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Taking the lymphatic route: dendritic cell migration to draining lymph nodes

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Abstract In contrast to leukocyte migration through blood vessels, trafficking via lymphatic vessels (LVs) is much less well characterized. An important cell type migrating via this route is antigen-presenting dendritic cells (DCs), which are key for the induction of protective immunity as well as for the maintenance of immunological tolerance. In this review, we will summarize and discuss current knowledge of the cellular and molecular events that control DC migration from the skin towards, into, and within LVs, followed by DC arrival and migration in draining lymph nodes. Finally, we will discuss potential strategies to therapeutically target this migratory step to modulate immune responses.

Keywords Dendritic cells · Migration · Lymphatic vessels · Draining lymph node · CCL21

Introduction

Vaccination is considered one of the greatest public health achievements of the twentieth century and has contributed to a dramatic decline in mortality from infectious diseases. Antigen-presenting dendritic cells (DCs) are an important immune cell type that is activated during both vaccination and infection. Upon encountering a pathogen or a vaccine in peripheral tissues, DCs take up antigen, mature, and start to migrate via afferent lymphatic vessels (LVs) to draining LNs (dLNs), where they present the antigen to T cells for the

induction of adaptive immune responses. Since their discovery approximately 40 years ago [1], ample evidence has demonstrated the importance of DCs not only in the induction of adaptive immunity in the context of vaccination and infection but also for the maintenance of tolerance [2–4]. The recognition of the importance of DCs in the immune system was also prominently highlighted by the awarding of the 2011 Nobel Prize of Physiology and Medicine to the late Ralph Steinman “for his discovery of the dendritic cell and its role in adaptive immunity” [5].

Although the migratory pattern of DCs from peripheral tissues to draining LNs has been known for approximately 30 years, many details about the cellular and molecular events that govern DC migration via afferent LVs are only now starting to be unraveled. This is very much in contrast to leukocyte trafficking through blood vessels (BVs), which has been studied in greater detail over the past 30 years. The gap in knowledge can likely be attributed to the fact that also the lymphatic vascular system as a whole has been much less well studied in comparison to the blood vascular system. Although LVs were already described in the seventeenth century, and the embryonic development of lymphatics was extensively studied during the beginning of the twentieth century [6], the lack of specific molecular markers for long time hampered further investigation of the lymphatic vascular system. In fact, lymphatic markers, such as the vascular endothelial growth factor receptor-3 (VEGFR-3), the hyaluronan receptor LYVE-1, podoplanin (gp38), or the lymphatic-specific transcription factor Prox-1 have only been identified over the past 18 years [6, 7]. Their discovery has contributed to a true renaissance and explosion of lymphatic vascular research and to recent progress made in the field of leukocyte trafficking via LVs.

In this review, we will first give a brief introduction to LV and DC biology. We will particularly focus on the skin and skin-dLNs, as these are the organs in which DC migration via

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LVs has mainly been studied. In a next step, we will introduce some of the tools, which are commonly used to experimentally study DC migration via LVs *in vivo*. We will then summarize and discuss different aspects of DC migration in the order of the DC's physiologic itinerary, namely its migration from peripheral tissues towards, into, and within lymphatic vessels (LVs), followed by its arrival and migration in the dLN. Finally, we will discuss emerging experimental and clinical approaches to modulate DC migration for the improvement of DC-based vaccines or for the prevention of transplant rejection.

Characteristic features of afferent LVs

The lymphatic vascular system is essential for fluid drainage from peripheral tissues, and for the uptake of dietary fats in the intestine [8]. Moreover, it fulfills important immune functions by mediating the transport of soluble antigens to dLNs as well as leukocyte trafficking to and from LNs. With the exception of the brain, LVs are present in virtually all vascularized tissues. The afferent lymphatic network begins in form of blind-ended lymphatic capillaries, which are considerably wider than BVs. Lymphatic capillaries then merge into collecting vessels, which may span long distances and finally connect the afferent lymphatic network with a dLN (Fig. 1a). Afferent LVs connect with the collagen-rich capsule of the LN and drain lymph directly into the space below the capsule, which is known as subcapsular sinus (SCS). The bottom of the SCS is lined by LYVE-1⁺-positive lymphatic endothelial cells (LECs), which are interspersed with CD169⁺ macrophages. After passing through the SCS, lymph flows into the surface-rich and highly branched medullary sinus. In the paracortex, in proximity to high endothelial venules (HEVs) and the T cell area, blind-ended LYVE-1⁺ cortical sinuses begin and represent the sites of lymphocyte egress from LNs [9]. Like the SCS, also the cortical sinuses merge with the medullary sinus, which finally converges into an efferent LV that exits the LN [10] (Fig. 1a). Particularly in larger mammals, lymph is often transported sequentially through many LNs that are organized in chains. In this setup, an efferent LV can at the same time represent the afferent LV of the subsequent LN [11]. In this review, however, we will explicitly refer to the initial LVs in peripheral tissue, which are composed of both capillaries and collectors, when mentioning the term afferent LVs. After passing through one or several LNs, the collecting LVs finally merge in the thorax to form a single conduit named thoracic duct, which releases its content (i.e., lymph) into the blood vascular circulation at the level of the left subclavian vein.

The morphology of afferent LVs is ideally adapted to their function, namely the uptake and transport of tissue fluids and leukocytes: Blind-ended lymphatic capillaries are surrounded by a very thin and highly fenestrated basement membrane,

which is composed of collagen IV, laminin, perlecan, and nidogen [12, 13] (Fig. 1a). LECs in lymphatic capillaries have a unique oak leaf shape [14]. Neighboring oak leaf-shaped LECs partially overlap and are connected to each other by discontinuous cell–cell junctions (Fig. 1b), which are arranged in “button-like” associations of adhesion molecules. Such buttons contain vascular endothelial cadherin and tight junction-associated molecules such as claudin-5, occludin, or junctional adhesion molecule-A [14]. This unique pattern of tight junctions and partially overlapping LECs generates characteristic flaps (also called primary valves), which are permissive to the passage of fluids and macromolecules [14]. Moreover, the flaps are thought to be the prime site where leukocytes enter into LVs [12, 14] (Fig. 1b). While lymphatic capillaries are ideally suited for the uptake of lymph components, lymphatic collectors are uniquely adapted to the transport of lymph: Similarly to endothelial cells in BVs, LECs in collecting LVs adopt an elongated shape and are surrounded by a continuous lining of cell–cell junctions, which renders the collecting LVs less fluid-permeable [14] (Fig. 1a, b). Collecting LVs also contain specialized valves that impede retrograde lymph flow and divide the vessel into segments, which are called lymphangions (Fig. 1a). Compared to lymphatic capillaries, lymphatic collectors are surrounded by a much thicker and less fenestrated layer of basement membrane. Moreover, lymphatic collectors are covered by smooth muscles cells, which account for the rhythmical contractions of collecting vessels. These contractions mediate the propagation of lymph from one lymphangion to the next, in downstream direction.

