



Initial afferent lymphatic vessels controlling outbound leukocyte traffic from skin to lymph nodes

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Tissue drains fluid and macromolecules through lymphatic vessels (LVs), which are lined by a specialized endothelium that expresses peculiar differentiation proteins, not found in blood vessels (i.e., LYVE-1, Podoplanin, PROX-1, and VEGFR-3). Lymphatic capillaries are characteristically devoid of a continuous basal membrane and are anchored to the ECM by elastic fibers that act as pulling ropes which open the vessel to avoid edema if tissue volume increases, as it occurs upon inflammation. LVs are also crucial for the transit of T lymphocytes and antigen presenting cells from tissue to draining lymph nodes (LN). Importantly, cell traffic control across lymphatic endothelium is differently regulated under resting and inflammatory conditions. Under steady-state non-inflammatory conditions, leukocytes enter into the lymphatic capillaries through basal membrane gaps (portals). This entrance is integrin-independent and seems to be mainly guided by CCL21 chemokine gradients acting on leukocytes expressing CCR7. In contrast, inflammatory processes in lymphatic capillaries involve a plethora of cytokines, chemokines, leukocyte integrins, and other adhesion molecules. Importantly, under inflammation a role for integrins and their ligands becomes apparent and, as a consequence, the number of leukocytes entering the lymphatic capillaries multiplies several-fold. Enhancing transmigration of dendritic cells en route to LN is conceivably useful for vaccination and cancer immunotherapy, whereas interference with such key mechanisms may ameliorate autoimmunity or excessive inflammation. Recent findings illustrate how, transient cell-to-cell interactions between lymphatic endothelial cells and leukocytes contribute to shape the subsequent behavior of leukocytes and condition the LV for subsequent trans-migratory events.

Keywords: dendritic cell, T cell, lymphatic vessel, migration, inflammation

INTRODUCTION

The lymphatic vascular system is composed by a one-direction system of conduits interrupted by lymph nodes (LN) that run in parallel to the blood vascular system.

Up to 50% of the protein that extravasates from blood vessels is reabsorbed by the lymphatic network (1). Besides, lymphatic vessels (LVs) in the gut are also devoted to the transport of absorbed lipids from the diet. One of the main functions of LVs is to conduit immune cells from tissues to the LN. This last function of the LVs constitutes the focus of this review. The cellular mechanisms and molecules involved in leukocyte transit across blood vessels have been studied in more detail and are generally assumed to be similar in the LVs (2). However, it must be stressed that although there are partial parallelisms in cell transit across these two types of vasculature, the diverse structure of the lymphatic capillaries may explain non-overlapping trans-migratory mechanisms. Even more, these peculiarities offer us new opportunities for selective therapeutic intervention to modulate leukocyte transit across the lymphatic capillaries.

The majority of the leukocyte populations that travels via the lymphatics to the LN are CD4⁺ lymphocytes, including effector memory and regulatory T cells (3–5). Myeloid cells also use

the same conduits (6, 7), the majority of them being dendritic cells (DC) (8, 9). Myeloid cells are present in the lymph in lower amounts in homeostasis respect to T lymphocytes but their quantities significantly increase under inflammatory conditions.

Dendritic cells are key elements of the adaptive immunity that patrol peripheral tissues in search of pathogens or damage signals. Their main mission is to recognize and process foreign antigens in peripheral tissues and ferry them to the LN where they are presented to naïve T cells and then trigger an effective immune response.

In consequence, DC traffic toward LN via LVs has been extensively studied by immunologists in the recent years. Due to their abundance in the skin and its accessibility, DC transit in this tissue in homeostasis and inflammation has been the model used for most of the experimental studies. DCs from the skin include epidermal resident CD206⁺ Langerhans cells as well as dermal resident DC. Importantly, both cell types migrate at different times to the LN after contacting pathogen [for review Ref. (10)]. DC migration to the LN under steady-state conditions is constant and occurs at modest intensity (11) being instrumental to preserve peripheral tolerance to self-antigens (12–15). In contrast, under inflammation, DC migration toward the LN is significantly

increased in response to chemotactic signals induced by inflammatory products (16). The amount of DC and specific subpopulation of antigen presenting DC (APC) entering into in the LN from peripheral tissues is important not only to elicit but also to sustain proper adaptive immune responses against pathogens. Therefore, the existence of regulation mechanisms for leukocyte-egress routes from the peripheral tissue is reasonable. Such mechanisms as consequence regulate leukocyte entrance into LN (16).

