-cadherin** (polyclonal)	eBioscience	MBS158
Mouse anti-Human **α-tubulin** (DM1A)	Cell Signaling	3873
Goat anti-Hamster AF546 (Polyclonal)	Invitrogen	A-21111
Goat anti-Hamster AF488 (Polyclonal)	Invitrogen	A-21110
Goat anti-Rabbit AF647 (Polyclonal)	Invitrogen	A-21245
Purified Syrian Hamster IgG Isotype (SHG-1)	BioLegend	402002
Goat anti-Rabbit IgG-HRP	Cell Signaling	7074
Horse anti-Mouse IgG-HRP	Cell Signaling	7076

Table 3. Biological samples used in experiments

Biological Samples	Supplier
Normal Goat Serum (NGS)	Invitrogen
Normal Rat Serum (NRS)	Own production
Fetal Bovine Serum (FBS)	BioWhittaker
Bovine serum albumin (BSA)	Euromedex

Table 4. Chemicals, peptides and kits used in experiments

Chemicals, Peptides, Kits	Supplier
Streptavidin APC conjugate	eBioscience
Streptavidin AF647 conjugate	Invitrogen
Streptavidin FITC conjugate	BD Pharmingen
7-Amino-Actinomycin D (7-AAD)	BD Pharmingen
4′,6-Diamidino-2-Phenylindole (DAPI)	Roche
Fixable Viability Dye eFluor 450	eBioscience
Phalloidin ATTO 565	Sigma-Aldrich
FcR Blocking Reagent mouse	MACS Miltenyi Biotec
Collagenase D	Roche
DNase I	Roche
Dispase II	Roche
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
OCT Embedding Matrix	Cell Path
Acetone	VWR Chemicals
Paraformaldehyde	Sigma-Aldrich
Mowiol® 4-88	Carl Roth GmbH + Co. KG
Pierce™ BCA Protein Assay Kit	Thermo Scientific

Pefablock	Sigma-Aldrich
Aprotinin	Sigma-Aldrich
Leupeptin	Sigma-Aldrich
Phosphatase inhibitor cocktail 3	Sigma-Aldrich
Clarity™ Western ECL Blotting Substrate	BioRad

1 Mice

C57BL/6 (Charles River Laboratories France) and congenic B6.SJL-*Ptpcr*^{a} Pepc^{b}/BoyJ (B6 Cd45.1) (Jackson Laboratory, Bar Harbor, Maine, US) mice, designated CD45.1 hereinafter, were kept and bred under pathogen-free conditions. Both female and male adult mice with an age of 8-18 weeks and age-matched littermates as controls were used. All experiments were carried out in conformity to the animal bioethics legislation approved by and according to national guidelines of the CREMEAS (Comité Régional d'Ethique en Matière d'Expérimentation Animale de Strasbourg).

2 LecB injections

Recombinant LecB was prepared, fluorescently labeled and provided by the group of Prof. Dr. Winfried Römer (BIOSS, Freiburg, Germany) as described in (Landi et al., 2019). Dilutions of the LecB stock were prepared in sterile PBS (BioWhittaker, Lonza, Basel, Switzerland) and injected with a volume of 25 µL per injection site. Control mice were injected with the same volume of PBS (BioWhittaker). Mice were anesthetized with isoflurane for the time of injections. For analysis of LecB target cells, mice were injected intradermally into the ear pinna and subcutaneously into the hind footpad with 12.5 µg of fluorescently labelled LecB and 4 h later, auricular and popliteal lymph nodes (LNs) were dissected. For experiments with unlabeled LecB, 12.5 µg of LecB was injected in the same way and auricular and popliteal LNs were taken 24 h later. In some experiments, brachial LN were additionally dissected and served as non-drained LN control. For adoptive transfer and

lymph node entry blockage experiments, mice were injected with LecB in the ear and hind footpad on one side and contralaterally with PBS, and PBS-draining LNs served as internal control.

3 Adoptive transfer

For adoptive transfer of lymphocytes, auricular, axillary, inguinal, brachial and popliteal LNs and spleens of CD45.1 mice were dissected. LNs and spleens were cut into halves and fourths, respectively, before being crushed separately in PBS with a tissue grinder to isolate cells. The obtained LN cell suspension was passed twice through a 40 µm Nylon cell strainer (Falcon) to remove remaining tissue and to dissociate cell aggregates, and the strainer was rinsed twice with PBS. The spleen cell suspension was centrifuged at 1500 rpm for 5 min and the cell pellet incubated in Ammonium-Chloride-Potassium (ACK) lysing buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA; filtered 0.22 µm) for 4 min for lysis of red blood cells. The spleen cell suspension was then washed with PBS to remove the ACK lysing buffer and passed twice through a 40 µm Nylon cell strainer (Falcon) that was rinsed twice with PBS. To determine the cell count, cells were diluted 1:10 in Trypan Blue (BioWhittaker, Lonza, Basel, Switzerland) and viable, unstained cells were counted using a Malassez counting chamber. The cell suspensions were pooled and 30 x 10^6 cells each were injected intravenously into C57BL/6 (CD45.2) mice that were 1 to 4 hours prior to cell transfer injected intradermally into the ear pinna or subcutaneously into the hind footpad with 12.5 µg of LecB and contralaterally with the same volume of PBS. Twenty-four hours after adoptive cell transfer, the brachial, auricular and popliteal LNs were dissected for flow cytometric analysis.

4 Lymph node entry blockage

Mice were injected intravenously with 100 µg of anti-CD62L (Bio X Cell) and 6 hours later they were injected with 12.5 µg LecB and contralaterally with the same volume of PBS intradermally into the ear pinna and subcutaneously into the hind footpad. Eighteen hours later the draining auricular and popliteal LNs as well as the brachial LNs as negative control were dissected for flow cytometric analysis.

5 Isolation of lymph node cells

For flow cytometric analysis, the dissected LNs were cut into small pieces with the aid of a scalpel blade and, depending on the experiments, the two LNs of the same type of one mouse were either pooled or not and transferred into centrifuge microtubes containing 700 µL and 400 µL, respectively, of freshly made digestion medium comprised of RPMI-1640 (BioWhittaker, Lonza, Basel, Switzerland) containing 2% (v/v) fetal calf serum (FCS; Dutscher, Brumath, France), 1 mg/mL Dispase (Roche, Basel, Switzerland), 1 mg/mL Collagenase D (Roche, Basel, Switzerland), and 0.1 mg/mL DNAse I (Roche, Basel, Switzerland). The samples were incubated on a shaker (Thermomixer comfort, Eppendorf, Hamburg, Germany) at 37° C and 1000 rpm for 45 minutes to ensure the contents were well-mixed. The LN digest was then aspired and expirated several times using a 1-mL pipette to break aggregates before stopping the digestion by addition of 4 and 7 µL, respectively, of 0.5 M EDTA (Sigma-Aldrich, St. Louis, MO, US) per tube. To obtain a cell suspension free from undigested tissue and cell clumps, the digest was passed through a 100 µm Nylon cell strainer (Falcon) that was then rinsed with PBS (BioWhittaker, Lonza, Basel, Switzerland) containing 2% (v/v) FCS and 2.5 mM EDTA. After centrifugation of the single cell suspensions at 1500 rpm for 5 min, the cells were resuspended in 500 µL PBS (BioWhittaker, Lonza, Basel, Switzerland) containing 2% FCS and 2.5 mM EDTA. To determine the cell count, cells were diluted 1:10 in Trypan Blue (BioWhittaker, Lonza, Basel, Switzerland) and viable, unstained cells were counted using a Malassez counting chamber.

6 Flow cytometry

For flow cytometric analysis of macrophages, dendritic cells and lymphocytes, 0.5 to 1 x 10^6 cells of the LN cell suspensions each were used, whereas for stromal cell analysis, all isolated cells were used. All reactions were, if not otherwise stated, performed at 4°C in PBS (BioWhittaker, Lonza, Baser, Switzerland) containing 2% (v/v) FCS and 2.5 mM EDTA. Cells were incubated with antibodies for 20 to 30 min and washed twice in PBS (BioWhittaker, Lonza, Basel, Switzerland) containing 2% (v/v) FCS and 2.5 mM EDTA. When a fixable viability dye was used, cells were washed twice in PBS and incubated with the dye diluted in PBS for 15-30 min prior to antibody incubation. When DAPI was used for exclusion of dead cells, cells were resuspended in DAPI (Roche, 0.1 µg/mL) after incubation with antibodies.

For analysis of LN macrophages, unspecific antibody binding and binding to Fc receptors was prevented by incubating cells with a FcR blocking reagent (MACS Miltenyi Biotec, dilution 1:200) and 5% (v/v) rat serum, together with the Fixable Viability Dye eFluor780 (FVD eF780, eBioscience, dilution 1:2000) for 30 min. Cells were then stained with anti-CD169-PE (Siglec-1) (BioLegend, dilution 1:200), anti-CD11c-PE-Cy7 (BD Pharmingen, dilution 1:400), anti-CD11b-PerCP-Cy5.5 (BioLegend, dilution 1:400) and anti-F4/80-APC (BioLegend, dilution 1:100).

For analysis of LN dendritic cells, the Fixable Viability Dye eFluor 450 (eBioscience, dilution 1:1000) was used as a dead cell marker. Binding of antibodies to Fc receptors was blocked with FcR Blocking Reagent (MACS Miltenyi Biotec, dilution 1:100) and cells were stained with anti-CD103-PE (BD Pharmingen, dilution 1:100), anti-CD11c-PE-Cy7 (BD Pharmingen, dilution 1:200) and anti-I-A/I-E-AF700 (BioLegend, dilution 1:400). Cells were washed with PBS and fixed in BD Cytofix^{TM} Fixation Buffer (BD Biosciences) for 20 min and washed in BD Perm/Wash^{TM} Buffer (BD Biosciences) for intracellular staining with Langerin-AF647 (Novus Biologicals, dilution 1:100 in BD Perm/Wash^{TM} Buffer).

For analysis of lymphocytes, cells were, depending on the experiment, stained with anti-CD45.2-APC (BD Pharmingen, dilution 1:200) and anti-CD45.1 APC-eFluor780 (eBioscience, dilution 1:100) or anti-CD45-APC/Cy7 (BioLegend, dilution 1:500) alone, and with anti-CD45R/B220-FITC (BD Pharmingen, dilution 1:200), anti-CD3e-PE or –APC (both BD Pharmingen, dilution 1:200). 7-Aminoactinomycin (7-AAD) (BD Pharmingen, dilution 1:50) was used for exclusion of dead cells and added to the cells just before flow cytometric analysis.

For analysis of myeloid cells, binding of antibodies to Fc receptors was blocked with FcR Blocking Reagent (MACS Miltenyi Biotec, dilution 1:100) and cells were stained with anti-CD45-APC/Cy7 (BioLegend, dilution 1:500), anti-CD11b- PerCP Cy5.5 (BioLegend, dilution 1:400), anti-Ly6C-PE (BD Pharmingen, dilution 1:200), and anti-Ly6G-APC (eBioscience, dilution 1:200). The Fixable Viability Dye eFluor 450 (eBioscience, dilution 1:1000) was used as a dead cell marker.

For analysis of LN stromal cells, cells were stained with anti-CD45-APC/Cy7 (BioLegend, dilution 1:400) and TER-119-APC-eF780 (eBioscience, dilution 1:400) to gate out $CD45^+$ and $TER\text{-}119^+$ hematopoietic cells into a dump channel, and with anti-CD31-PE (BioLegend, dilution 1:200), anti-gp38/Podoplanin-PE/Cy7 (BioLegend, dilution 1:500), and, depending on the experiments, with anti-PNAd-biotin (Biolegend, dilution 1:200), anti-mCLCA1 (a kind gift from Andy Farr, University of Washington, Seattle, USA, dilution 1:10), anti-

VCAM-1-APC (BioLegend, dilution 1:500), anti-ICAM-1-APC (BioLegend, dilution 1:300), and anti-MAdCAM-1-biotin (eBioscience, dilution 1:200). The corresponding secondary antibodies included Streptavidin-APC (eBioscience, dilution 1:1000), goat anti-Hamster IgG-A488 (Invitrogen, dilution 1:1000), and Streptavidin-FITC (BD Pharmingen, dilution 1:1000), respectively. DAPI (Roche, 0.1 µg/mL) was used to exclude dead cells.
Flow cytometry and analysis was performed using a Gallios Flow Cytometer (Beckman Coulter, Idianapolis, USA) and the software package FlowJo (Tree Star, Inc., Ashland, OR, USA), respectively.

7 Immunofluorescence of lymph nodes

Dissected LNs were embedded in OCT Embedding Matrix for Frozen Sections (Cell Path, Newton, Poys, UK) and frozen in liquid nitrogen. If not directly used, frozen LNs were stored at -80°C. Sections of 7-8 µm were cut on a cryostat (Leica CM3050S, Wetzlar, Hessen Germany) and loaded on Superfrost^{TM} Plus Gold Slides (Thermo Scientific, Waltham, MA, US). Sections were dried on air for at least 2 hours, and then fixed in acetone for 20 min on ice. After drying, sections were stored at -80°C if not used immediately.
For immunofluorescence staining of LN sections, sections were thawed at room temperature if stored at -80°C before and were then circumscribed with the hydrophobic barrier pen ImmEdge PEN (Vector Laboratories, Burlingame, CA, USA), providing a hydrophobic barrier that keeps all reagents localized on the tissue sections. During the whole staining procedure, slides with the tissue sections were kept in a humidified chamber to prevent the evaporation of the reagents and drying-out of the sections. All reactions were performed at room temperature in PBS (BioWhittaker, Lonza, Basel, Switzerland) containing 2% (v/v) fetal calf serum or 2% (w/v) BSA and were followed by three washing steps with PBS (BioWhittaker, Lonza, Basel, Switzerland) for 5 minutes each. To block unspecific binding of the primary antibodies, LN sections were first incubated with PBS (BioWhittaker, Lonza, Basel, Switzerland) containing 2% (v/v) fetal calf serum for at least 30 min. Sections were then incubated with primary antibodies including anti-CD169-AF594 (BioLegend, dilution 1:1000), anti-mCLCA1 (a kind gift from Andy Farr, University of Washington, Seattle, USA, dilution 1:10), anti-CD31-biotin (eBioscience, dilution 1:200) and anti-PNAd-biotin (BioLegend, dilution 1:50) for 2 hours, followed by an incubation with the corresponding secondary antibodies, including anti-hamster IgG-AF546 (Invitrogen, dilution 1:1000) and

Streptavidin AF647 (Invitrogen, dilution 1:1000), together with DAPI (Roche, 0.05 µg/mL) to stain nuclear DNA for 20-30 min. Coverslips were mounted onto the slides with Fluoromount-G™ (Invitrogen). The edges of the coverslips were sealed with nail polish to prevent air pockets from forming and slides were stored at 4°C in the dark until microscopic examination.