CCL21 expression

A molecule with key relevance for DC migration via LVs is the chemokine CCL21, which is constitutively expressed by LVs [15–17] (Fig. 2a, b). In response to activating stimuli, tissue-resident DCs upregulate the CC-chemokine receptor 7 (CCR7) [2, 18], which initiates their migration towards CCL21-expressing LVs and to dLNs. Indeed, in mice, blockade of CCL21 [15] or genetic deletion of CCR7 [19, 20] was shown to severely compromise DC migration to dLNs. CCL21 comprises a highly positively charged C-terminal motif, which accounts for its immobilization on heparan sulfates present on cell surfaces or in the extracellular matrix (ECM) surrounding BVs and LVs [17, 21, 22]. Moreover, CCL21 was shown to bind with nanomolar affinity to LEC-expressed podoplanin, a mucin-type glycoprotein [23]. In mice, CCL21 is encoded by two genes, which give rise to two gene products: CCL21-Leu and CCL21-Ser differ in only one amino acid but display gross differences in their tissue distribution [24]. CCL21-Leu is the main isoform expressed by LVs [24], whereas CCL21-Ser is mainly expressed by HEVs and fibroblastic reticular cells (FRCs) in secondary

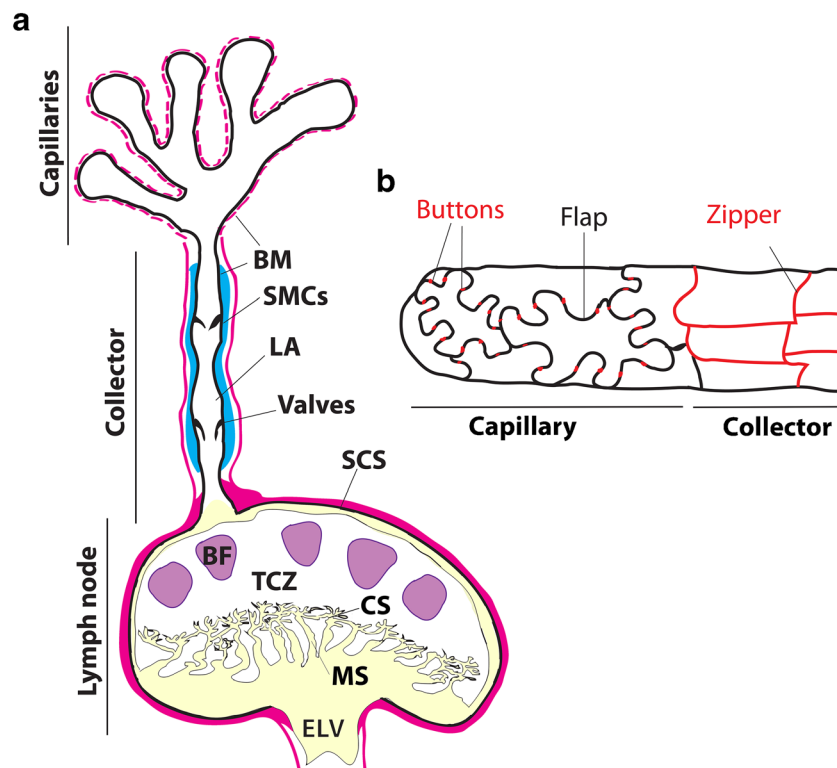


Fig. 1 Structure of afferent LVs and the LV network in dLNs. **a** Afferent LVs begin as blind-ended capillaries, which merge into collecting vessels and connect with dLNs. In contrast to lymphatic collectors, lymphatic capillaries have a thin and a highly fenestrated basement membrane (BM). Lymphatic collectors are surrounded by smooth muscle cells (SMCs) and contain valves. A lymphangion (LA) is defined as the segment between two valves. The lymphatic network in the dLN is organized into the subcapsular sinus (SCS), the medullary sinus (MS),

and the cortical sinuses (CS). Further abbreviations used: T cell zone (TCZ); B cell follicle (BF), efferent lymphatic vessel (ELV). **b** LECs in lymphatic capillaries are oak leaf shaped and display a discontinuous, “button-like” distribution of junctional adhesion molecules (red dots). Adjacent oak leaf-shaped LECs partially overlap, thereby creating open flaps, which are also called primary valves. LECs in lymphatic collectors have an elongated shape and are connected by continuous, “zipper-like” cell junctions (red lines)

lymphoid organs (SLOs) [25]. Substantial insights into these expression patterns and their functional consequences have come from the analysis of a naturally occurring mutant mouse strain, the so-called *plt* (paucity of lymph node T cells) mice. *plt* mice have a defect in the production of CCL19 (the second ligand of CCR7) and CCL21-Ser but retain expression of CCL21-Leu in peripheral LVs [24, 26]. As a result, DCs in *plt* mice are still able to enter into dermal afferent LVs, but their entry and interstitial migration in the dLN are impaired [25, 26].

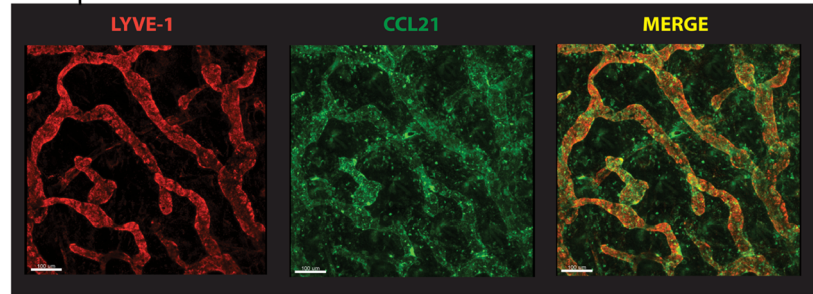
Dendritic cells

DCs are important immune sentinels that form a functional bridge between innate and adaptive immunity. Being particularly abundant in tissues, which form the border with the environment—such as the skin, intestinal tissues or the respiratory tract—DCs readily come into contact with pathogens or other noxious stimuli that breach the body’s barriers. DCs are highly phagocytic and derive their name from the fact that they possess many dendritic processes [1], which allow them to