LN sections were examined and images of LN sections acquired with a 20x air objective (Plan-Apochromat, NA 0.8, DIC, Zeiss) using the Axio Observer.Z1 (Carl Zeiss, Oberkochen, Germany) with a CSU spinning disk head (Yokogawa Musashino, Tokyo, Japan) and a Metamorph software (Metamorph, Nashville, TN, USA). Higher resolution and magnification images were acquired with a 20x air objective (Plan-Apochromat, NA0.8, DIC, Zeiss) and 63x oil immersion objective (Plan-Apochromat, NA 1.4, DIC; Zeiss) using the Axio Observer.Z1 with a confocal LSM 780 head (both Carl Zeiss, Oberkochen, Germany) and the software Zen (Carl Zeiss, Oberkochen, Germany). Analysis of the microscopic images was performed using the open source software ImageJ.

8 Cell culture

Human Umbilical Vein Endothelial Cells (HUVECs) from pooled donors were purchased from PromoCell (PromoCell GmbH, Heidelberg, Germany) and grown in Endothelial Cell Growth Medium 2 (PromoCell GmbH, Heidelberg, Germany) at 37° C in a humidified incubator containing 5% CO_2. Cells at passages 3 to 6 were used for the experiments.

9 Immunofluorescence of HUVECs

HUVECs, seeded on glass coverslips and grown to confluence, were stimulated with 5 µg/mL of fluorescently labeled LecB (LecB-AF488) for 1, 3 and 5 h at 37° C. Cells were then washed twice with PBS (Gibco) and fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich) for 15 min. Fixed cells were incubated in 50 mM NH_4Cl for 5 min to quench free aldehyde groups to prevent PFA-induced background fluorescence. After permeabilization in SAPO buffer (PBS supplemented with 0.2% BSA and 0.02% saponin) for 30 min, cells were incubated with anti-CD144/VE-cadherin (eBioscience, dilution 1:1000 in SAPO buffer) for 1 hour. Cells were then washed three times with PBS (Gibco) and incubated with the corresponding anti-rabbit AF647 (Invitrogen, dilution 1:200 in Sapo buffer), DAPI (supplier,

1 µg/mL) for nuclear staining and Phalloidin ATTO 565 (supplier, dilution 1:1000) for actin staining for 30 min. Coverslips were washed 4-6 times with PBS (Gibco) and dipped in ddH_2O to remove salts. Coverslips were mounted on glass slides with Mowiol® 4-88 containing DABCO (Carl Roth). Images of stained cells were acquired with a 60x oil immersion objective (CFI Apochromat TIRF, NA 1.49, Nikon) using the Nikon Eclipse Ti-E inverted microscope, equipped with a Nikon A1R confocal laser scanning system (Nikon, Tokyo, Japan) and the NIS-Elements software (Nikon, version 4.20). Acquired images were analyzed using the open source software ImageJ.

10 Western Blot

HUVECs were grown to confluence and stimulated with 5 µg/mL of LecB for 1, 3, and 5 h at 37°C. Cells were then washed twice in ice-cold PBS (Gibco), lysed in RIPA buffer containing phosphatase and protease inhibitors (200 µM pefablock 0.8 µM aprotinin, 11 µM leupeptin, 1% (v/v) phosphatase inhibitor cocktail 3 (Sigma-Aldrich)) for 45 min on ice, and centrifuged to remove cell debris. Cell lysates were stored at -80°C if not used immediately. The protein concentration of the cell lysates was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's protocol. Twenty-five µg of protein of each sample was separated on 8% SDS-polyacrylamide gels and transferred on nitrocellulose membranes by semi-dry blotting. The membranes were blocked in 3% BSA for 1h at room temperature and incubated with anti-CD144/VE-cadherin (eBioscience, dilution 1:1000) and anti-α-tubulin (Cell Signaling, dilution 1:4000) as loading control overnight at 4°C. Membranes were then incubated with anti-Rabbit IgG-HRP (Cell Signaling, dilution 1:1000) and anti-Mouse IgG-HRP (Cell Signaling, dilution 1:4000) for 1 h at room temperature. The Clarity™ Western ECL Blotting Substrate (BIO RAD) was used according to the manufacturer's protocol for signal development and chemiluminescence was detected using the Fusion FX chemiluminescence imager (Vilber Lourmat, Marne-la-Vallée, France). Densitometric quantification of blots was performed using ImageJ and protein levels were normalized to α-tubulin.

11 Statistical analysis

Descriptive and statistical analyses were performed using the software Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). Data were expressed as means ± SEM. Statistical significances were determined using the Mann-Whitney or Kruskal-Wallis test and a P value of < 0.05 was considered significant, ns $p > 0.05$, *$p \leq 0.05$, **$p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

RESULTS

Results

1 Subcutaneous LecB is captured by the lymphatic sinus-associated macrophages of draining lymph nodes

It is known that lymph-borne antigens and pathogens transported via the lymph to the draining lymph nodes (LNs), are captured by $CD169^+$ macrophages lining the subcapsular sinus floor (Junt et al., 2007) and by resident dendritic cells (DCs) in close proximity to the lymphatic sinuses (Gerner et al., 2015). Thus, we considered the possibility that $CD169^+$ macrophages and DCs may capture LecB from the luminal space of the lymphatic sinuses. Therefore, to gain insights into the LN target cells of LecB, we injected AF488-fluorescently labelled LecB intradermally into the ear pinna and into the hind footpad of C57BL/6 mice **(Figure 21A)**. We first used flow cytometric analysis to identify LN macrophages as $MHC\text{-}II^{int}CD11c^{low}$ cells among the live $CD11b^+CD11c^+$ cells. By staining for CD169 and F4/80 we could distinguish between the different LN macrophage subsets, namely the double-positive ($CD169^+F4/80^+$) medullary sinus macrophages (MSMs), the CD169 single-positive ($CD169^+F4/80^-$) subcapsular sinus macrophages (SSMs), the F4/80 single-positive ($CD169^-F4/80^+$) and the double-negative ($CD169^-F4/80^-$) macrophages **(Figure 21B)**. We found that four hours after LecB injection, all cell subsets have captured LecB-AF488, but MSMs and SSMs displayed the highest median fluorescence intensity. Indeed, the median fluorescence intensity was highest in MSMs, followed by SSMs with a 0.5-fold difference, and with a 2 to 4-fold difference to $CD169^-F4/80^+$ macrophages and double negative ($CD169^-F4/80^-$) macrophages, respectively **(Figure 21B, 22B)**. To confirm these findings, we visualized LecB-AF488 by microscopy on LN cross-sections in costaining with CD169. Among other cells, the $CD169^+$ SSMs were LecB-$AF488^+$, validating the binding of LecB to $CD169^+$ MSMs and SSMs in LecB-drained popliteal LNs **(Figure 21C)**.

These data show that skin-draining LecB is taken up by all LN macrophage subsets but in particular by the sinus-associated MSMs and SSMs.

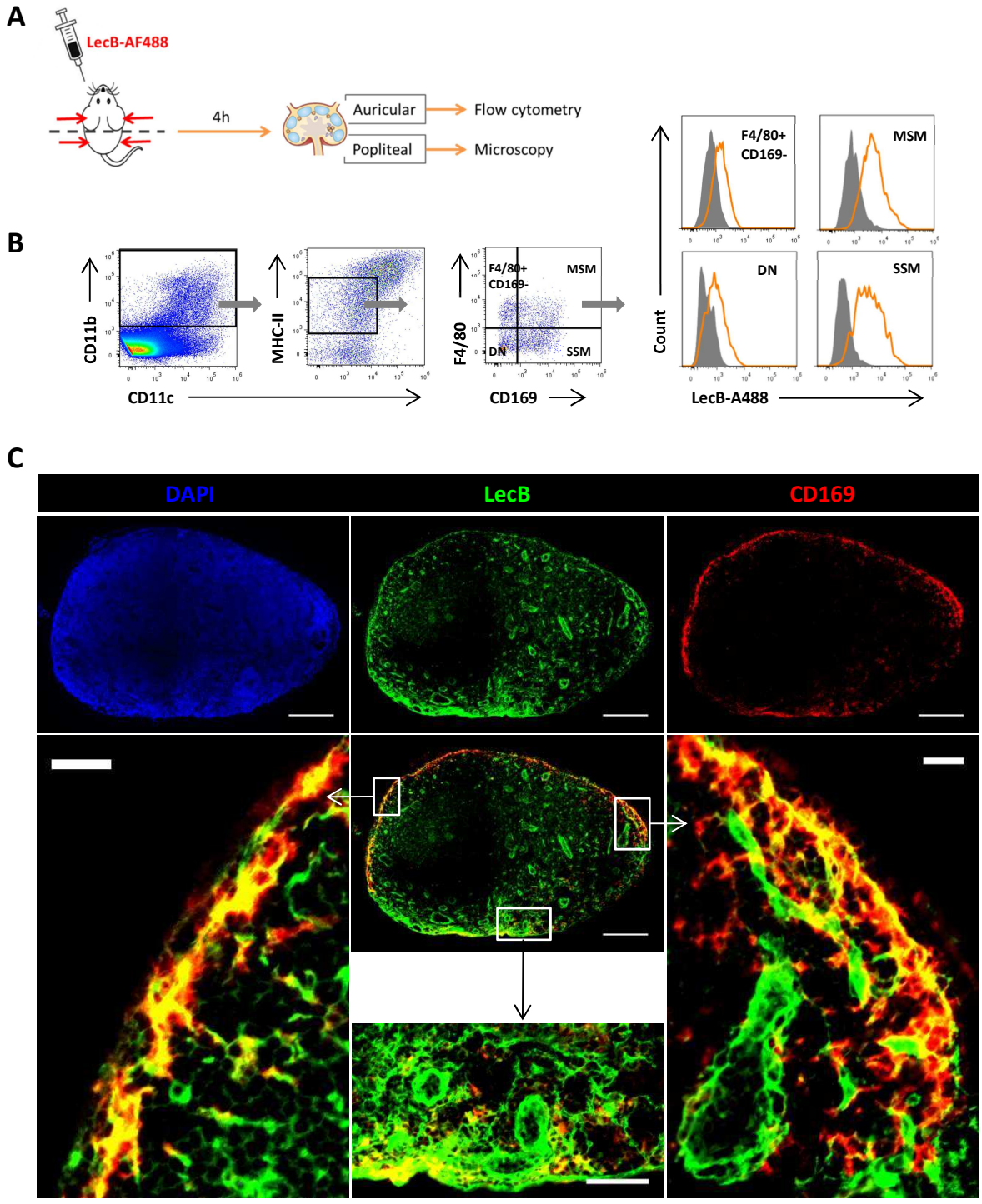

Figure 21. LecB is captured by lymphatic sinus-associated macrophages within draining lymph nodes

(A) Fluorescently labeled LecB (12.5 µg, LecB-AF488) was injected intradermally into the ear pinna and subcutaneously into the hind footpad in wild-type mice, and draining auricular and popliteal lymph nodes (LNs) were analyzed 4 h post injection for LecB binding among LN macrophages by flow cytometry and microscopy, respectively.

(B) Flow cytometry gating strategy to identify LN macrophage subsets for analysis of LecB binding. Histograms show LecB-AF488 fluorescence of LN macrophages in PBS-drained (grey filled) and LecB-drained (orange lined) LNs. Each point represents the results of two pooled auricular LNs of one mouse.

(C) Representative microscopy images of popliteal LN section 4 h after LecB-AF488 injection stained for $CD169^+$ LN macrophages. Magnified images of the white boxed regions are shown in the lower panel on the right and the left. Scale bars represent 200 µm in whole LN images and 50 µm in magnified images.

Next, we identified dendritic cells (DCs) by flow cytometry among the live $CD11c^+MHC\text{-}II^+$ cells **(Figure 22A)**. We could distinguish conventional $MHC\text{-}II^{int}$ lymphoid-tissue resident DCs (hereinafter referred as LN-resident DCs) from $MHC\text{-}II^{hi}$ peripheral tissue-derived migratory DCs (hereinafter referred as skin-derived DCs), composed of $CD207/Langerin^-$ dermal DCs, $CD207/Langerin^+CD103^-$ Langerhans cells (LCs) and $CD207^+CD103^+$ dermal DCs (hereinafter referred as $CD103^+$ dDCs). We found all DC subsets to be $LecB\text{-}AF488^+$, displaying similar median fluorescence intensities among each other **(Figure 22A, B)**. Compared with the LecB median fluorescence intensities of the $CD169^+$ LN macrophages, they were however 3.5 to 9 times lower **(Figure 22B)**. B and T lymphocytes, found among the live $CD11c^-MHC\text{-}II^{int}$ and $CD11c^-MHC\text{-}II^-$ cells, respectively, showed only a slight staining for LecB-AF488. The LecB-AF488 median fluorescence intensity was on average 45-fold lower compared with that of MSMs **(Figure 22A, B)**. Together, these results indicate that LecB drains into the subcapsular sinus and medullary sinuses of the peripheral LNs, where it is captured by the sinus-associated subcapsular and medullary sinus macrophages. To a lesser extent, the lectin is also taken up by other macrophage subsets and by skin-derived migratory DCs.

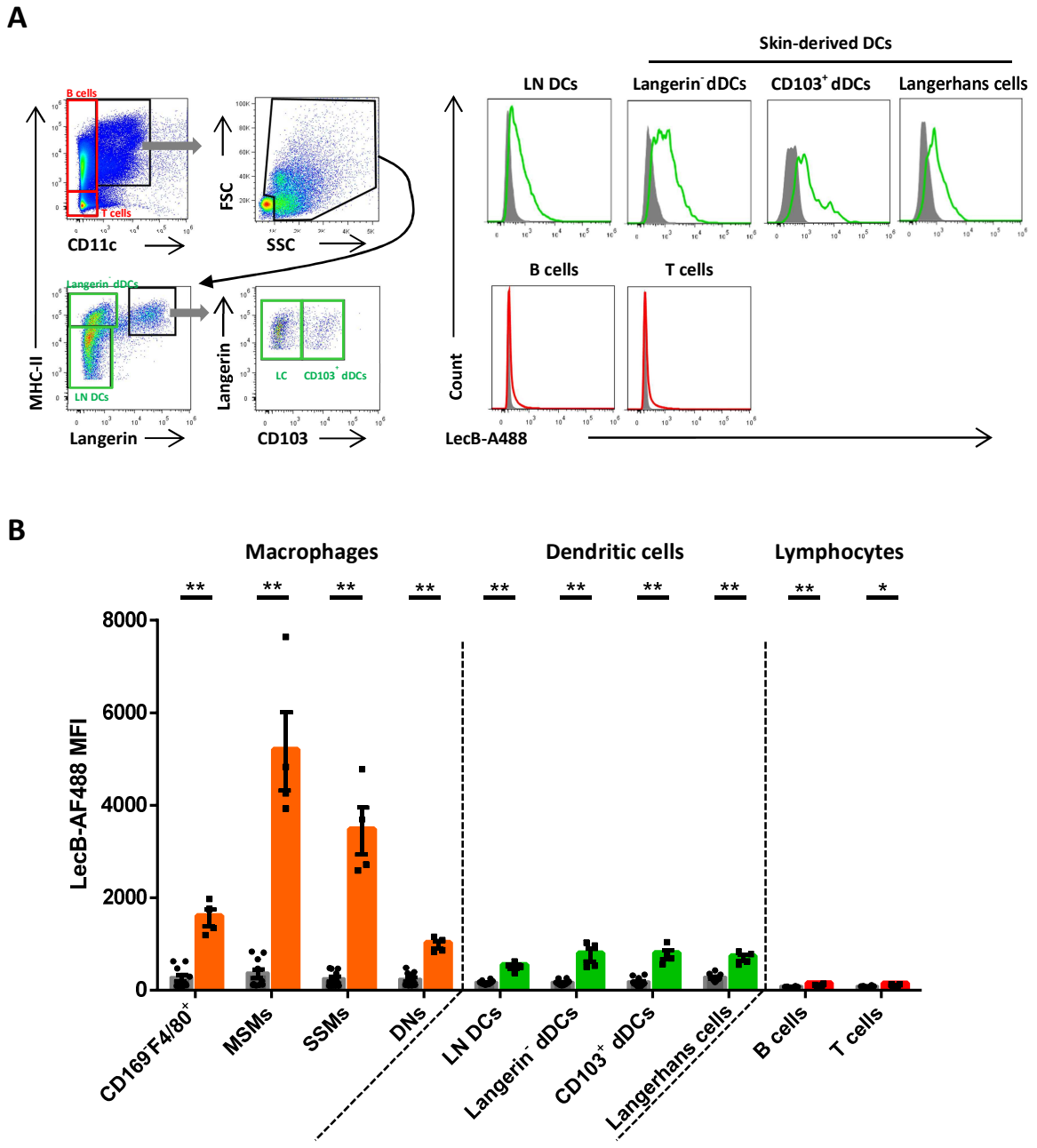

Figure 22. LecB binds mainly to lymphatic sinus-associated macrophages within draining lymph nodes

Mice were injected with 12.5 µg of LecB-AF488 or the same volume of PBS as control into the ear pinna, and draining auricular lymph nodes (LNs) were dissected 4 h later for flow cytometric analysis.

(A) Flow cytometry gating strategy to identify LN-resident and skin-derived migratory dendritic cell subsets and B and T lymphocytes (left panel). Histograms (right panel) show LecB-AF488 fluorescence among the indicated cells in PBS- (grey filled histograms) and LecB-drained (colored lined histograms) LNs.

(B) LecB-AF488 median fluorescence intensities (MFIs) of macrophages, dendritic cells, B and T lymphocytes within the PBS-drained (grey bars) and LecB-drained (colored bars) LNs assessed by flow cytometry. Each point represents the result of two pooled auricular LNs of one mouse (PBS control: n=10, LecB-AF488-treated: n=4). Mean values and standard error of the mean are shown by bars and error bars, respectively. *p < 0.05, **p < 0.01; Mann-Whitney-U test.

2 LecB drains the lymphatic sinuses and reaches blood endothelial cells via the conduits

Small lymph-borne factors are known to flow with the lymph into the subcapsular sinus and through the medullary sinuses of draining LNs towards the high endothelial venules (HEVs) (Gretz et al., 2000; Sixt et al., 2005). To examine whether LecB follows a similar distribution pattern within the LN when draining with the lymph, we analyzed popliteal LNs by microscopy. Our results revealed the presence of AF488-labeled LecB on lymphatic endothelial cells (LECs), lining both the subcapsular and medullary sinuses **(Figure 23A, B and C, arrows).** Strikingly, only the LECs of the subcapsular sinus floor exhibited a LecB-AF488 staining while the LECs of the subcapsular sinus ceiling were devoid of LecB **(Figure 23B)**. Such distribution suggests the binding of LecB to a specific structure only found on floor LECs. Indeed, the ceiling LECs were the only cells among all LECs within the LN lacking binding of LecB, indicating a phenotypical and thus functional distinction between the ceiling and the remaining LECs. Additionally, we observed a fluorescence signal of LecB on conduits extending from the floor of the subcapsular sinus to the underlying $CD31^{+}PNAd^{+}$ HEVs **(Figure 23B, 24)**, suggesting that LecB moves within the reticular fibers to reach the HEVs. The distribution pattern of LecB within the LN was however not continuous but rather showed a gradient from the subcapsular sinus to the HEVs, with HEVs in the deeper cortex being devoid of LecB **(Figure 23A)**.

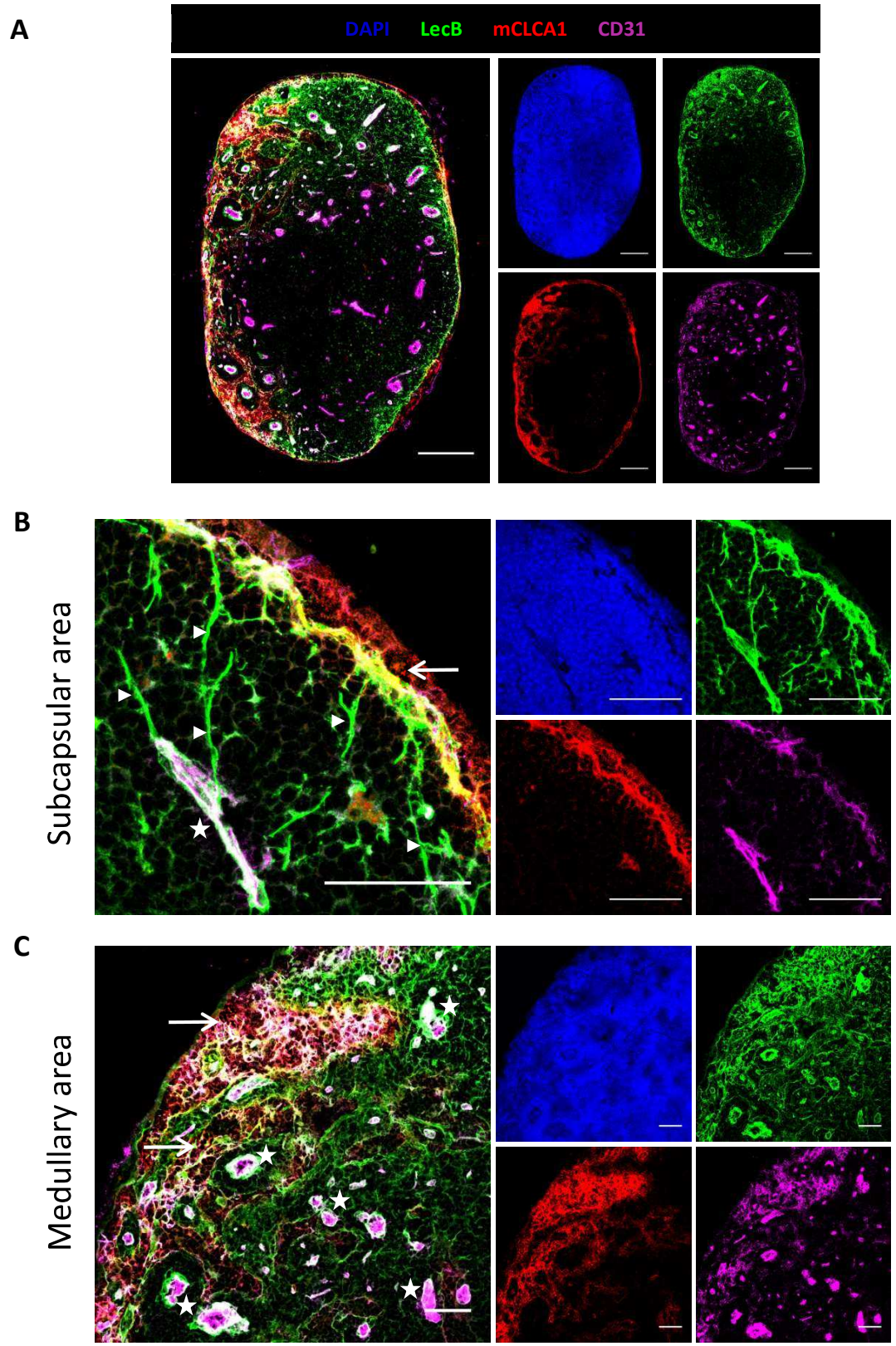

Figure 23. LecB drains the lymphatic sinuses and reaches blood endothelial cells via the conduits

(A) Representative image of a popliteal lymph node (LN) section 4 h after subcutaneous injection of 12.5 µg LecB-AF488 into the footpad, stained for endothelial cells (CD31) and lymphatic endothelial cells (mCLCA1). (B) Z-projection of confocal image stack of the subcapsular region of the popliteal LN.

(C) Z-projection of confocal image stack of the medullary region of popliteal LN.
Arrows and asterisks indicate lymphatic and blood endothelial cells, respectively, while fibroblastic reticular cell conduits are highlighted by arrow heads. Scale bars represent 200 µm in whole LN images and 50 µm in magnified images.

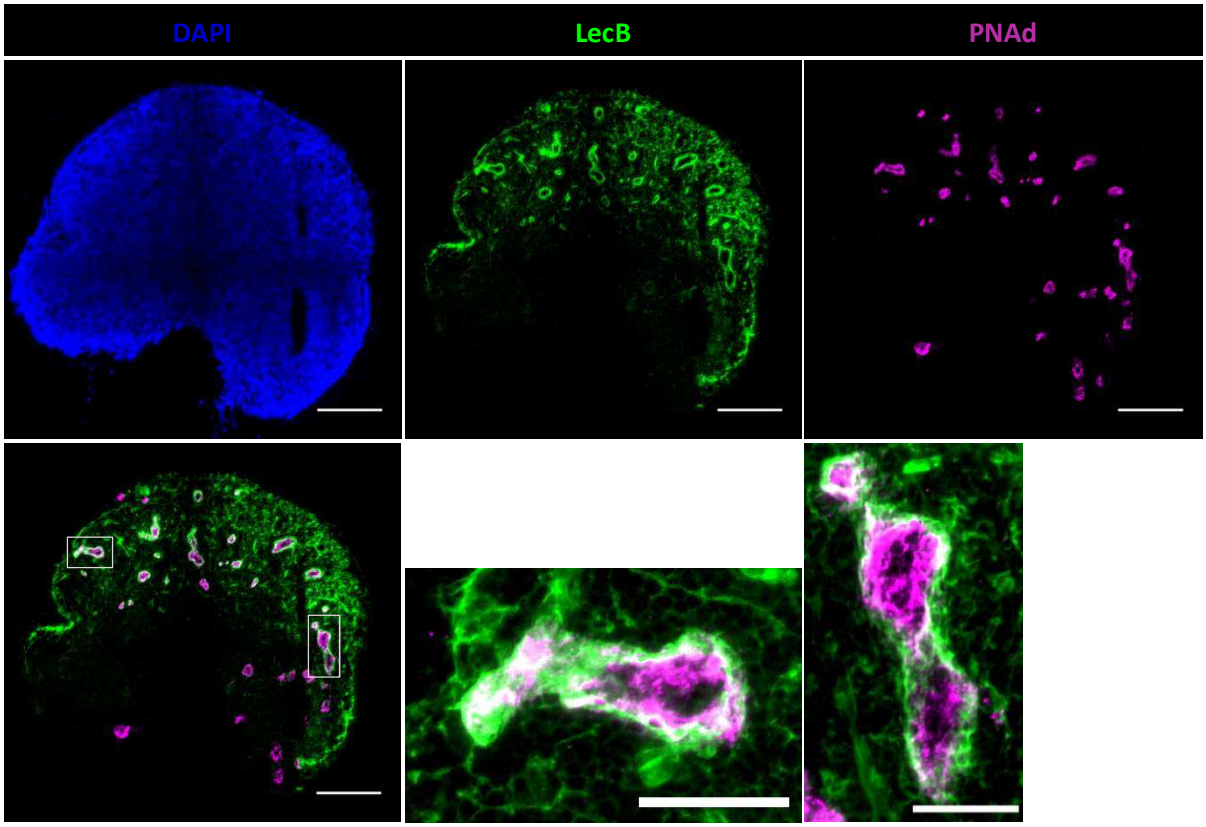

Figure 24. LecB drains the lymphatic sinuses and reaches PNAd+ high endothelial venules via the conduits
Representative image of a popliteal LN section 4 h after subcutaneous injection of 12.5 µg LecB-AF488 into the hind footpad, stained for $PNAd^+$ high endothelial venules. Magnified images of the white boxed regions are shown in the lower panel on the right. Scale bars represent 200 µm in whole LN images and 50 µm in magnified images.

In order to confirm these results, we analyzed the stromal cell compartment of popliteal LNs by flow cytometry **(Figure 25A)**. We identified stromal cells among the live $CD45^-TER\text{-}119^-$ non-hematopoietic cells. By staining for gp38/podoplanin and CD31 we could discriminate between the $gp38^+CD31^-$ fibroblastic reticular cells (FRCs), the $gp38^+CD31^+$ lymphatic endothelial cells (LECs), the $gp38^-CD31^+$ blood endothelial cells (BECs), among which the

Peripheral Node Addressin $(PNAd)^+$ high endothelial venules (HEVs), and the remaining double-negative (DN) cells. Consistent with the microscopic analysis, the flow cytometric analysis revealed a signal of AF488-labeled LecB in all stromal cell subsets, with the highest LecB median fluorescence intensity in LECs, followed by BECs, HEVs and FRCs **(Figure 25B)**. LECs were the only cells, among all stromal cell subsets, being nearly completely LecB-$AF488^+$ (90-98%) **(Figure 25A)**. The binding of LecB to fibroblastic reticular cells (FRCs) suggests that the reticular structures observed by microscopy were indeed reticular conduits, ensheathed by FRCs. Together, these data indicate that LecB drains the lymphatic sinuses, thereby binding to the lining lymphatic endothelial cells, and reaches the high endothelial venules via the fibroblastic reticular cell conduits.