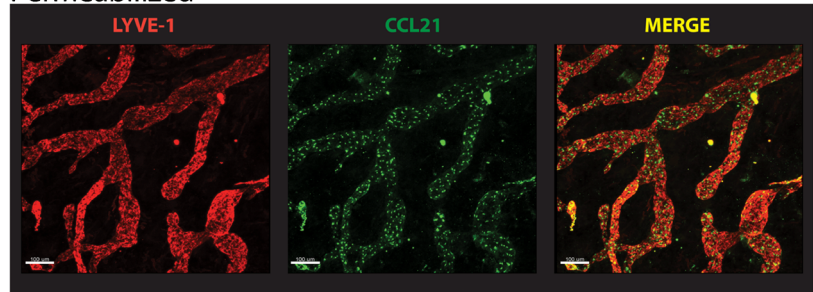
constantly sample their environment. They express a plethora of receptors that are able to recognize pathogen-associated molecular patterns, including Toll-like receptors (TLRs) and RIG and Nod-like receptors. Moreover, DCs can be indirectly activated by recognition of damage-associated molecular patterns or by inflammatory cytokines produced in the context of tissue inflammation [2]. DC activation initiates a series of phenotypic changes that are summarized as maturation [2]. During the maturation process, DCs cleave ingested antigen into peptides for presentation on major histocompatibility complex molecules and also upregulate co-stimulatory molecules, which are essential for the subsequent activation of naive CD4⁺ and CD8⁺ T cells. Besides enhancing the antigen presentation capacity of DCs, maturation also induces profound changes in the DC’s migratory behavior. Importantly, DCs downregulate inflammatory chemokine receptors and upregulate CCR7 and CXCR4 chemokine receptors [18]. While DC migration is strikingly enhanced in the presence of infection and other forms of tissue inflammation, also a minor but constant migration of DCs to LNs occurs in uninfamed, steady-state conditions. In fact, steady-state DC migration was shown to be important for the maintenance of

Fig. 2 Expression of CCL21 in LVs. The expression of CCL21 (green) in LYVE-1⁺ LVs (red) was analyzed in tissue whole mounts prepared from murine ear skin. **a** Staining of the extracellular fraction of CCL21 (performed under unfixed conditions, as described in [17]) reveals a diffuse CCL21 staining pattern that largely co-localizes with LYVE-1⁺ LVs. **b** Staining performed under PFA-fixed and permeabilizing conditions (as described in [16]) suggests the presence of punctuate, intracellular deposits of CCL21 in LECs. Scale bar, 100 μ m

a Non-permeabilized



b Permeabilized



peripheral tolerance [27, 28]. In particular, mice that lack the CCR7 receptor have been shown to develop symptoms of autoimmunity in various organs [29].

DCs present in peripheral organs like the skin do not form a homogenous population but exist in many different subsets of functionally related cells [2, 4, 30]. In the skin, the organ in which DC migration has been best studied, DCs can be very rudimentarily divided into Langerhans cells (LCs), which are found in the epidermis, and the various DC subsets present in the dermis [31, 32] (Fig. 3a–c). LCs developmentally originate from the bone marrow (BM) but have the capacity to self-renew in the epidermis under steady-state conditions [33]. However, under inflammatory conditions, new BM-derived cells are recruited to the epidermis and differentiate into LCs [34]. LCs are characterized by the expression of CD207/langerin, epithelial cell adhesion molecule (EPCAM) and CD1a [32]. Both in mice and in humans, the dermis is populated by various subsets of DCs that are referred to as dermal DCs. Under steady-state conditions, murine dermal DCs are replaced approximately every 10–14 days by precursors from the BM [4]. In the context of inflammation, the dermal DC pool is additionally increased by recruited inflammatory monocytes that can differentiate into monocyte-derived DCs [4]. Simply spoken, dermal DCs can be divided into two subsets, depending on their expression of CD207/langerin and CD103, or the expression of CD11b [4, 35]. All skin-resident DC subsets can migrate to LNs, but the exact contribution of the individual subsets to immune responses is not yet completely understood [4, 35, 36].

Tools to study DC migration in vivo

Experiments to investigate the involvement of a gene product in DC migration to dLNs rely on the availability of knock-out mice or blocking antibodies. Traditionally, such experiments have involved the adoptive transfer of DCs into recipient mice [37–41], or the performance of so-called fluorescein isothiocyanate (FITC) painting experiments [16, 41–44]. In adoptive transfer studies, DCs are typically isolated from donor mice or generated in vitro from BM cultures. Upon fluorescent or radioactive labeling, DCs are injected into the skin, e.g., the footpad of a recipient mouse, and their arrival in the dLNs is analyzed and quantified 16–48 h later. In FITC painting experiments, on the other hand, FITC dissolved in dibutyl phthalate is applied onto the skin of mice. FITC is a contact sensitizer and rapidly penetrates the skin, leading to its uptake by skin-resident LCs and dermal DCs. The painting process also induces DC mobilization to dLNs, where DCs are subsequently quantified based on their green fluorescent signal. More recently, also confocal- and multiphoton-based time-lapse microscopy [45] has started to become a useful tool for elucidating DC migration via LVs. In contrast to the above-mentioned migration experiments, which address the overall involvement of a molecule in the migratory process, imaging studies allow the investigation of DC migration in situ and with cellular resolution. Consequently, imaging experiments identify the step in the migratory process a particular candidate gene participates in, i.e., whether this is in the migration towards, into, or within afferent LVs, or migration within

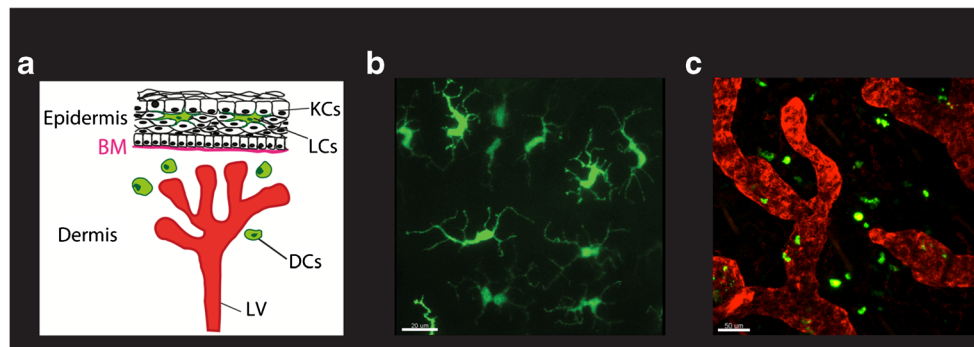


Fig. 3 Morphology of LCs and DCs in murine skin. **a** Schematic representation of the epidermis and dermis: Langerhans cells (LCs) and keratinocytes (KCs) are shown in the epidermis and dermal DCs and an afferent LV in the dermis. BM, basement membrane. **b**, **c** Visualization of **b** Langerhans cells (*green*) and **c** dermal dendritic cells (*green*) and LVs

(*red*) in the skin of transgenic mice expressing yellow-fluorescent protein (YFP) under the control of the CD11c promoter [108]. Confocal images were acquired in whole mounts prepared from murine ear skin, which were additionally stained with the LV marker LYVE-1 (*red*). Scale bars are 20 μm (**b**) and 50 μm (**c**)

dLNs. Time-lapse microscopy experiments to study DC migration via LVs have been performed *in vitro* in dermal tissue explants [12, 13, 17]. Moreover, intravital microscopy (IVM) experiments performed in the skin and LNs of anesthetized mice have been reported [43, 46–48]. Both setups rely on the simultaneous fluorescent visualization of LVs and DCs in the tissue. In the case of DCs, this has either been accomplished by imaging endogenously fluorescent DCs in transgenic reporter mice [43, 46, 47] or by imaging *in vitro*-generated fluorescent DCs after their injection into the tissue *in vivo* [43, 46, 48] or application onto dermal tissue explants *in vitro* [12, 13, 17]. Similarly, LVs have been visualized by performing imaging in gene-targeted mice expressing a fluorescent protein in LVs [43] or by labeling LVs *in situ* with injected fluorescent antibodies [12, 13, 17, 46–48].

Interstitial DC migration

The migratory behavior of DCs in the dermis appears to greatly differ from the one of LCs in the epidermis. This can likely be attributed to the vast differences in the cellular composition and density between these two compartments. LCs exhibit numerous dendritic processes that closely intermingle with neighboring epidermal keratinocytes [47, 49] (Fig. 3a, b), and a rate limiting step in LC migration appears to be their detachment from keratinocytes. The mechanisms that govern LC detachment are not well known, but this step was recently shown to depend on the expression of the adhesion molecule EPCAM [50] and the upregulation of CXCR4, which directs LCs towards dermal sources of CXCL12 [51]. Moreover, matrix metalloproteinases have been implicated in LC migration from the epidermis and across the dense epidermal basement membrane, which separates the epidermis from the dermis [52, 53]. Thus, LC migration in the epidermis appears to be largely regulated by adhesive interactions with neighboring cells, *i.e.*, with keratinocytes. By contrast, DC

migration in the dermis, which displays a much lower cellular density, is less restricted to cell–cell adhesive contacts. In fact, under steady-state conditions, DC migration from the dermis to the dLN was shown to occur completely independent of integrin-mediated cell adhesion [13]. DC migration in the dermis—but also in other tissues like SLOs—is generally described as an amoeboid-type of migration [54]. This type of movement predominantly occurs independently of the molecular composition of the tissue and allows the cell to migrate autonomously through the interstitial space, without the need of following preformed routes [54].

Molecules involved in amoeboid DC migration

Rather than utilizing adhesive interactions, amoeboid movement is achieved by “squeezing and flowing” of the actin cytoskeleton [54]. This process is generally characterized by two events taking place at the cellular front and rear: while F-actin polymerization at the front leads to the formation of protrusions in forward direction, actomyosin-mediated contractions in the rear support uropod detachment, nuclear contraction, and propulsion of cytoplasmic matter [54]. Actin polymerization is mediated by Rac1 and Rac2, and DCs deficient in the latter enzymes were shown to display a rounded up phenotype and were unable to migrate from skin to dLNs *in vivo* [55]. More recently, other molecules involved in actin cytoskeleton remodeling have been shown to be important for DC migration *in vivo*, namely the Rho GTPase family member *cdc42* [56], the actin capping protein *eps8* [57], and the Wiskott–Aldrich syndrome protein [58]. Actomyosin-mediated cellular contraction, on the other hand, is particularly relevant when cells need to squeeze through narrow openings, as present in the ECM, or for transmigration through endothelial cell–cell junctions, which require contraction of the cell’s bulky nucleus [59]. This process is mainly driven by members of the non-muscle-myosin II family. Although several kinases reportedly regulate non-muscle myosin II activity

[59], so far only the Rho-associated protein kinase (ROCK) was shown to impact DC migration: IVM experiments performed by our group revealed that pharmacologic blockade of ROCK significantly reduced the velocity of interstitial migration of DCs in ear skin and the overall process of DC migration to dLNs [43].

Insights from IVM

Important new insights into the behavior and migration of DCs in the skin have recently emerged from IVM experiments [47, 49, 60]. These analyses have, for instance, revealed that under steady-state conditions, LCs are immotile in comparison to the significantly more motile dermal DCs [47, 49]. In contrast to the numerous dendritic processes of LCs, dermal DCs display fewer but thicker dendrites, which they use to actively probe the environment [47, 60]. One IVM study reported that upon encountering a pathogen (*Leishmania major*) or a danger stimulus (LPS), dermal DCs rapidly became immotile, retracted their dendritic processes, and only regained motility after 6–8 h [60]. By contrast, another study reported that DCs became more motile as early as 20 min after exposure to a contact sensitizer or to various adjuvants and started to migrate directedly towards LVs [47]. LC mobilization in response to inflammatory stimuli or physical trauma, on the other hand, appears to only occur 24–48 h after stimulation [47, 49]. This is in line with recent studies demonstrating that, upon stimulation with contact sensitizers, LCs arrive at significantly later time points in dLNs as compared to dermal DCs [49, 61]: While the arrival of dermal DCs peaked around 24 h after sensitization, LCs only started to arrive several days after sensitization [49, 61].

Role of CCL21

Time-lapse microscopy has also contributed to a better understanding of how interstitially migrating DCs approach LVs. Specifically, time-lapse microscopy experiments revealed that the directedness, but not the velocity of interstitial DC migration, was dependent on G-protein-coupled receptor signaling [60], in particular on CCR7 [17, 46]. Thus, while wild-type DCs moved directedly towards LVs, CCR7^{-/-} DCs only rarely reached LVs by random migration [17, 46]. Current data regarding the pattern of CCL21 distribution and DC chemotaxis towards LVs are still somewhat controversial. One recent study reported on the existence of punctuate extracellular CCL21 deposits in the perilymphatic basement membrane, which DCs reportedly used as docking sites prior to intravasation into LVs [46]. Other findings suggest that extracellular CCL21 displays a rather diffuse staining pattern, which largely co-localizes with and surrounds LVs [17] (Fig. 2a). Interestingly, stainings performed under permeabilizing conditions have revealed that a great

proportion of CCL21 is present within LECs, in intracellular stores that belong to the trans-Golgi network [16, 17, 62] (Fig. 2b). In support of the existence of a peri-lymphatic CCL21 gradient, ex vivo time-lapse microscopy revealed that DCs in vicinity of less than 100 µm of a LV started to increase the directedness of their migration towards the LV [17]. Moreover, the latter study showed that directed DC migration towards LVs depended on the immobilization of CCL21 on heparan sulfates, which are present on the LEC surface or in the surrounding basement membrane: treatment of dermal tissue explants with heparitinase abolished the perilymphatic extracellular CCL21 staining pattern and eliminated directed migration of DCs towards LVs [17]. Similarly, DC migration to dLNs was shown to be reduced in mice with an endothelial-specific defect in heparan sulfate synthesis, further indicating that heparan sulfates are important for the establishment of a peri-lymphatic CCL21 gradient [22].