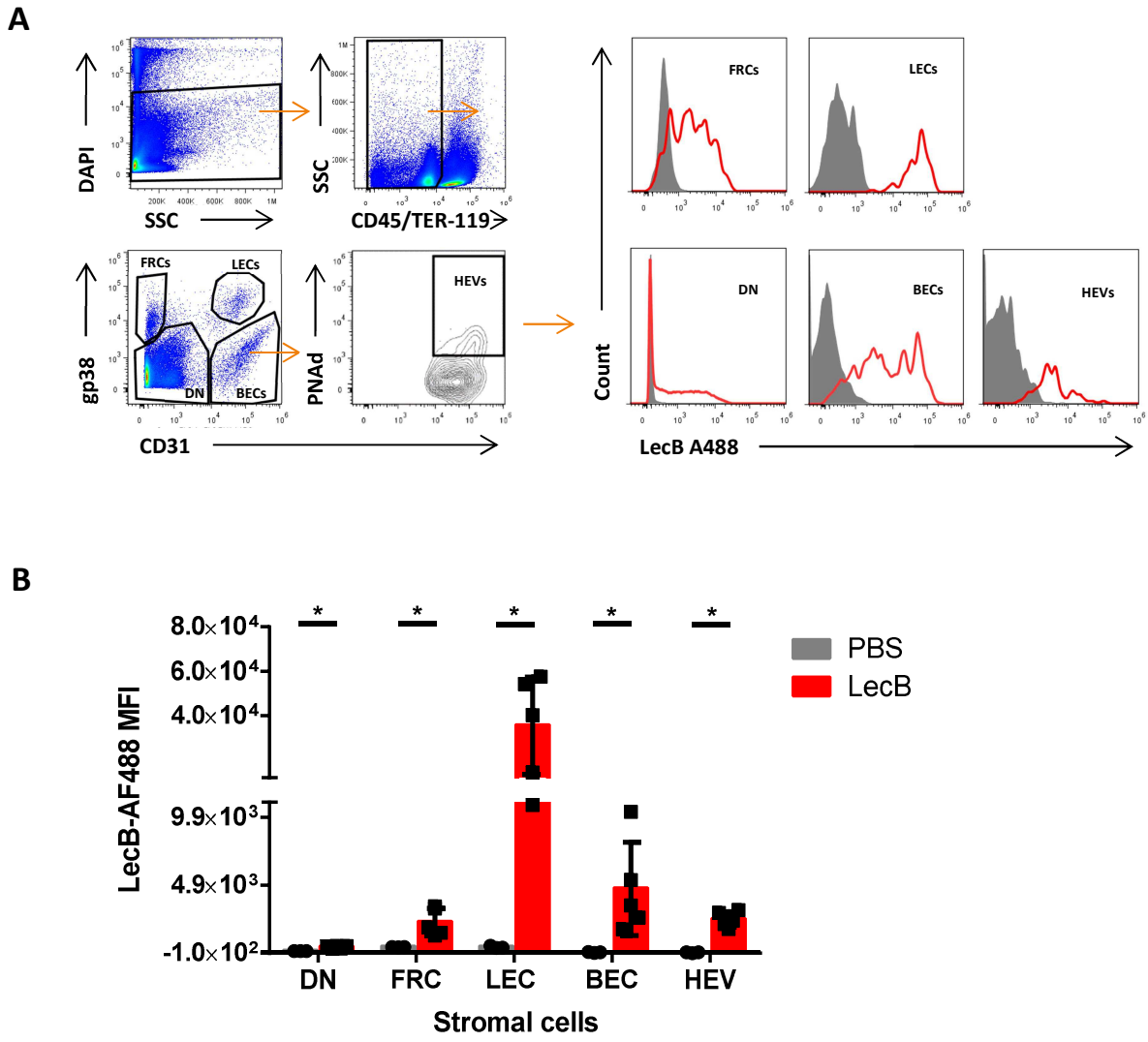

Figure 25. LecB drains the lymphatic sinuses and reaches PNAd$^+$ high endothelial venules via the conduits

(A) Flow cytometry gating strategy to identify LN stromal subsets among live CD45$^-$TER-119$^-$ non-hematopoietic cells in popliteal LNs (left panel). Histograms (right panel) show LecB-AF488 fluorescence among LN stromal cells of PBS-drained (grey filled) and of LecB-drained (red lined) LNs 4 h after subcutaneous injection of 12.5 µg LecB-AF488 or PBS into the hind footpad.

(B) LecB-AF488 median fluorescence intensity (MFI) of the different LN stromal cell subsets assessed by flow cytometry. Each point represents the result of two pooled popliteal LNs of one mouse (PBS control: n=3; LecB-treated: n=5). Mean values and standard error of the mean are shown by bars and error bars, respectively.

*p < 0.05, **p < 0.01; Mann-Whitney-U test.

DN, double negative cells; FRC, fibroblastic reticular cells; LEC, lymphatic endothelial cells; BEC, blood endothelial cells; HEV, high endothelial venules.

3 LecB does not increase lymphocyte homing to draining lymph nodes

High endothelial venules mediate the entry of circulating, naïve lymphocytes from the blood into the lymph node (Mionnet et al., 2011). Since LecB bound to these specialized endothelial cells, we investigated whether this binding affects the entry of lymphocytes. We first tested whether LecB was inflammatory, which would lead to rapid recruitment of innate immune cells such as neutrophils to the draining LN (Woytschak et al., 2016). We therefore injected LecB intradermally into the ear pinna and subcutaneously into the hind footpad on one side of the mice and contralaterally PBS to control for interindividual differences among mice **(Figure 26A)**. Twenty-four hours later, a time point sufficient for inflammation-induced LN enlargement (Acton et al., 2014), we analyzed the skin-draining auricular and popliteal LNs and the brachial LNs as negative control by flow cytometry. By macroscopic examination of the LNs we observed an increase in the size of both the auricular and popliteal LecB-drained LNs compared to the PBS-drained LNs **(Figure 26B)**. This size increase could be ascribed to a one-fold increase in the total cell number in the auricular LNs (0.5×10^7 vs. 1×10^7 cells on average in PBS vs. LecB-drained LNs, respectively) and a two-fold increase in the total cell number in the popliteal LecB-drained LNs compared to the PBS-drained control LNs (0.7×10^6 vs. 2×10^6) **(Figure 26C)**. It is notable that the LecB-dependent rise in total cell number **(Figure 26C)** was more pronounced in the popliteal than in the auricular LNs. In the non-draining brachial control LNs, we could observe no difference in cellular counts between the left and right-sided LN, indicating that LecB induces a local immune response solely in the draining LNs.

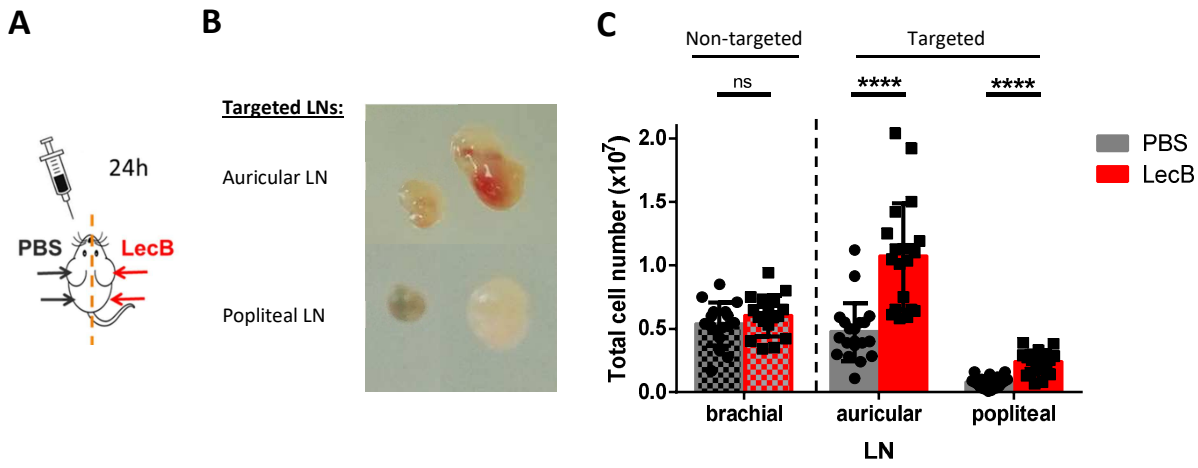

Figure 26. LecB increases cellularity in draining lymph node

(A) Mice were injected with 12.5 µg of purified LecB intradermally into the ear pinna and subcutaneously into the hind footpad on one side and contralaterally with the same volume of PBS as control and LN were dissected 24 h later.

(B) Pictures of dissected PBS-drained (left) and LecB-drained (right) auricular and popliteal LNs 24 h after the respective injection.

(C) Draining auricular and popliteal LNs as well as non-draining brachial LNs as control (dot pattern bars) were analyzed for cell numbers (PBS control: n=20, LecB-treated: n=17-20). Each point represents the result of one LN. Mean values and standard error of the mean are shown by bars and error bars, respectively. ns $p > 0.05$, ****$p < 0.0001$; Mann-Whitney-U test.

Additionally, we analyzed the myeloid cell compartment of the LN by flow cytometry. By staining for Ly6-G, which is exclusively present on neutrophils (Daley et al., 2008), and Ly6-C, highly expressed in inflammatory monocytes (Galli et al., 2011), we identified neutrophils among the live $CD45^+CD11b^+Ly6G^+$ cells and the inflammatory monocytes among the live $CD45^+CD11b^+Ly6G^-Ly6C^{hi}$ cells **(Figure 27A)**. Our results indicated a significant increase in the numbers of neutrophils: almost two-fold in the auricular and three-fold in the popliteal LecB-drained LNs, when compared to the PBS-drained LNs **(Figure 27B)**. The infiltration of inflammatory monocytes was more prominent, with a nine-fold increase for the auricular and a twenty-two-fold increase in the popliteal LecB-drained LNs **(Figure 27C)**. These data indicate that LecB is inflammatory, inducing an infiltration of neutrophils and inflammatory monocytes within the draining LNs.

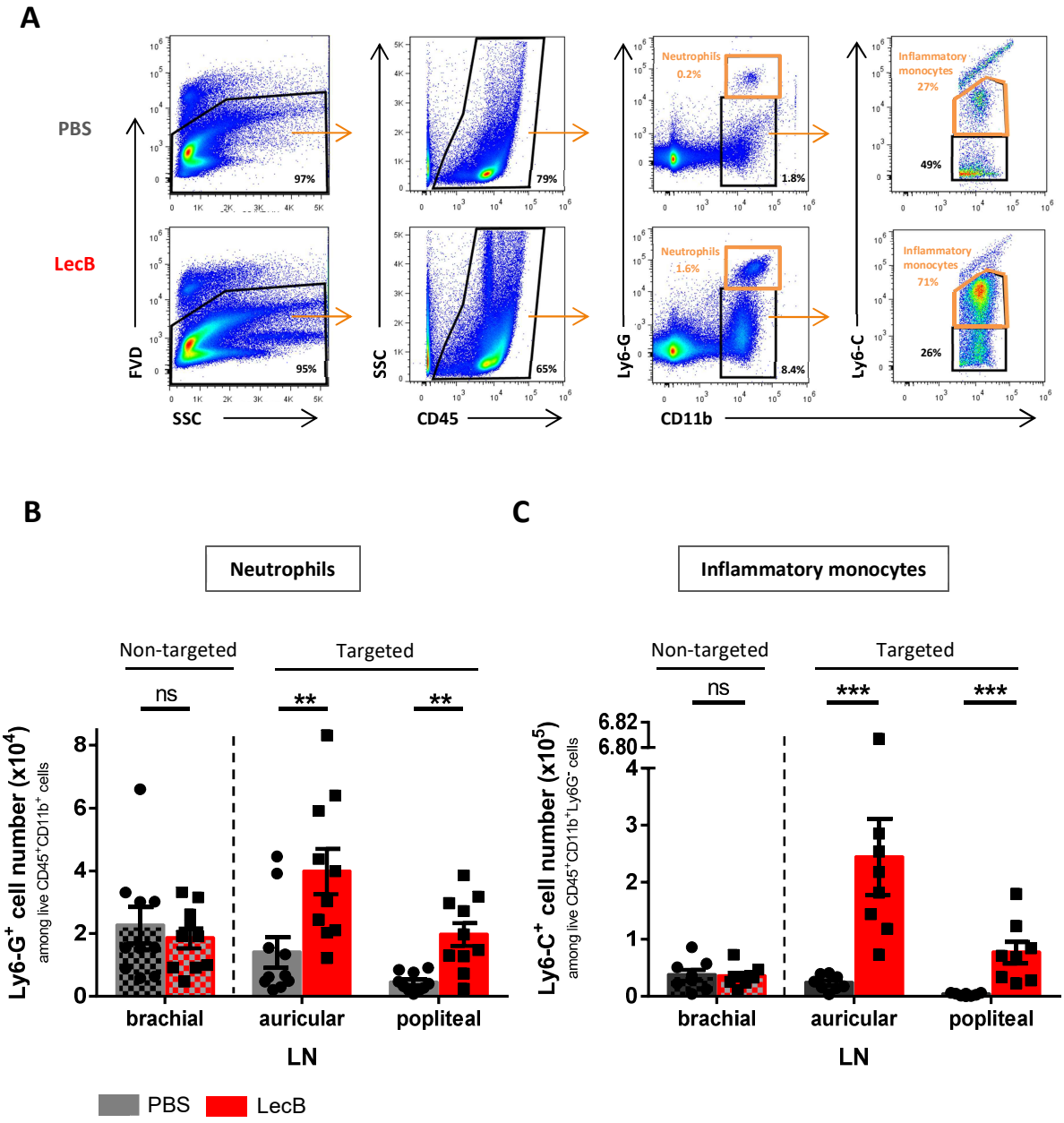

Figure 27. LecB induces neutrophil and inflammatory monocyte accumulation within draining lymph nodes

Mice were injected with 12.5 µg of purified LecB intradermally into the ear pinna and subcutaneously into the hind footpad on one side and contralaterally with the same volume of PBS as control. After 24 h neutrophils and inflammatory monocytes were analyzed by flow cytometry within draining auricular and popliteal LNs as well as non-draining brachial LNs as control.

(A) Flow cytometry gating strategy to identify neutrophils and inflammatory monocytes (highlighted in orange boxes).

(B) Neutrophil numbers within the indicated LNs (PBS control: n=10; LecB-treated: n=10).

(C) Inflammatory monocyte numbers within the indicated LNs (PBS control: n=8; LecB-treated: n=8).

Each point represents the result of one LN. Mean values and standard error of the mean are shown by bars and error bars, respectively. ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Mann-Whitney-U test.

As the neutrophils and inflammatory monocytes constituted only a fraction of the total LN cellularity (less than 8%), we next asked whether the LN size increase would be related to an increased recruitment of B and T lymphocytes. Therefore, we performed an adoptive transfer of lymphocytes with mice that carry two different alleles, CD45.1 and CD45.2, of the common antigen CD45 expressed in all leukocytes. Lymphocytes isolated from spleens and peripheral LNs of CD45.1 donor mice were intravenously transferred into LecB-treated CD45.2 host mice shortly after LecB injection **(Figure 28A)**. Twenty-four hours later, when the blood-circulating donor lymphocytes are expected to have entered the LN, we enumerated the donor and the host $CD3\varepsilon^+$ T and $B220^+$ B lymphocytes in the LNs **(Figure 28B)**. We observed an important increase in host T and B lymphocyte numbers in auricular and popliteal LecB-drained LNs compared to control LNs. The increase in cell numbers was more pronounced for T lymphocytes compared to B lymphocytes (~4-fold vs. 1.4-fold increase, respectively, for popliteal LNs) and consistent with the preceding findings, this increase was more marked in the popliteal LNs compared to the auricular LNs (e.g., ~4-fold vs. ~2-fold increase, respectively, for T lymphocytes) **(Figure 28C)**. Concerning the $CD45.1^+$ donor cells, we could observe no significant difference in T and B lymphocyte numbers between LecB- and PBS-drained LNs, except for the popliteal LNs, in which T lymphocyte numbers were significantly increased with LecB. There was, in fact, a tendency of a decline in B lymphocyte numbers in response to LecB compared to PBS in auricular LNs **(Figure 28D)**. Together, these data demonstrate that LecB induces an accumulation of LN cells, of which T and B lymphocytes are the main constituent. While increased recruitment of blood-derived inflammatory monocytes can partly account for LN growth, the rise in B and T lymphocyte numbers is not fully reflected in an accelerated entry from the blood stream into the organ.

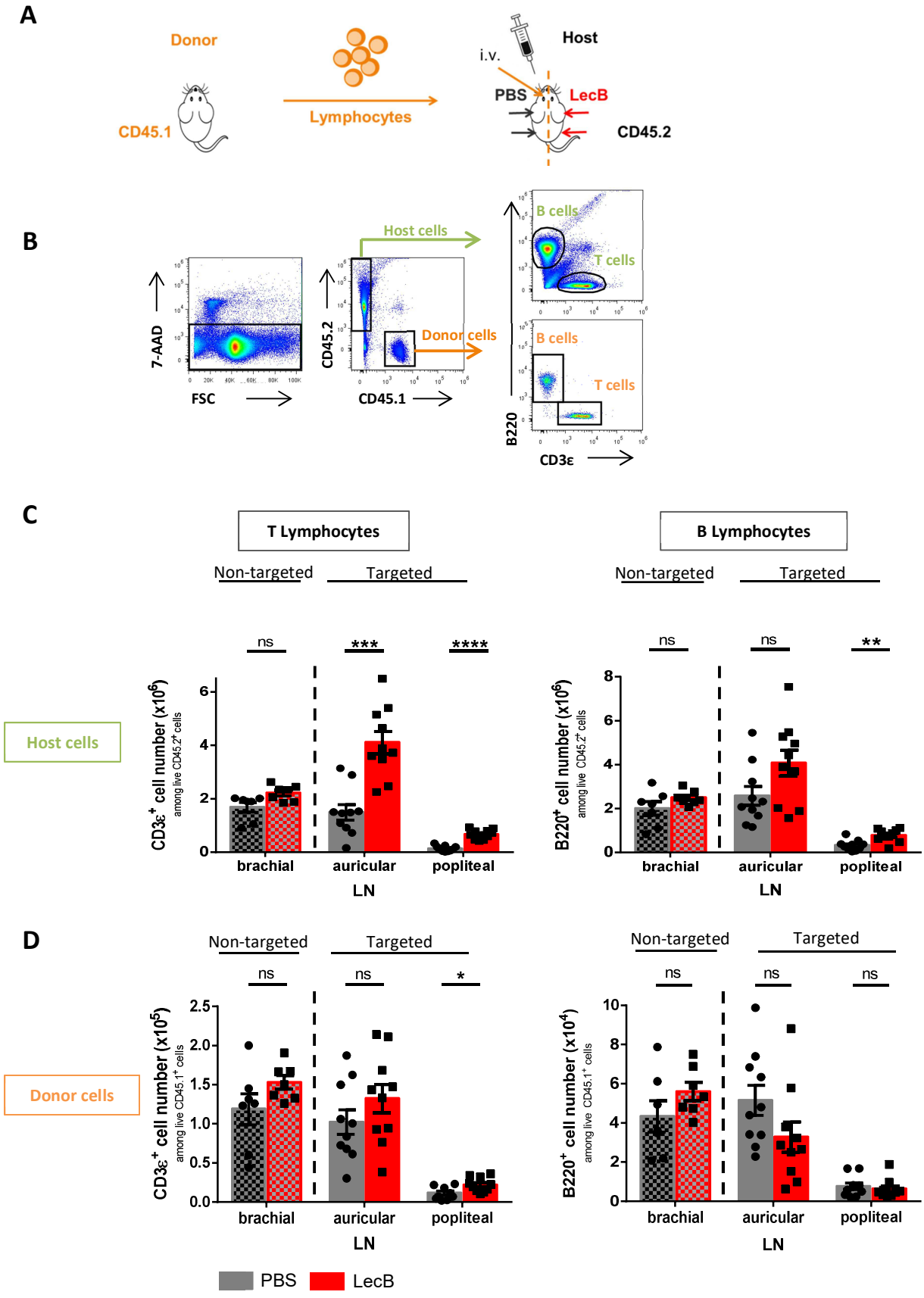

Figure 28. Enhanced entry from blood alone cannot account for increased lymphocyte numbers in draining LNs

(A) CD45.2 host mice were injected with 12.5 µg of purified LecB intradermally into the ear pinna and subcutaneously into the hind footpad on one side and contralaterally with the same volume of PBS as control. One to four hours later, $CD45.1^+$ lymphocytes were transferred into host mice and 24 h later, $CD45.1^+$ donor and $CD45.2^+$ host lymphocytes in indicated LNs were analyzed by flow cytometry.
(B) Flow cytometry gating strategy to identify $CD45.2^+$ host and $CD45.1^+$ donor lymphocytes.
(C) Host T and B lymphocyte numbers in indicated LNs (PBS control: n=7-10; LecB-treated: n=7-10).
(D) Donor T and B lymphocyte numbers in indicated LNs (PBS control: n=7-10; LecB-treated: n=7-10).
Each point represents the result of one LN. Mean values and standard error of the mean are shown by bars and error bars, respectively. ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; Mann-Whitney-U test.

4 LecB inhibits lymphocyte exit

Immune response-induced lymphocyte accumulation within the LN may result from increased lymphocyte entry from the blood into the LN, increased lymphocyte proliferation within the LN, or a reduced exit of lymphocytes from the LN. As the increase in cell numbers could not fully be ascribed to an enhanced entry of lymphocytes from the blood circulation, we hypothesized that a limited exit contributed to the rise in lymphocyte numbers. To test if LecB binding to lymphatic endothelial cells affected exit, we first administered intravenously an anti-CD62L antibody prior to subcutaneous LecB injection in order to block the ingress of cells from the blood into the LN (Gallatin et al., 1983; Mionnet et al., 2011). Eighteen hours after LecB or PBS injection, we measured the lymphocyte numbers within the draining LNs **(Figure 29A)**. Should LecB block egress, we would expect the maintenance of lymphocyte numbers in spite of CD62L-mediated inhibition of entry. Indeed, while the administration of the anti-CD62L antibody resulted in a significant decrease in B and T lymphocytes numbers in the PBS-treated mice compared to the untreated controls, their numbers remained by and large stable when LecB was co-administered with the CD62L-blocking antibody **(Figure 29B, C)**. These data are therefore in support of the conclusion that LecB negatively regulates lymphocyte egress.

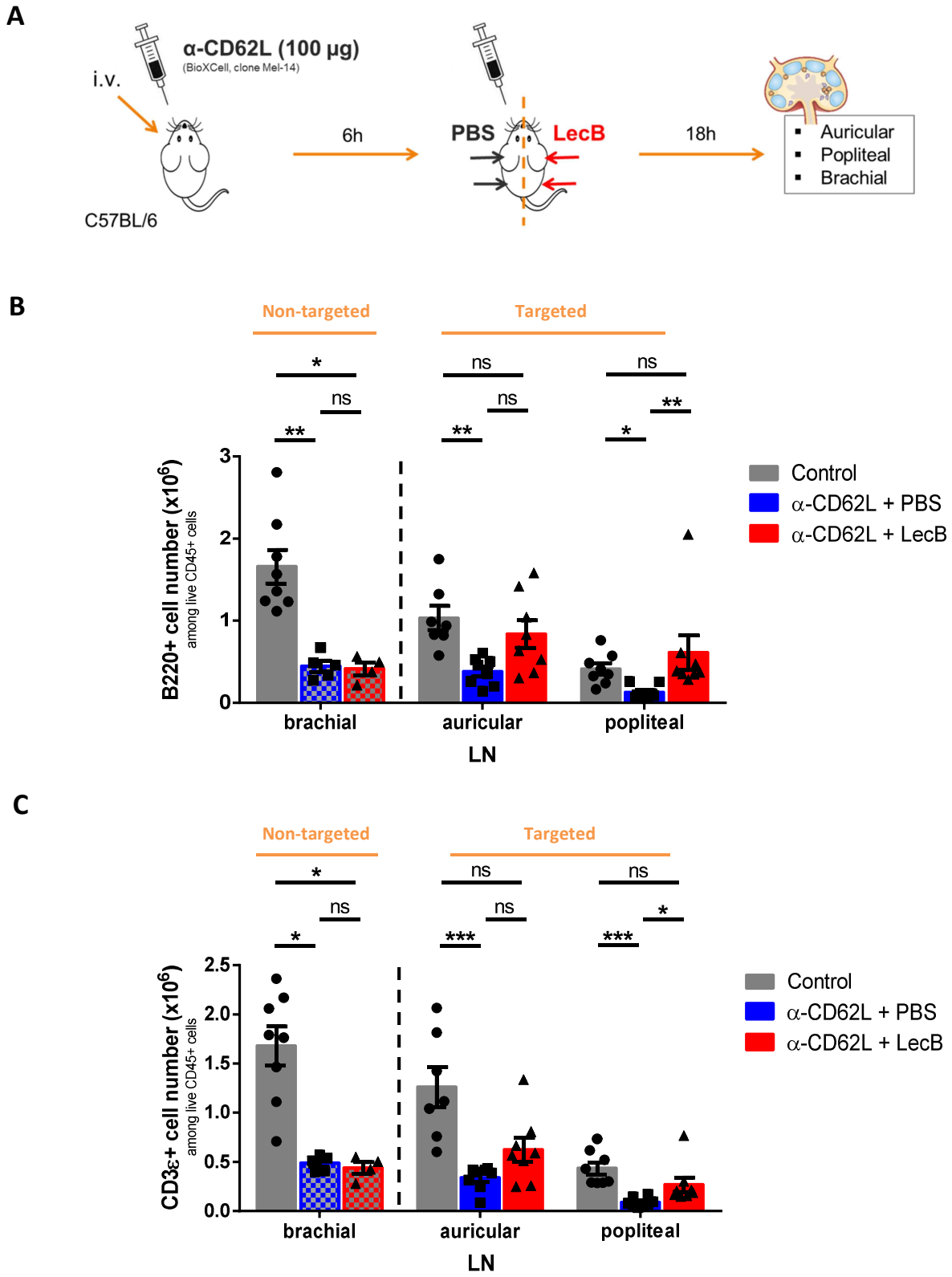

Figure 29. LecB inhibits lymphocyte egress

(A) Mice were injected intravenously with 100 µg of an anti-CD62L antibody to block lymphocyte entry into the LNs. Six hours later mice were injected with 12.5 µg of LecB intradermally into the ear pinna and subcutaneously into the hind footpad on one side and contralaterally with the same volume of PBS. Draining

auricular and popliteal LNs, and non-draining brachial LNs as control were analyzed for B and T lymphocytes by flow cytometry.
(B) B lymphocyte numbers in indicated LNs (Control: n=7-8; PBS control: n=5-8; LecB-treated: n=4-8).
(C) T lymphocyte numbers in indicated LNs (Control: n=7-8; PBS control: n=5-8; LecB-treated: n=4-8).
Each point represents the result of one LN. Mean values and standard error of the mean are shown by bars and error bars, respectively. ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Kruskal-Wallis test.

5 LecB induces phenotypical changes in lymphatic endothelial cells

To elucidate how LecB impacts endothelial cell function, we analyzed adhesion molecules involved in the migration of lymphocytes within lymph nodes. Interestingly, examination of LecB-drained LNs by microscopy revealed a loss of the cell surface protein murine chloride channel calcium activated 1 (mCLCA1) **(Figure 30A)**. Our flow cytometric analysis confirmed this result, showing a significant decline in the median fluorescence intensity of mCLCA1 on lymphatic endothelial cells in LecB-drained LNs compared to PBS-drained LNs **(Figure 30B, C)**. Surprisingly, the median fluorescence intensity of mCLCA1 was significantly increased on the blood endothelial cells with LecB compared to PBS control **(Figure 30C)**. We observed no change in MAdCAM-1 fluorescence intensity in any stromal cell subset in LecB-drained LN compared to PBS-drained LNs. However, the median fluorescence intensity of VCAM-1 increased significantly in lymphatic endothelial cells (LECs), blood endothelial cells (BECs) and fibroblastic reticular cells (FRCs) (approximately one-fold) **(Figure 30C)**. Additionally, we observed a one-fold decline in the median fluorescence intensity of ICAM-1 in lymphatic endothelial cells with LecB compared with PBS, but no remarkable difference in the other stromal cells **(Figure 30C)**.