Role of other molecules

Besides CCL21, also CCL19, the other known ligand of CCR7, has been implicated in DC migration to dLNs. In contrast to CCL21, CCL19 lacks a positively charged C-terminal moiety and therefore is exclusively found in a soluble form in tissues [21]. Interestingly, CCL19 does not appear to be expressed in LECs, but is produced by activated DCs. It has been suggested that, due to interstitial flow, DC-produced CCL19 might act in an autocrine manner by directing DC migration into the direction of fluid drainage, i.e., towards draining LVs. However, this concept of “autologous chemotaxis” [41] still awaits conclusive in vivo proof. While an early study observed that antibody-mediated blockade of CCL19 reduced DC migration from skin to dLNs [63], no migration defect was recently reported for CCL19-deficient mice [64]. Another chemoattractant with a well-documented role in DC migration via LVs to dLNs is the endogenous lipid mediator sphingosine-1-phosphate (S1P). S1P is well known for its indispensable role in directing lymphocytes egress from LNs into efferent LVs [10]. Maturation was shown to upregulate S1P receptors (S1PRs), in particular S1PR₁ and S1PR₃, in DCs, and to induce their chemotaxis towards S1P [65, 66]. Moreover, S1P receptor 1 (S1P₁)^{-/-} DCs as well as DCs in mice treated with the S1P analog FTY720 displayed reduced migration from skin to dLNs [65, 66]. In LNs, LECs have been identified as an important source of S1P, thereby establishing the S1P gradient between nodal tissue and lymph, which guides egressing lymphocytes [67]. Although not formally shown, it is conceivable that a similar gradient exists between the interstitial space in peripheral tissues and lymph and therefore could support directed DC migration towards afferent LVs. Alternatively, S1P could be important for keeping DCs within LVs, once they have transmigrated into the LV lumen.

DC transmigration across lymphatic endothelium

DC transmigration across lymphatic endothelium has traditionally been investigated in *in vitro* experiments performed with cultured LEC monolayers. In a typical setup, LECs are grown to confluence in transwell inserts and DC transmigration towards a chemotactic stimulus present in the lower well is assessed [42, 44, 62, 68]. A general drawback of *in vitro* transmigration experiments might be the fact that LEC monolayers *in vitro* form continuous, zipper-like cell–cell junctions that resemble the junctional arrangement in lymphatic collectors [14] (Fig. 1b), whereas DC immigration into LVs reportedly occurs through the button-like cell junctions present in LV capillaries [12, 14]. Nevertheless, *in vitro* assays have identified various molecules involved in the transmigration process, namely the LEC-expressed adhesion molecules CD31 [69], CD99 [69], VCAM-1 [42], ICAM-1 [42], and L1CAM [44] (Fig. 4b). Many of these molecules were subsequently validated in *in vivo* migration experiments [42, 44] or in DC crawl-out experiments performed with human skin explants [69, 70].

Insights from IVM

Also DC transmigration into lymphatic capillaries has recently been investigated by time-lapse imaging experiments. Experiments performed in murine ear skin explants [12] have revealed that DCs first need to penetrate the thin and discontinuous basement membrane that surrounds lymphatic capillaries. Transmigration preferentially occurs through preexisting pores. These pores get widened during the transmigration process, thereby facilitating the subsequent passage of more DCs through the same portal [12]. Once in direct contact with the lymphatic endothelium, DCs transmigrate into the LV lumen by passing through the characteristic flaps present between oak leaf-shaped LECs in lymphatic capillaries (Fig. 1b). The flaps generate openings of approximately 2–3 μm in diameter, which DCs appear to traverse without altering the “button”-like pattern of tight junctions between neighboring LECs [12, 14]. Besides experiments performed in skin explants, DC transmigration into the LVs has also been investigated by IVM: upon arrival at the LV, DCs were observed to dock and probe the vessel surface for several minutes, followed by transmigration into the vessel lumen [46, 47]. Also during IVM, DCs were frequently observed to successively transmigrate at the same point into the LV lumen, possibly through the same portal and flap [43, 46]. Furthermore, IVM experiments revealed that the entire transmigration process takes between 30 and 60 min [43, 46, 47]. It is currently not clear whether DCs may also enter LVs *in vivo* by migrating through zipper-like junctions of lymphatic collectors. Notably, the basement membrane of collecting vessels was shown to be thicker and less fenestrated [12], which

additionally might render transmigration into collectors more difficult.