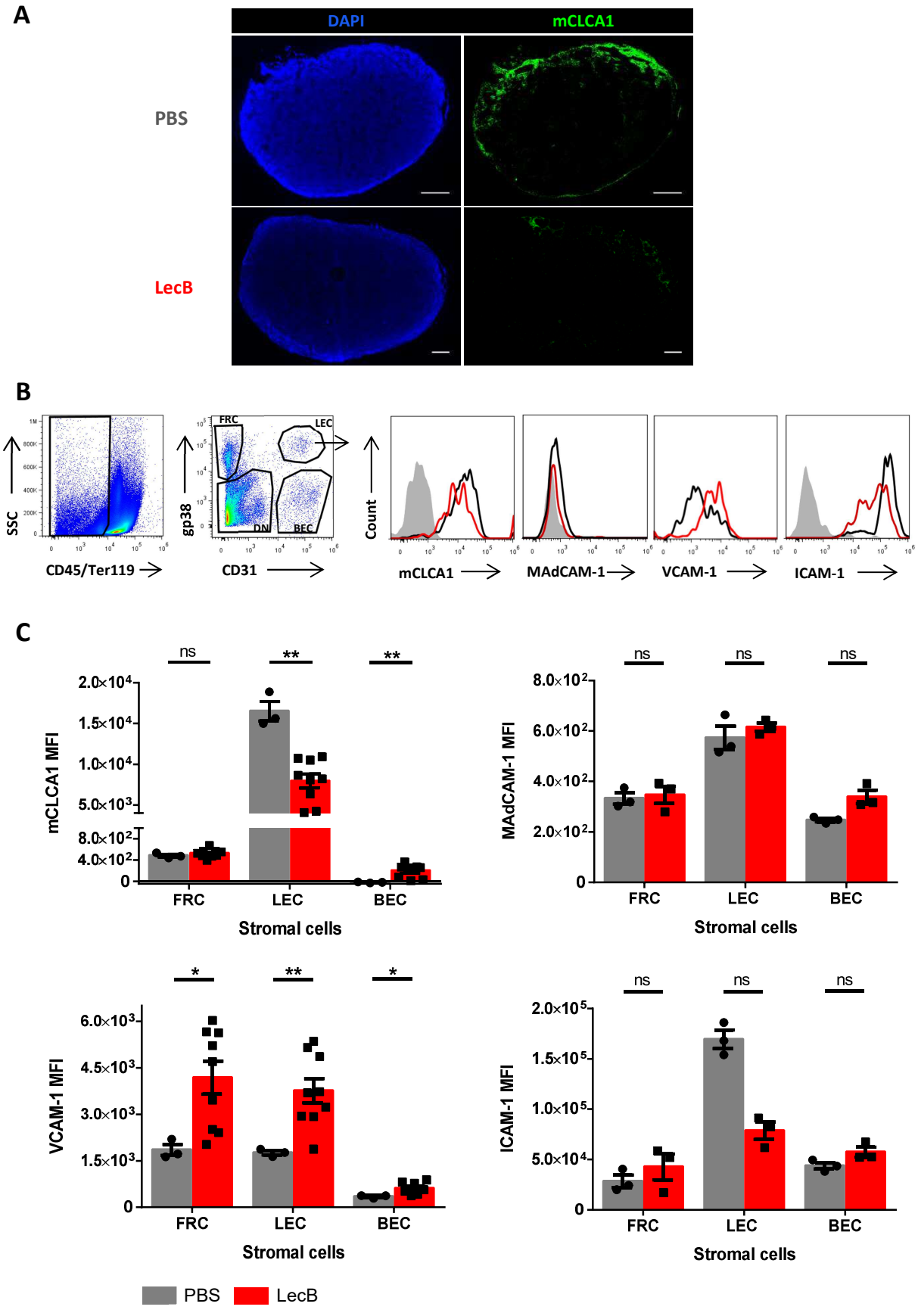

Figure 30. LecB induces phenotypical changes in lymphatic endothelial cells

(A) Representative microscopy images of sections of PBS- and LecB-drained popliteal LNs 24 h after injection, stained for mCLCA1. Scale bars represent 200 µm.
(B) Representative flow cytometry dot plots of stromal cells of two pooled popliteal LNs. Histograms show fluorescence of the indicated proteins in PBS-drained popliteal LNs (black lined), LecB-drained (red lined) and FMO control (light grey filled).
(C) Median fluorescence intensities (MFIs) of the indicated proteins in stromal cells of PBS- and LecB-drained LNs assessed by flow cytometry (mCLCA1/VCAM-1: PBS control: n=3, LecB-treated: n=9; MAdCAM-1/ICAM-1: PBS control: n=3, LecB-treated: n=3). Each point represents the results of two pooled popliteal LNs of one mouse. Mean values and standard error of the mean are shown by bars and error bars, respectively. ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$; Mann-Whitney-U test.
DN, double negative cells; FRC, fibroblastic reticular cells; LEC, lymphatic endothelial cells; BEC, blood endothelial cells.

Surprisingly, our flow cytometric analysis of the stromal cell compartment revealed a decline in the stromal cell numbers in the LecB-drained LNs compared to the PBS-drained control LNs **(Figure 31A)**. Indeed, four hours after LecB injection, numbers of lymphatic endothelial cells, blood endothelial cells and high endothelial venules decreased by a factor of 3, 0.8 and 2.6, respectively, compared to PBS-drained LNs **(Figure 31B, left panel)**. These differences did not reach statistical significance though. After twenty-four hours of LecB injection, we observed a significant difference only in blood endothelial cell numbers, which have halved **(Figure 31B, right panel).** The proportion of live cells, however, did not differ in LecB- and PBS-drained LNs after four or twenty-four hours of LecB injection **(Figure 31C)**.
Together, these results demonstrate that LecB impacts endothelial cells within the draining LNs and changes their phenotype.

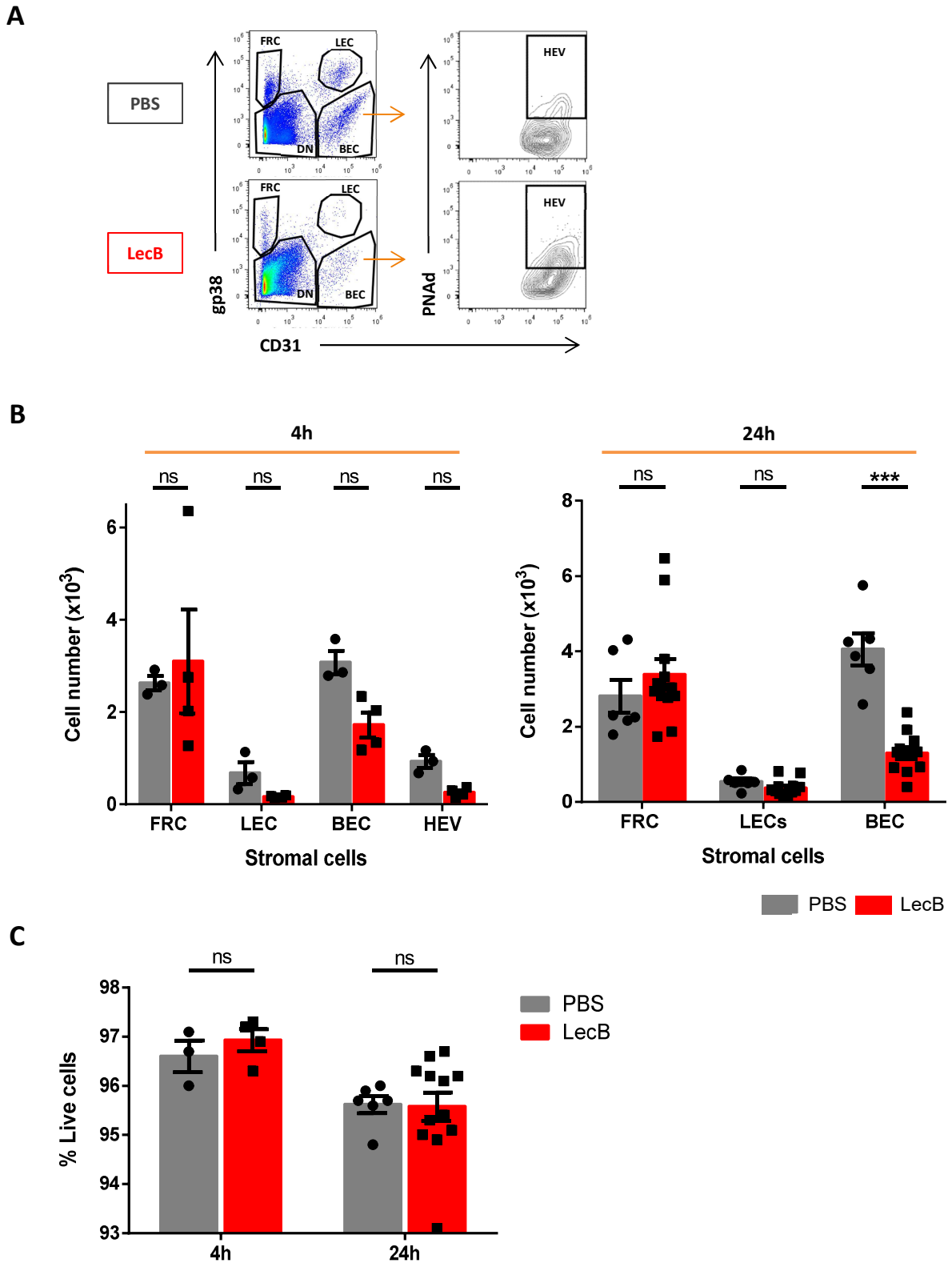

Figure 31. LecB reduces endothelial cell numbers in draining LNs

(A) Flow cytometry dot plots of stromal cells of two pooled draining popliteal LNs of one mouse 4 h after LecB and PBS injection.

(B) Cell numbers of stromal cell subsets 4 h (PBS control: n=3, LecB-treated: n=4) (left panel) and 24 h (PBS control: n=6, LecB-treated: n=12) (right panel) after LecB and PBS injection.
(C) Proportions of live cells 4 h (PBS control: n=3, LecB-treated: n=4) and 24 h (PBS control: n=6, LecB-treated: n=12) after LecB and PBS injection. Each point represents the proportion of live ($DAPI^-$) cells of two pooled popliteal LNs of one mouse assessed by flow cytometry.
Mean values and standard error of the mean are shown by bars and error bars, respectively. ns $p > 0.05$, ***$p < 0.001$; Mann-Whitney-U test. DN, double negative cells; FRC, fibroblastic reticular cells; LEC, lymphatic endothelial cells; BEC, blood endothelial cells; HEV, high endothelial venules.

6 LecB binds to human umbilical vein endothelial cells and is internalized in a time-dependent manner

To gain more insights into how LecB could alter endothelial cell functions, we cultured human umbilical vein endothelial cells (HUVECs) and treated them with LecB. Consistent with the preceding *in vivo* findings in mice, we observed binding of LecB-AF488 to HUVECs. This binding occurred at the intercellular junctions in a time-dependent manner: after one hour, we observed LecB at the intercellular junctions while after three hours, we found it clustered at the edges of the cells and internalized by the cells. Intracellularly, it was located close to the nucleus in big clusters, which have increased in size after five hours **(Figure 32)**.

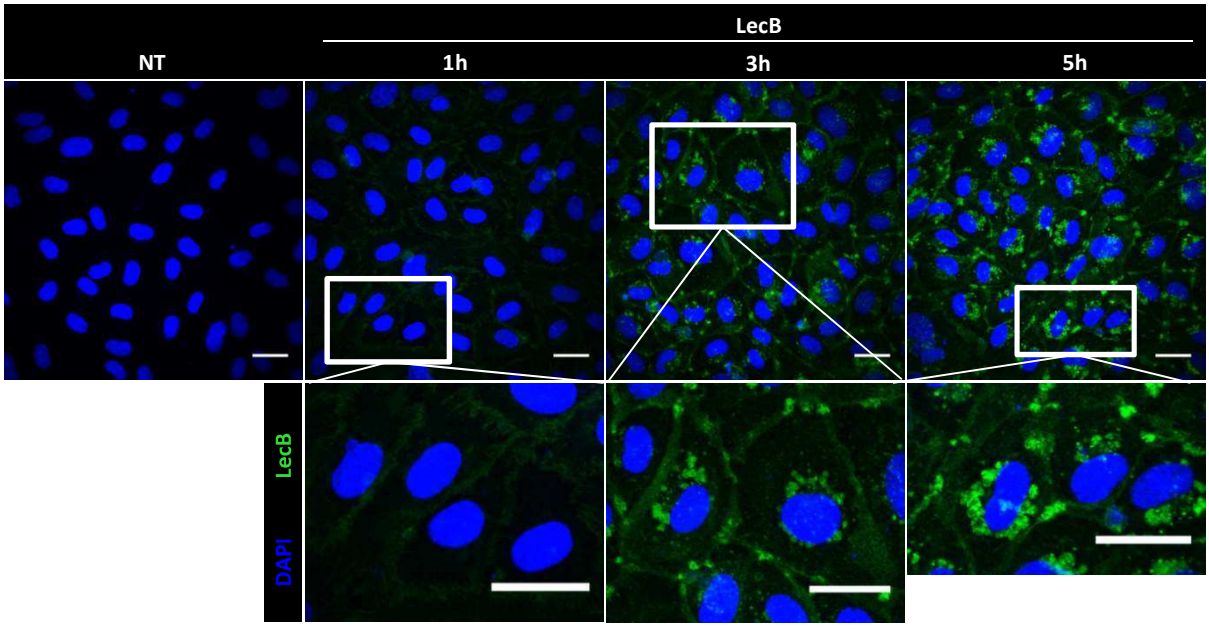

Figure 32. LecB binds to and is internalized by human umbilical vein endothelial cells in a time-dependent manner

Human umbilical vein endothelial cells (HUVECs) were cultured to confluence and treated with 5 µg/mL of fluorescently labeled LecB (LecB-AF488) during the indicated time periods. Representative confocal microscopy images are shown. Magnified images of the white boxed regions are shown in the lower panel. Scale bars represent 25 µm. NT, non-treated.

7 LecB reduces VE-cadherin protein level and causes cortical actin ring formation in human umbilical vein endothelial cells

Since we found LecB binding to the intercellular junctions in HUVECs, we analyzed whether LecB affects the major endothelial adhesion molecule VE-cadherin, which controls cellular junctions. By confocal microscopy we found a time-dependent decline in the fluorescence signal of VE-cadherin. We observed this reduction already after one hour of LecB stimulation in HUVECs and after three to five hours the staining of VE-cadherin became diffuse **(Figure 33A)**. By western blot analysis of VE-cadherin in treated HUVECs we confirmed the time-dependent decrease in the protein level of VE-cadherin, which has halved after five hours **(Figure 33B)**. Surprisingly, we observed a reorganization of the actin filaments with the LecB treatment of HUVECs. In the untreated cells, we found many actin filaments within the cells that started to reorganize after one hour of LecB treatment. Starting from three hours of LecB treatment, we could observe cortical actin rings and only few actin fibers within the cells **(Figure 33C)**. Consistent with these findings, the treated HUVECs became more adherent to the culture dishes. Together, these results indicate that LecB impacts endothelial cell junctions and induces a reorganization of actin fibers into cortical actin rings.

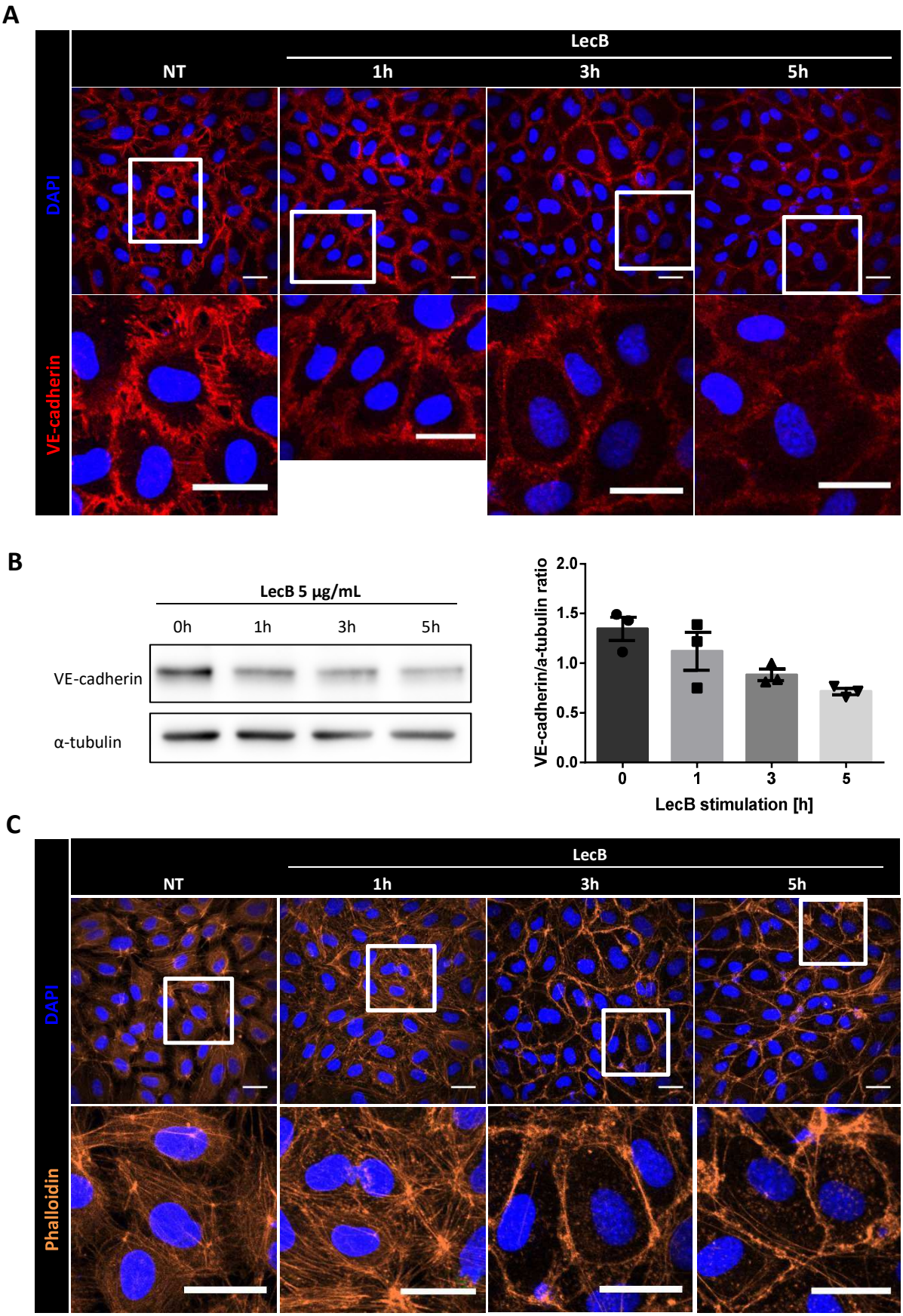
A
LecB
NT
1h
3h
5h
DAPI
VE-cadherin
B
LecB 5 µg/mL
0h
1h
3h
5h
VE-cadherin
α-tubulin
VE-cadherin/a-tubulin ratio
2.0
1.5
1.0
0.5
0.0
0
1
3
5
LecB stimulation [h]
C
LecB
NT
1h
3h
5h
DAPI
Phalloidin

Figure 33. LecB reduces VE-cadherin protein level and causes cortical actin ring formation in human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVECs) were cultured to confluence and treated with 5 µg/mL of purified LecB during the indicated periods

(A) Representative confocal microscopy images of HUVECs stained for VE-cadherin. Magnified images of the white boxed regions are shown in the lower panel. Scale bars represent 25 µm.

(B) Western blot analysis of VE-cadherin in cell lysates from HUVECs treated with LecB.

(C) Representative confocal microscopy images of HUVECs stained for actin (Phalloidin). Magnified images of the white boxed regions are shown in the lower panel. Scale bars represent 25 µm.

NT, non-treated.

DISCUSSION

Discussion

Many virulence factors of *Pseudomonas aeruginosa* are involved in the evasion of the host immune response (Johnson, 2018), allowing the pathogen to persist at the infection site, which may lead to untreatable, chronic infections. Hence, virulence factors are in the focus of research on alternative treatment strategies, some of which turned out to be promising (Chatterjee et al., 2016). However, the exact role of all virulence factors in the pathophysiology of *P. aeruginosa* infections and evasion of the host immune response is still far from being fully elucidated. A better knowledge of the functions of virulence factors may not only contribute to the overall comprehension of the pathophysiology, but may also provide new targets for the development of preventative and/or therapeutic approaches for the management of *P. aeruginosa* infections. The *P. aeruginosa* lectin LecB has been described to play an important role in the adhesion of the bacterium to host epithelial cells (Boukerb et al., 2014; Chemani et al., 2009; Noskova et al., 2015) and to be involved in biofilm formation (Passos da Silva et al., 2019; Tielker et al., 2005). Its influence on cells of the immune system, however, is only poorly investigated (Avichezer and Gilboa-Garber, 1987). Here, we investigated for the first time to our knowledge the activity of exogenously administered LecB in the skin-draining lymph node in mice. By flow cytometry and fluorescence microscopy, we identified lymphatic endothelial cells as one of the main LecB target cells within the draining LN and showed that twenty-four hours after subcutaneous or intradermal injection, LecB caused an accumulation of lymphocytes within the skin-draining LNs. While injection of traceable lymphocytes revealed that LecB did not enhance the recruitment of lymphocytes into the LN, we showed instead in lymphocyte entry-blocking experiments that LecB impeded lymphocyte egress. We demonstrated that LecB modifies lymphatic endothelial cells *in vivo* and *in vitro*, suggesting a reinforced endothelial barrier function and a possible role for LecB in disturbing lymphocyte circulation between the blood and the LN.

Consistent with earlier studies of soluble, low molecular weight molecules (Gretz et al., 2000; Sixt et al., 2005), injected LecB drained into the subcapsular sinus and medullary sinuses of the skin-draining LNs and to the HEVs via FRC-conduits. While imaging clearly showed lectin binding to FRCs and HEVs, quantitative analysis by flow cytometry revealed that only a fraction of these cells were stained by LecB. In line with this, in the LN sections we observed a LecB gradient from the SCS to the HEVs, with those HEVs in the deeper cortex being devoid of LecB. Similar distribution patterns have been reported on the plant lectins

wheat germ agglutinin (WGA), pisum sativum agglutinin (PSA) and lens culinairs agglutinin (LCA) in LN sections after administration into the footpad of mice (Gretz et al., 2000). The limited access of these lectins to deeper LN structures possibly results from interactions with proteoglycans and glycosaminoglycans (GAG) associated with the collagens of conduits and conduit-associated cells (Gretz et al., 2000).

HEVs function as entry site of naïve lymphocyte from the blood into the LN (Warnock et al., 1998). We observed LecB binding to these vessels, which led us hypothesize that this binding could interfere with lymphocyte entry. Subcutaneous and intradermal LecB injection induced an inflammatory response twenty-four hours after injection with enlargement of the draining LN and a strong infiltration of myeloid cells, i.e., neutrophils and inflammatory monocytes, and an increase in lymphocyte numbers. Since inflammation is accompanied by an increased blood flow through the LN (Soderberg et al., 2005) and an expansion of the LN vasculature by VEGF-induced proliferation of lymphatic and blood endothelial cells (Kumar et al., 2012), which results in an increased lymphocyte influx, we expected an enhanced recruitment of lymphocytes responsible for the increase in lymphocytes. However, by performing an adoptive transfer of donor CD45.1^{+} lymphocytes into host CD45.2^{+} mice, we detected no accelerated B cell entry after LecB injection. We rather observed a slight decrease in donor B cell numbers within the draining LN compared to the PBS control.

The data are not incompatible with LecB impeding lymphocyte entry into the LN. In fact, HEVs express large amounts of peripheral node addressins (PNAds) on their cell surface, which are required for lymphocyte homing (Berg et al., 1991; Kanda et al., 2004; Umemoto et al., 2006). These sulfated glycoproteins share a highly sialylated, fucosylated and sulfated structure on O-glycans, the 6-sulfo sialyl Lewis^{x} tetrasaccharide (Lowe, 2002), and thus provide a potential binding site for LecB on HEVs. Indeed, Lewis^{x} and siayl-Lewis^{x} have both been reported as LecB ligands (Dupin et al., 2018; Marotte et al., 2007; Topin et al., 2013). Moreover, sialyl-Lewis^{x} and 6-sulfo-sialyl Lewis^{x} structures have been described as preferential ligands for *P. aeruginosa* (Scharfman et al., 1999; Scharfman et al., 2000) and in severely infected patients suffering from CF, bronchial mucins appeared more sialylated and contained more sialyl-Le^{x} epitopes than mucins from less infected patients (Davril et al., 1999; Lo-Guidice et al., 1994). The L-selectin on lymphocytes specifically recognizes 6-sulfo-sialyl-Lewis^{x} and fucosylation and sulfation have been shown to be required for binding (Lowe, 2002). Accordingly, mice doubly deficient for the α-1,3-fucosyltransferases FucT-VI and FucT-VII have extremely small peripheral LNs comparable to those in L-selectin-lacking

mice (Arbonés et al., 1994) and are devoid of naïve lymphocytes (Homeister et al., 2001). Thus, LecB binding to 6-sulfo sialyl Lewis^x might prevent the binding of L-selectin to the HEV endothelial cell wall, thereby reducing lymphocyte homing to draining LNs.

Nevertheless, while we can exclude an increased influx as cause for the accumulation of lymphocytes within the LN after LecB injection, we cannot conclude with the present data whether or not LecB inhibits lymphocyte entry. Contrary to donor T cells numbers, donor B cell numbers tended to be lower with LecB compared to PBS control, which was suggestive of a cell entry inhibitory effect of LecB. The differential effect of LecB on B and T cell entry may arise from the distinct efficiency of B and T cell homing to lymphoid tissues. B cell migration into peripheral lymphoid tissues has been reported to be less efficient than that of T cells (Okada et al., 2002; Tang et al., 1998). This is because B cells express L-selectin at 50 to 100% lower levels than T cells (Tang et al., 1998). In addition, T cells have been shown to express ~10-fold higher surface level of CCR7 than B cells, and to be more responsive to CCL19 and CCL21 than B cells in *in vitro* chemotaxis assays (Okada et al., 2002). It is possible that a LecB-induced inhibition of lymphocyte entry counterbalanced the inflammation-induced increased influx of cells to the basal level. This would negatively affect the encounter of lymphocytes with their cognate antigen and thus reduce the development of a *P. aeruginosa*-specific protective, adaptive immune response. Thus, future studies are required to address the hypothesis that LecB inhibits the migration of lymphocytes through HEVs.

Since an enhanced entry of lymphocytes into the LN could not account for the LecB-induced accumulation of lymphocytes, we first assumed that LecB stimulated directly the proliferation of lymphocytes. Several plant lectins, including the phytoheamagglutinin (PHA), Concanavalin A (Con A) and the pokeweed mitogen have been reported to exhibit mitogenic properties toward mammalian cells, including B and T cells (Gorelik et al., 2001). Stimulation of T cell proliferation has been shown to result from lectin binding to and activation of the T cell receptor (Gorelik et al., 2001). Similarly, LecB has been reported to be mitogenic toward human peripheral blood lymphocytes and murine splenic lymphocytes *in vitro* (Avichezer and Gilboa-Garber, 1987) and in a recent study, LecB has been shown to bind to and activate primary murine B cells *in vitro* (Wilhelm et al., 2019). However, we only found a minor LecB staining on B and T cells in skin-draining LN by flow cytometry after injection of fluorescently labeled LecB. Moreover, the intercellular LecB staining in the cortex was very low in intensity in the LN sections, indicating that the access of LecB to

lymphocytes of the cortical parenchyma was restricted. Therefore, a direct mitogenic effect of LecB toward lymphocytes within the LN is unlikely. This is further supported by the fact that after adoptive transfer of $CD45.1^+$ donor lymphocytes into $CD45.2^+$ mice, we observed no increase in the donor population while host lymphocytes numbers significantly augmented. If LecB induced lymphocyte proliferation, we would expect that transferred donor cells had also proliferated twenty-four hours after LecB injection.

Alternatively, LN-resident macrophages and DCs could have activated lymphocytes. Indeed, we observed LecB staining on all LN macrophage subsets and on all skin-derived migratory and LN-resident DCs within the LN. The sinus-associated SSMs and MSMs captured LecB most strongly among all macrophages and DCs. These results are in line with those of Ingulli et *al.*, reporting that four hours after subcutaneous injection of fluorochrome-labeled ovalbumin (OVA) (MW ~45 kDa), LN DCs contained low levels of OVA, whereas LN macrophages contained the largest amount (Ingulli et al., 2002). Similar to LecB, *P. aeruginosa* has been shown to be present in the cortical and particularly in the medullary sinuses of the skin-draining LN after subcutaneous footpad injection in mice (Kastenmüller et al., 2012). The importance of LN macrophages in *P. aeruginosa* infections has also been demonstrated in the same study, reporting that local depletion of these macrophages in the draining LN led to a 10-fold increase in the local and systemic bacterial loads after subcutaneous injection of *P. aeruginosa* (Kastenmüller et al., 2012). SSMs are known to capture lymph-borne antigens and pathogens for subsequent transfer to the underlying B cell follicles (Phan et al., 2007). Consequently, SSMs could quickly sample LecB from the SCS and relay it to B cells for their activation. The fact that SSMs preferentially capture large lymph-borne particles (> 70 kDa), such as viruses and bacteria (Junt et al., 2007; Kastenmüller et al., 2012; Roozendaal et al., 2009), may explain why MSMs sampled LecB most strongly. Indeed, MSM dispose a broad range of membrane receptors, which bind to any lymph-borne particulate (Kuka and Iannacone, 2014).

Skin-derived migratory DCs have been reported to capture tissue-deposited antigens and to migrate after eighteen hours to four days, depending on the DC subset, to draining LNs for T cell priming (Itano et al., 2003; Kissenpfennig et al., 2005; Tomura et al., 2014). We analyzed LecB binding to DCs four hours after intradermal and subcutaneous injection. Consequently, skin-derived migratory DCs must have acquired LecB in transit to the LN or within the LN. Even though we observed LN macrophages and DCs having captured LecB, we can exclude that lymphocyte activation and clonal expansion of effector cells led to the LecB-induced

lymphocyte accumulation within the LN. Several studies have reported a lag-time of more than twenty-four hours between the initial antigenic stimulation and the first round of lymphocyte division (van Stipdonk et al., 2001; Veiga-Fernandes et al., 2000; Yoon et al., 2010). We found, however, an increase in LN cellularity already after four hours (data not shown) and twenty-four hours of LecB injection, pointing to a different mechanism.

In our experiments, we found the observed effects of LecB to be more pronounced and to be more homogenous in popliteal LNs compared to auricular LNs. This may be due to the route of LecB administration. While we injected LecB intradermally into the ear to target the draining auricular LNs, we administered it subcutaneously into the footpad to target the draining popliteal LNs. In contrast to the subcutaneous tissue, which is poor in antigen-presenting cells, the dermis is rich in DCs (Bonnotte et al., 2003; Igyártó et al., 2011). Accordingly, skin DCs may capture intradermally injected LecB and thereby reduce the amount of LecB draining into the LN, while subcutaneously administered LecB probably drain nearly completely into the LN. Moreover, the ease of subcutaneous injections ensures invariably, unlike intradermal injections, the administration of the full dose of LecB, which probably results in more homogenous results.