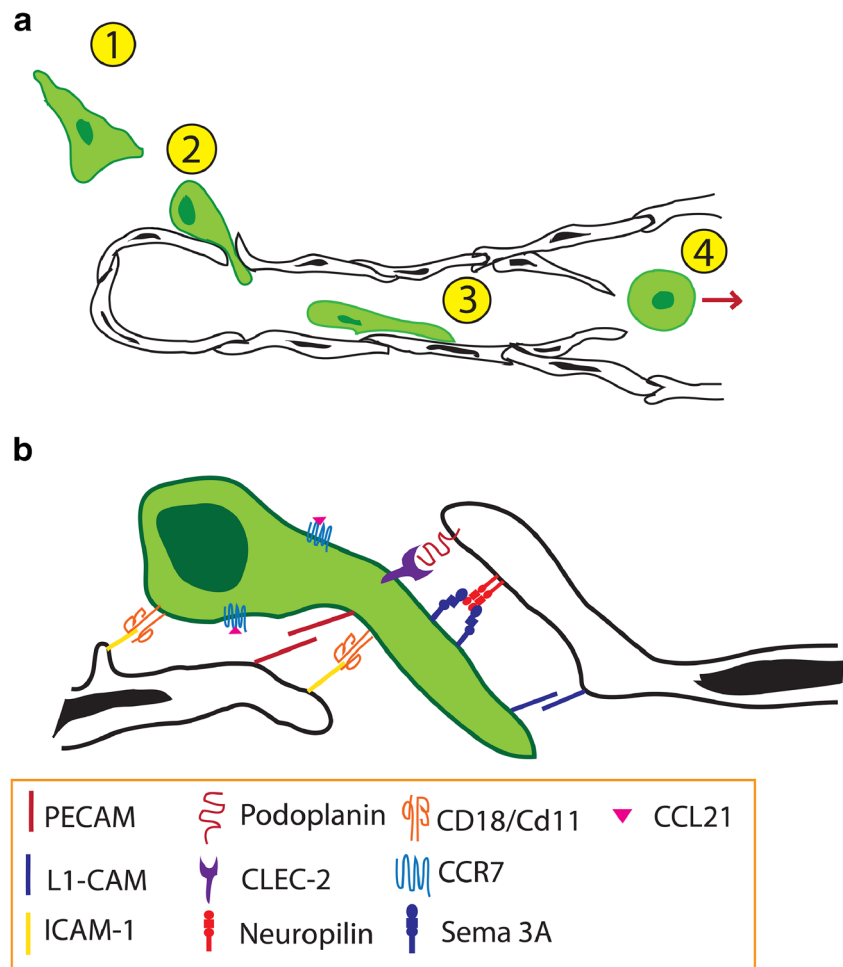
Involvement of integrins

In agreement with the fact that migration of adoptively transferred, integrin-deficient DCs from uninflamed skin to dLNs was not compromised [13], time-lapse microscopy experiments did not detect any defect in the entry process of integrin-deficient DCs into afferent LVs [13]. Thus, under uninflamed, steady-state conditions, DC entry into LVs does not require integrins. Intriguingly, various studies have, however, revealed that in the context of inflammation, DC and LC migration was compromised in mice deficient for ICAM-1, the ligand of the DC-expressed integrins Mac-1 ($\alpha_M\beta_2$) and LFA-1 ($\alpha_L\beta_2$), or in mice treated with ICAM-1 or LFA-1 blocking antibodies [39, 42, 71]. These controversial findings most likely are explained by the fact that LECs in uninflamed, steady-state skin express extremely low levels of ICAM-1 and VCAM-1, whereas these molecules are strongly upregulated in the context of inflammation [16, 43]. Indeed, performing a side-by-side comparison of DC migration from inflamed and from uninflamed tissues, a recent study demonstrated that blockade of LFA-1 only reduced DC migration from inflamed but not from uninflamed skin [39]. Notably, in contrast to LECs found in uninflamed skin *in vivo* [16, 43], *in vitro* cultured LECs express high basal levels of VCAM-1 and ICAM-1, which are further induced upon treatment with inflammatory cytokines [42]. *In vitro* studies also revealed that ICAM-1 and VCAM-1 form enriched structures in inflamed LECs, which facilitated the adhesion and transmigration of DCs across the lymphatic endothelium [39].

Further molecules involved in transmigration

Although afferent lymphatic capillaries contain open flaps, which are thought to facilitate leukocyte entry and fluid flow, the lymphatic vasculature nevertheless appears to pose a physical barrier to immigrating leukocytes. Similarly to extravasation out of BVs, also DC transmigration into LVs appears to depend on actomyosin-mediated contraction of the cell's bulky nucleus [40, 43, 72]. This process could on one hand be needed for squeezing through the narrow pores in the basement membrane that surrounds LVs [12] (Fig. 1a). Alternatively, nuclear contraction could be required for the passage through the endothelial flaps, where the approximate diameter of 2–3 μm likely still represents a barrier that cannot be passed without active cellular contraction [59, 73] (Fig. 1b). In support of this assumption, the axonal guidance molecule semaphorin 3A (Sema3A), which is highly expressed in LVs but not in BVs, was recently shown to mediate DC transmigration across lymphatic endothelium [72]. The authors demonstrated that Sema3A production in

Fig. 4 Model of DC migration towards, into, and within afferent LVs. **a** (1) DCs approach LVs in a CCR7/CCL21-dependent manner. (2) Transmigration across the lymphatic endothelium occurs through the characteristic flaps present in lymphatic capillaries. (3) Once within lymphatic capillaries, DCs actively crawl towards downstream collecting vessels, (4) from where they are passively transported to the dLN by flow. **b** Schematic summary of molecules shown to mediate DC migration across lymphatic endothelium



LECs promoted actomyosin-mediated cellular contraction in DCs, thereby enhancing DC transmigration across lymphatic endothelium. Consequently, DC migration to dLNs was significantly reduced in *Sema3A*^{-/-} mice or in mice deficient in plexin-A1, which together with neuropilin-1 forms the Sema3A receptor on DCs [72]. On the other hand, also the C-type lectin CLEC-2, which is upregulated on mature DCs, was shown to promote actin cytoskeleton rearrangements and to support DC transmigration into LVs [40]. The cellular ligand of CLEC-2 on LECs during this process is the small transmembrane glycoprotein podoplanin, a well-known lymphatic marker gene [6]. CLEC-2 deficiency impaired DC migration on podoplanin substrates in vitro, as well as DC migration into LVs and to dLNs in vivo [40].

Involvement of CCL21

Besides attracting DCs towards LVs, CCL21 has also been implicated in DC migration on cellular substrates and hence in transmigration across lymphatic endothelium. CCL21 immobilized on the surface of FRCs was recently shown to support haptotactic movement of DCs by triggering integrin-

mediated adhesion [74]. Moreover, CCL21 was shown to enhance DC transmigration across in vitro-cultured lymphatic endothelium in an ICAM-1-dependent manner [62]. However, given the fact that LECs in vivo in uninflamed tissues express much lower ICAM-1 levels, the supportive role of CCL21 in mediating DC transmigration across ICAM-1-expressing LECs is likely only relevant in the context of tissue inflammation.

Impact of inflammation-induced changes in LECs on DC migration

In addition to inducing the upregulation of the integrin ligands ICAM-1 and VCAM-1, tissue inflammation and inflammatory mediators have also been shown to induce the expression of CCL21 and other chemokines in LECs [16, 37, 39, 42, 62]. Furthermore, it was shown that increased lymph flow, which typically accompanies inflammatory processes, can upregulate CCL21 and ICAM-1 levels in LECs [68]. It has been suggested that an inflammation-induced increase in CCL21 expression could contribute to the enhancement of DC migration, which is typically observed in the context of

inflammation [16, 37, 62]. Indeed, *in vitro* studies have revealed that inflammatory cytokines like TNF α enhance the secretion of CCL21 from its intracellular stores [62], but is still not clear how tissue inflammation affects CCL21 secretion *in vivo*. When comparing DC migration in two different models of tissue inflammation, our group recently observed that a higher upregulation of lymphatic CCL21 and ICAM-1, which was observed in one of the models, did not actually correlate with a higher induction of DC migration [16]. Thus, it seems more likely that the ability of an inflammatory stimulus to promote DC maturation and to increase the DC's responsiveness for CCR7 ligands is the rate-limiting factor in DC migration to dLNs.

Although many other chemokine are upregulated in LECs in the context of tissue inflammation [16, 42], only CXCL12 [75] and CX3CL1 [38] have thus far been implicated in DC migration to dLNs. Inflammation-induced CXCL12 was shown to enhance the migration of CXCR4-expressing dermal DCs and LCs to dLNs [75]. Moreover, LEC-expressed CX3CL1 (fractalkine) reportedly supports DC transmigration across lymphatic endothelium and the overall trafficking process from inflamed tissue to dLNs [38]. On the other hand, experiments performed with CCR7^{-/-} DCs also have revealed that in the context of tissue inflammation DC migration to LNs remains highly CCR7-dependent [16]. Overall, these data indicate that CCR7 remains the key mediator of DC migration during inflammation, but that other chemokines like CXCL12 and CXCL3 contribute to this process, possibly by affecting distinct steps in the migration cascade.