Immune response-induced lymphocyte accumulation within the LN may result from increased lymphocyte entry from the blood into the LN, increased lymphocyte proliferation within the LN or a reduced exit of lymphocytes from the LN. Since we could not show that increased entry and proliferation were responsible for the rise in LN cellularity, we assumed that LecB restricted the egress of lymphocytes. By blocking the cell entry with a CD62L (L-selectin)-blocking antibody we observed indeed that lymphocyte numbers remained by and large stable when LecB was co-administered contrary to the PBS-treated mice, in which lymphocyte numbers were significantly decreased compared to the untreated controls. Although these data are in support of the conclusion that LecB negatively regulates lymphocyte egress, we cannot exclude the possibility that the limited egress actually resulted from inflammatory mediators rather than from LecB directly. Indeed, under inflammatory conditions with high pro-inflammatory cytokine levels, lymphocytes express CD69 on their cell surface upon activation. CD69 has been shown to interact with S1PR1 on the lymphocyte membrane (Bankovich et al., 2010; Shiow et al., 2006). The subsequent down-modulation of S1PR1 and decreased responsiveness of lymphocytes to S1P produced by lymphatic endothelial cells leads to reduced lymphocyte egress from inflamed LNs (Bankovich et al., 2010; Shiow et al., 2006). Thus, in order to elucidate the cause for the restricted lymphocyte

egress, future studies should monitor lymphocyte numbers within the draining LNs over a prolonged period of time after LecB injection.

LECs were the only stromal cell subset being nearly completely labeled with LecB after its injection, as assessed by flow cytometry. Previous studies on carbohydrate ligands of LecA and LecB on mammalian tissues have shown that binding to endothelial cells is restricted to LecA. Accordingly, in mink lung and pancreas tissue sections, LecB has been shown to bind to epithelial cells only, while LecA bound additionally to endothelial cells (Kirkeby et al., 2007) and to endothelial cells in kidney, heart and adrenal gland from mouse tissue sections (Kirkeby et al., 2006). Gilboa-Garber et *al*., however, reported that 125I-labeled LecB injected intravenously in mice exhibits the highest affinity to spleen (lymphoid tissue) and kidney, followed by lung and liver (Gilboa-Garber et al. 2002). Although the administration route in this study differed from ours and the target cells of LecB within the spleen have not been reported, the finding that LecB bound most strongly to lymphoid tissue is in line with our results. Nevertheless, we provide clear evidence of LecB binding to mouse (lymphatic) endothelial cells within the skin-draining LN and to human umbilical vein endothelial cells. The presence of fucose residues on HUVECs as well as on various human tissue sections has indeed been reported by Holthöfer et *al*., suggesting the Ulex europaeus I agglutinin, a lectin specific for some alpha-L-fucose-containing glycocompounds, as a histologic marker for endothelium in human tissues (Holthofer et al., 1982). Interestingly, the LECs of the subcapsular sinus ceiling were the only cells among all LECs within the LN being devoid of LecB, which indicates that LecB binds to LECs via a receptor or structure that is not commonly shared by these cells. In addition to CCRL1 (ACKR4), the atypical chemokine receptor for CCL19 and CCL21, that is uniquely expressed on the SCS ceiling lining LECs (Ulvmar et al., 2014), our group previously showed that integrin alpha 2b (ITGA2b, CD41) is expressed by all LECs except for the ceiling LECs (Cordeiro et al., 2016). These findings emphasize phenotypical and functional differences between the SCS ceiling and floor LECs. Even if these results already give an indication about a potential LecB binding site, so far we have not identified any LecB counter-receptor on LECs and further studies are needed to address this issue.

To assess whether LecB induced phenotypical changes in LECs, which may explain the restricted lymphocyte egress, we analyzed different adhesion molecules on LECs. Surprisingly, we observed a loss of the cell surface protein murine chloride channel calcium activated 1 (mCLCA1) in LN sections twenty-four hours after LecB administration as well as

a decline in ICAM-1 on LECs as assessed by flow cytometry. While there was no difference in MAdCAM-1 staining, VCAM-1 was significantly increased on all stromal cell subsets. mCLCA1 is specifically and constitutively expressed on the lymphatic endothelium within the LN (Furuya et al., 2010; Ruddell et al., 2003). Unlike ICAM-1 and VCAM-1, which are constitutively expressed at low levels on the resting endothelium and strongly upregulated upon stimulation with proinflammatory cytokines, such as TNF-α and IL1-β (Boscacci et al., 2010; Furuya et al., 2010; van Buul et al., 2010), the expression of mCLCA1 is not affected by these cytokines (Furuya et al., 2010). So far, endothelial ICAM-1 has been known to strongly interact with LFA-1 expressed on lymphocytes, thereby promoting firm arrest of the latter on the HEV surface for subsequent transmigration (Boscacci et al., 2010). However, Furuya et *al*. reported LEC mCLCA1 as new binding partner of LFA-1 (Furuya et al., 2010). Indeed, in adhesion assays using neutralizing antibodies, they reported that mCLCA1-LFA-1-mediated adhesion of lymphocytes to the endothelium predominated over ICAM-1-mediated adhesion, even under conditions where ICAM-1 was upregulated by cytokine treatment (Furuya et al., 2010). Even though these data support a role of mCLCA1 in lymphocyte adhesion to the lymphatic endothelium and we observed a decline in mCLCA1 on LECs with LecB, which may indeed impact the interaction of B and T cells with the lymphatic endothelium, these results have been obtained in *in vitro* assays and the relevance *in vivo* remains to be confirmed. Whether the interaction of mCLCA1 with LFA-1 is involved in lymphocyte egress and may be relevant for the LecB-induced restriction of lymphocyte egress is arguable since Lo et *al*. reported that α4 and β2 integrins are not essential for lymphocyte egress from LNs (Lo et al., 2005).

The LecB-mediated sequestration of lymphocytes together with a possible inhibition of LN homing of naïve lymphocytes from blood would greatly impair the adaptive immune response to *P. aeruginosa* and thereby hinder the effective clearance of the pathogen. Indeed, studies on RAG-2$^{-/-}$ mice that lack mature lymphocytes, showed that lymphocytes play an important role in *P. aeruginosa* infection. Following intranasal infection, these mice demonstrated an increased mortality compared to the wild-type controls (Koh et al., 2009; Nieuwenhuis et al., 2002). Thus, further functional studies are needed to investigate whether the initiation of the adaptive immune response is hindered by LecB, i.e., whether primed lymphocytes (effector T cells and antibody-producing plasma cells) are sequestered within the LN by LecB.

Besides the observed decline in mCLCA1 abundance on LECs, we found some untypical mCLCA1 staining on BECs in the LecB-drained LNs by flow cytometry analysis. Moreover,

we found a decline in the stromal cell numbers, while the proportion of live cells in LecB-drained LNs did not differ from the one in PBS-drained LNs four and twenty-four hours after injection. Therefore, it is likely that the expression of the markers used to identify the different stromal cell subsets, i.e., gp38 (podoplanin) and CD31 (PECAM-1), were also reduced by LecB. As LEC and BEC numbers (both identified by means of CD31 expression) were more affected than FRC numbers, expression of CD31 might be particularly concerned. In favor of this hypothesis, we showed in cultured HUVECs a time-dependent decrease in the protein level of VE-cadherin upon LecB exposure. CD31 and VE-cadherin are both endothelial cell junction proteins and have been shown to be co-regulated by several factors, including S1P-S1PR1 signaling (Krump-Konvalinkova et al., 2005) and CD44 (Tsuneki and Madri, 2014) in endothelial cells for maintaining endothelial barrier integrity. Thus, a possible LecB-induced loss of CD31 and/ or gp38 on stromal cells should be verified for further stromal analysis and LecB-insensitive stromal cell markers should be used to ensure the right identification of the different stromal cell subsets.

The time-dependent decline in the VE-cadherin protein level in HUVECs upon exposure to LecB and the diffuse VE-staining observed by confocal microscopy point to a LecB-induced loss of endothelial integrity. The importance of VE-cadherin in maintaining endothelial integrity is emphasized by a study conducted by Corada et *al*., showing that injection of the BV13 function-blocking VE-cadherin antibody in mice led to dispersion of VE-cadherin away from endothelial cell junctions of blood vessels and fatal leakage and hemorrhage within hours (Corada et al., 1999). A similar study by Baluk et *al*. reported not only a BV13 antibody-induced dispersion of VE-cadherin in endothelial cells of initial lymphatics and blood vessels but also a dispersion of CD31 in these vessels (Baluk et al., 2007). Hence, it is possible that indeed LecB not only affects VE-cadherin but also CD31. These findings are in line with those of Laughlin et *al*., reporting LecA to negatively impact the intestinal epithelium. LecA has been shown to reduce the transepithelial electrical resistance and to increase the epithelial paracellular permeability in cultured Caco-2 monolayers, which probably resulted from disrupted tight junctions as protein levels of the tight junctional proteins Zonula occludens-1 (ZO-1) and occludin were reduced (Laughlin et al., 2000). A loss of the endothelial barrier integrity would favor the entry of *P. aeruginosa*-produced virulence factors or the invasion of the bacteria into the LN parenchyma and/or blood vessels, promoting bacterial systemic dissemination.

However, in contrast to the decline in VE-cadherin, suggestive for a loss of endothelial integrity, we found HUVECs concomitantly denser, with cortical actin enrichment and a reduction in the amount of central stress fibers, when stimulated with LecB compared to the non-treated control cells, which is suggestive for a reinforced endothelial barrier (Garcia-Ponce et al., 2015). We observed LecB binding to HUVECs initially at the intercellular junctions, and at later time points clustered at the cell edges. It is possible that LecB binding to endothelial cell junctions not only led to dispersion of the adherens junctions, i.e., VE-cadherin, but also crosslinked the endothelial cells, thereby stabilizing their barrier integrity. The structural organization of LecB as a tetramer with each monomer containing one carbohydrate binding site, localized on opposing ends of the protein (Loris et al., 2003), would allow crosslinking of adjacent cells. Indeed, LecB and LecA have both recently been shown to crosslink adjacent giant unilamellar vesicles (GUVs), engineered protocells, which have been functionalized with lectin-specific ligands, to form protocellular junctions (Villringer et al., 2018).

It remains to be investigated whether the LecB-induced dispersion of VE-cadherin and the potential reinforced endothelial barrier integrity negatively impact the transmigration of leukocytes across the endothelium. The possible crosslinking of the endothelial cells by LecB at the intercellular junctions could indeed interfere with paracellular transmigration of leukocytes. However, the tightness of the endothelial barrier has been shown to not affect immune cell trafficking as lymphocytes can switch their transmigration route preference in settings of differing inter-endothelial adhesive strength and independently of changes in the level or distribution of endothelial adhesion molecules (Martinelli et al., 2014).

We investigated the role of purified and exogenously administered LecB in the skin-draining LNs. To date we do not know to what extent this approach and the results we obtained are physiologically relevant in settings of infection with the whole bacteria. *P. aeruginosa* dispose a wide array of virulence factors, which may work synergistically with or in dependence of LecB, such as type IV pili, whose normal assembly relies on LecB (Sonawane et al., 2006). Even though LecB has been shown to be exposed on the surface of biofilm cells, from which it can be released by the addition of L-fucose (Funken et al., 2012), it remains to be determined whether LecB is secreted and also the range of LecB concentration at the infection site. Bajolet-Laudinat et *al.* reported LecA to be present in *P. aeruginosa* culture medium at concentrations higher than 20 µg/mL for a bacterial inoculum of 10^9 bacteria per

mL as well as in infected sputum of CF patients (Bajolet-Laudinat et al., 1994). Thus, it is supposable that this applies similarly to LecB.

In conclusion, our results provided new insights into the *in vivo* function of the lectin LecB in the host immune evasion of *P. aeruginosa*. We showed LecB to bind to endothelial cells within the skin-draining LNs and to modify these cells phenotypically, thereby disturbing lymphocyte circulation between the blood and the LN, which is essential for immunosurveillance and the establishment of protective adaptive immune responses.

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APPENDIX

Appendix

Publication

Camara A, Cordeiro OG, Alloush F, **Sponsel J**, Chypre M, Onder L, Asano K, Tanaka M, Yagita H, Ludewig B, et al. (2019). Lymph Node Mesenchymal and Endothelial Stromal Cells Cooperate via the RANK-RANKL Cytokine Axis to Shape the Sinusoidal Macrophage Niche. Immunity 50, 1467-1481.e1466.

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Host Immune Evasion by the *Pseudomonas Aeruginosa* Virulence Factor LecB

Résumé

Pseudomonas aeruginosa est l'une des bactéries multirésistantes les plus répandues. Bien que le rôle du facteur de virulence LecB, une protéine se liant au fucose, ait été montré comme important pour la fixation aux cellules hôtes, son interaction avec le système immunitaire reste à élucider. Nous montrons ici que LecB cible les cellules endothéliales (CE) dans les ganglions lymphatiques (GL) drainant après injection cutanée chez la souris. Vingt-quatre heures après l'injection, LecB provoque une accumulation de lymphocytes dans les GL drainant la peau. Bien que l'injection de lymphocytes traçables ait révélé que LecB n'accélère pas le recrutement des lymphocytes dans le GL, nous montrons plutôt dans les expériences de blocage de l'entrée des lymphocytes que LecB empêche la sortie de ces derniers. Nous démontrons que LecB modifie les CE in vivo et in vitro, ce qui suggère une fonction de barrière endothéliale renforcée et un rôle possible pour LecB dans la perturbation de la circulation des lymphocytes entre le sang et le GL ce qui est essentiel pour l'immunosurveillance et l'établissement des réponses immunitaires adaptatives protectrices.

Mots clés: *Pseudomonas aeruginosa*, lectine, LecB, ganglion lymphatique, cellules endothéliales

Résumé en anglais

Pseudomonas aeruginosa is one of the most common multidrug-resistant bacteria. The role of its virulence factor LecB, a fucose-binding protein, in the attachment to host cells has been shown. Its interaction with the immune system, however, is still to be elucidated. Here, we show that LecB targets endothelial cells within the draining lymph nodes (LNs) after cutaneous injection in mice. Twenty-four hours after injection, LecB causes an accumulation of lymphocytes within the skin-draining LNs. While injection of traceable lymphocytes revealed that LecB does not enhance the recruitment of lymphocytes into the LN, we show instead in lymphocyte entry-blocking experiments that LecB impedes lymphocyte egress. We demonstrate that LecB modifies endothelial cells *in vivo* and *in vitro*, suggesting a reinforced endothelial barrier function and a possible role for LecB in disturbing lymphocyte circulation between the blood and the LN, which is essential for immunosurveillance and the establishment of protective adaptive immune responses.

Key words: *Pseudomonas aeruginosa*, lectin, LecB, lymph node, endothelial cells