Intralymphatic DC migration

Recent IVM experiments from three different groups, including our own, have revealed that DCs that have transmigrated into lymphatic capillaries are not passively transported away by lymph flow, but actively crawl inside lymphatic capillaries [43, 46, 47]. These findings contrast with previous IVM data from rat mesenteric lymphatic collectors; in these experiments, leukocytes were observed to be passively propagated as rounded-up cells [76, 77]. Different from mesenteric collecting LVs, in which peak flow velocities of several millimeter/second can be reached [78, 79], intralymphatic flow velocities in capillaries reportedly range from 1 to 30 $\mu\text{m/s}$ [80, 81]. The observation that DCs actively crawl in lymphatic capillaries therefore suggests that the intracapillary hydrodynamic forces might not be sufficient to support passive drainage of cells right upon entry into the blind-ended capillaries. IVM also revealed that DCs detach and flow once within collecting vessels [43, 46], in line with the notion that lymph flow is stronger in collecting vessels. On the other hand, detachment in collectors could also be attributed to differences in adhesion molecule expression between

the capillary and the collecting vessel bed, respectively. In support of this hypothesis, we have observed by FACS analysis performed on skin single cell suspensions that ICAM-1 levels were consistently higher in LECs from lymphatic capillaries as compared to LECs from lymphatic collectors (data not shown). Although intralymphatic DCs displayed an overall movement in the direction of downstream LNs, IVM studies indicated that intralymphatic DCs frequently change direction and also crawl for some time in the opposite direction of the presumed lymph flow [43, 46]. It is presently not clear what determines the directionality of DC migration within lymphatic capillaries. In agreement with a previous study [46] we observed that upon PBS injection into the skin, to artificially enhance lymphatic flow, DCs migrated more directed within lymphatic capillaries [43]. However, in addition to more directed migration in downstream direction (i.e., towards the dLN), DCs also migrated more directed into the opposite, upstream direction, resulting in no net improvement of DC migration into the downstream direction of the dLN [43]. Thus, it is presently not clear to which extent directionality of intralymphatic DC crawling is determined by lymph flow or whether other signals, such as for example chemical cues deposited within LVs, might play a role in this process.

Molecules involved

Thus far, intralymphatic crawling of DCs has only been scarcely studied at the molecular level. Performing IVM, we have identified a role for ROCK in DCs in this process. As previously mentioned, ROCK initiates actomyosin-mediated nuclear contractions, which are important when DCs migrate through narrow openings, as present in the ECM of the interstitium *in vivo* [43]. Moreover, ROCK was also shown to mediate leukocyte crawling within BVs, since it facilitates de-adhesion of integrins from their endothelial cell-expressed ligands, in particular from ICAM-1 [73]. Interestingly, intralymphatic DC crawling was only weakly ROCK-dependent under steady-state conditions. By contrast, blockade of ROCK profoundly inhibited intralymphatic DC migration in the context of tissue inflammation, likely by inhibiting de-adhesion from inflammation-induced ICAM-1 [43]. The fact that under steady-state conditions integrin ligands like ICAM-1 are only weakly expressed in LVs [16, 43] and that intralymphatic crawling was only weakly ROCK dependent [43] supports the notion that also intralymphatic DC migration under steady-state conditions does not require integrins [13]. Nevertheless, it is well possible that other LEC- and DC-expressed cell surface molecules support intralymphatic DC adhesion and crawling: in this regard, molecules such as Sema3A and podoplanin, which thus far have mainly been implicated in the transmigration process [40, 72], could be interesting candidates. Overall, the observation that transmigrated DCs first crawl in lymphatic capillaries and

are only later on passively drained by flow indicates that this migratory step is more complex than previously appreciated.

DC migration across the lymphatic sinus of dLNs

Only few studies have thus far investigated the arrival and migration of DCs in dLNs. Experiments performed in plt mice, which lack expression of CCR7 ligands in LNs, have revealed that the process of DC migration into the LN T cell area is highly dependent on the expression of CCR7 ligands by FRCs of the T cell zone [25, 26]. DC translocation into the T cell area was also shown to be integrin independent, since adoptive transfer of WT and integrin-deficient DCs into the skin resulted in no differences in the localization and number of cells in the T cell areas of the dLN [13]. Further insights into the entry process of DCs into the nodal tissue have recently come from IVM experiments performed in popliteal LNs, which drain the skin of the footpad [48]. Upon injection into afferent LVs, DCs were observed to penetrate into the LN by crossing lymphatics at the level of subcapsular sinus. Surprisingly, this was in contrast to injected CD4 T cells, which preferentially entered the node across medullary sinuses [48]. The entrance and migration towards the T cell area was observed to completely depend on CCR7 expression [48]. Similarly, *in vitro* time-lapse microscopy performed on LN sections revealed that DCs migrated in a CCR7-dependent, directed manner from the LN periphery towards the T cell area [74]. CCL21 deposited on FRCs supported haptotactic DC migration, but the directionality of DC movement was determined by soluble chemokine gradients. Interestingly, the latter study also revealed that DCs in contact with surface-bound CCL21 can enzymatically cleave off its C-terminal heparan sulfate-binding moiety, thereby generating a soluble form of CCL21 [74]. While surface-bound CCL21 enhances cell migration along fibers or cellular scaffolds by triggering integrin activation and cell adhesion, truncated CCL21 can form soluble gradients that contribute to DC chemotaxis [74].

Inflammation is known to increase DC migration to dLNs [16, 37]. Interestingly, recent evidence suggests that this process might not only be regulated by changes occurring in DCs and in afferent LVs but also by modulation of the lymphatic network in the dLN. In particular lymphangiogenesis occurring in the SCS of LNs draining sites of inflammation was shown to partially account for the increase in DC migration observed in the context of inflammation [82, 83]. In a model of inflammation induced by CFA injection into the skin, DC accumulation in dLNs could be significantly reduced by anti-lymphangiogenic treatment [82]. DCs that have migrated from a peripheral tissue to a dLN typically remain in this node and do not exit via efferent LVs or migrate to further dLNs [11, 48]. Besides their prominent role in antigen presentation, also additional functions have recently been identified for DCs

within dLNs. Constitutive homing of DCs to dLNs was shown to contribute to LN homeostasis by controlling the phenotype of HEVs and the capacity of this specialized vascular bed to recruit lymphocytes [11, 84, 85]. In specific, activation of lymphotoxin beta receptor signaling in HEV endothelial cells by DC-expressed lymphotoxin was shown to induce the expression of functional trafficking molecules in HEVs, thereby contributing to lymphocyte homing [84].

Modulation of DC migration for therapy

Enhancing DC migration during vaccination

Over the last 20 years, there has been a great interest in targeting DCs for the development of therapeutic vaccines, in particular for the treatment of cancer [3]. Many of these vaccination approaches follow the classical vaccination scheme in which antigen and adjuvants are injected into the patient. At the same time, also many DC-based vaccines are currently being investigated [3]. The latter typically involve the generation of DCs from blood-derived monocytes or from CD34⁺ hematopoietic precursors, DC maturation and loading with tumor antigens, followed by DC injection into the patient [3, 86]. The first marketed cancer vaccine is Sipuleucel-T, a cell-based vaccine that was FDA-approved for the treatment of metastatic prostate cancer in 2010 [87]. Sipuleucel-T is generated from antigen-presenting cells derived from the patient's blood, which are cultured in presence of a fusion protein consisting of the DC maturation factor GM-CSF and the prostate cancer antigen prostate acid phosphatase. After culture, the cells are intravenously injected into the patient—a route of administration that is expected to preferentially target DCs to the spleen and peripheral organs like the liver or lung, but not directly to LNs [88]. Besides intravenous administration, other vaccination approaches involve injection of DCs into the skin [86, 88, 89] or directly into LVs or LNs [86], to enable immune priming in LNs. The route of administration continues to be a matter of debate and is thought to affect the quality of the T cell response induced as well as the trafficking behavior imprinted onto the effector T cells [86].

Imaging studies in patients injected intradermally with radiolabeled DCs have indicated that only a very small fraction of injected DCs, ranging from 1 to 8 %, typically arrives in dLNs [86, 89, 90]. This low number is similar to what is typically observed when performing adoptive transfer experiments in mice [37–39]. Consequently, several experimental approaches to enhance DC migration via LVs have recently been pursued. Given that the CCR7/CCL21 axis is of key importance for DC migration, a particular focus has been on enhancing CCR7 expression and CCR7 responsiveness in DCs. Lipid-based mediators, namely prostaglandin E2 (PGE-2) [91], leukotriene C4 [63], and leukotriene B4 [92],

have recently been shown to enhance DC migration to LNs, by increasing CCR7 expression levels and DC responsiveness for CCR7 ligands. In fact, one of the most commonly used maturation protocol for clinical grade DCs involves the incubation of monocyte-derived DCs in media supplemented with proinflammatory cytokines and prostaglandin E2 [93]. However, in contrast to the above mentioned studies, other studies have indicated that PGE-2 might negatively affect the immune-stimulatory capacity of DCs [94]. Interestingly, it was recently suggested that this might not be the case when maturing DCs in presence of leukotriene C4 [92]. Besides addition of lipid mediators, generation and maturation of DCs in presence of interferon alpha (IFN α) was shown to enhance DC migration in vitro and in vivo, by enhancing CCR7 expression and responsiveness, as well as DC adhesion and transmigration across lymphatic endothelium [95, 96]. Inclusion of IFN α during DC maturation has recently started to be investigated in clinical trials [97]. In addition to the manipulation of DCs to enhance their responsiveness for CCL21 ligands, also preconditioning of the skin prior to DC injection may augment DC migration to LNs. For example, in mice, inflaming the skin by local injection of TNF α was shown to enhance DC migration to dLNs, presumably by upregulating CCL21 expression in LVs [37]. Moreover, in mouse models as well as in human cancer patients, it was shown that induction of skin inflammation by topical application of the TLR7 agonist imiquimod prior to s.c. injection of immature DCs promoted DC maturation and subsequent migration to dLNs [98, 99].

Blocking DC migration to prevent transplant rejection

DC migration via LVs to dLNs may not only be a therapeutic target for boosting vaccination but also for modulating immunity in the context of transplant rejection [100]. Over the last 10 years, numerous studies have documented the involvement of inflammatory lymphangiogenesis in the graft rejection process [23, 83, 101, 102]. Blockade of lymphangiogenesis and, consequently, of the migration of graft-derived DCs to dLNs has been proposed as a potential approach for prolonging graft survival. This might be particularly attractive in the context of transplanted tissues like the cornea, which normally is avascular and only becomes infiltrated with blood and LVs under inflammatory conditions. In fact, in mouse models of cornea transplantation, blockade of lymphangiogenesis was shown to decrease DC migration from the graft to dLNs and to strongly increase allograft survival [101, 102]. Similarly, anti-lymphangiogenic treatment with sunitinib or VEGFR-3-blocking Abs promoted survival of pancreatic islets allografts transplanted under the kidney capsule [103]. Besides blocking lymphangiogenesis, also direct manipulation of DC trafficking from the allograft, by targeting the CCL21/CCR7 axis, has been investigated as a

potential strategy for prolonging graft survival. For example, in plt mice, the survival of islet allografts implanted under the kidney capsule was significantly prolonged [104]. Similarly, when islets allografts were cultured in medium containing CCL21 prior to transplantation, to induce the emigration of DCs from the graft, allograft survival was nearly doubled [105]. Moreover, treatment of mice with CCL19-Ig fusion protein enhanced the survival of kidney and cardiac allografts, likely by perturbing the migration CCR7-expressing DCs and T cells into SLOs as well as their co-localization within SLOs [106]. On the other hand, no prolongation of graft survival was observed in the context of cardiac allografts transplanted into plt mice or of islet allografts implanted into the liver of plt mice [104]. Thus, overall, approaches targeting DC migration have yielded varying results, depending on the type of organ transplanted and the site of grafting. An important confounding factor in these studies likely is the fact that LVs are not the only route by which DCs can emigrate from transplanted tissues: graft-derived antigen-presenting cells were also shown to induce alloresponses in the spleen, upon exiting the graft via BVs [100, 105, 107]. Therefore, blockade of DC migration via LVs will only prove to be a therapeutically useful strategy for those transplant situations, in which DC trafficking via lymphatics indeed represents the prime route for host sensitization.

Conclusions

Over the past 15 years, major progress has been made in our understanding of DC migration from peripheral tissues to dLNs. It is very clear by now that DC migration via lymphatics is not a random event but a tightly regulated process, in which CCL21/CCR7 signaling plays a dominant role. In fact, the CCR7/CCL21 axis was shown to be important for almost every step in DC migration via LVs; namely for DC migration towards and into afferent LVs and also for DC entry into the dLN and migration into the nodal T cell area. Another important recent insight to the field has been the discovery that DC migration via LVs in steady-state does not require integrins. By contrast, integrins are involved in DC migration in the context of inflammation, when integrin-ligands are induced on lymphatic endothelium. Most of the recent advances in our understanding of DC migration via LVs have come from in vitro and in vivo imaging experiments. The latter make it possible to study DC migration in situ and with cellular resolution. Besides many other new insights, IVM has revealed that DCs, which have entered into afferent LVs, actively migrate within lymphatic capillaries and are only passively transported by flow once they reach lymphatic collectors. In the future, it will be relevant to understand whether this behavior is only dictated by the hydrodynamic conditions in the vessel or whether intralymphatic DC

migration might have further biologic significance. Overall, the recent findings have revealed that DC migration via LVs is a by far more complex process than previously assumed. A major challenge for the years to come will be to optimally use the emerging knowledge about DC migration for the improvement of immune-modulatory therapies.

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