LYMPHATIC VESSELS UNDER STEADY-STATE AND PRO-INFLAMMATORY CONDITIONS

Leukocyte entry into the LVs is determined by the peculiar morphological features of these vessels: they are endowed with an intermittent basement membrane and their intercellular junctions are dispersed in button-like structures that leave small flaps of loose overlapping membrane extensions between individual LEC (17). Further, the LVs are attached to the extracellular matrix by anchoring fibers (18, 19) that stretch when tissue volume increases and lead to the opening the inter-endothelial flaps (19). It has been firmly established how under steady-state conditions DCs are able to penetrate into LVs via the preexisting pores (portals) of their basal membrane and subsequently migrate into LVs through inter-endothelial cell openings. This migration occurs in a process guided by chemokine gradients and mediated by contractions of the actin cytoskeleton, but is independent from integrin engagement, as it was shown in experiments performed in mice whose traceable DC are devoid of all integrins as bona fide pan-integrin knock-out DC mice (20).

It has recently been reported that during chronic inflammation or extensive lymphangiogenesis, there exists a transformation of the dispersed button-like adhesive structures of the mature lymphatic capillaries into ones more restrictive for cell transit featuring zipper-like contact adhesions similar to those present in collecting LVs and in blood capillaries. Importantly, this transformation is reversible and dependent on the activation of the glucocorticoid receptor by its phosphorylation (21). In fact, additional findings supported these observations. For example, it was reported how inflammatory cytokines (22) and pathogen-associated patterns (23) can promote VEGF-C production by the stromal cells and induce the formation of new LVs (lymphangiogenesis). In these models, inhibition of signaling across its receptor VEGFR-3 impaired the resolution of inflammation while its activation attenuated edema and induced the sprouting of new LVs (24–27). Besides, it has been extensively reported how increments in LVs facilitate the local resolution of the immune and inflammatory responses by augmenting DC transit across their boundaries (27, 28).

In line with this experimental evidence, it has been demonstrated how inflammatory mediators such as TNF α induce the up-regulation of integrin ligands on LVs surface such as VCAM and ICAM-1 and induce changes in the secretion of chemotactic cytokines both in *in vitro* and *in vivo* settings (29, 30). CCL21 is the main cytokine that drives DC migration to the LVs and its expression by LVs is strongly up-regulated upon exposure to pro-inflammatory cytokines such as TNF α (31). Nevertheless, other cytokines such as IFN γ limit LV proliferation (32) suggesting that transit of DCs across the lymphatic boundaries seems

to be a phenomenon highly controlled by inflammatory mediators, although the precise molecules at work in each situation seem to vary. Indeed, previous reports from Vigl et al. showed how different models of inflammation (i.e., contact hypersensitivity, CHS induction, or CFA injection) lead to diverse changes in LEC phenotypes in a stimulus-dependent manner (30). Other reports have demonstrated that the characteristic increment in transmural lymph flow that accompanies inflammation also results in greater CCL21 cytokine expression and leukocyte transmigration across LEC (33). Besides, to further support the role of this cytokine in DC migration Tal and co-workers have recently demonstrated by *in vivo* time-lapse microscopy that DC not only ingress the initial lymphatics through basal membrane deprived portals located in close proximity to CCL21 depots, but once inside the vessel these leukocytes crawl directionally on the luminal-side of the capillary. To crawl DC advance extending filopodia at their leading edges and retracting uropods formed at their rear end (34). These cells moved in a way that much resembles the inflammation-mediated integrin crawling of leukocytes inside the lumen of blood capillaries before their extravasation into tissues (35).

Peripheral inflammation is also able to promote effects in distant LN. Reports from Kinder and co-workers show how peripheral activated mast cells release micro particles that contain TNF α and other vasoactive mediators that facilitate leukocyte-egress toward the LN and induce the lymphangiogenesis of the lymphatic sinusoids in secondary lymphoid organs (36, 37). This new mechanism results in long distance-education of draining LN for the eventual reception of activated leukocytes (38, 39).

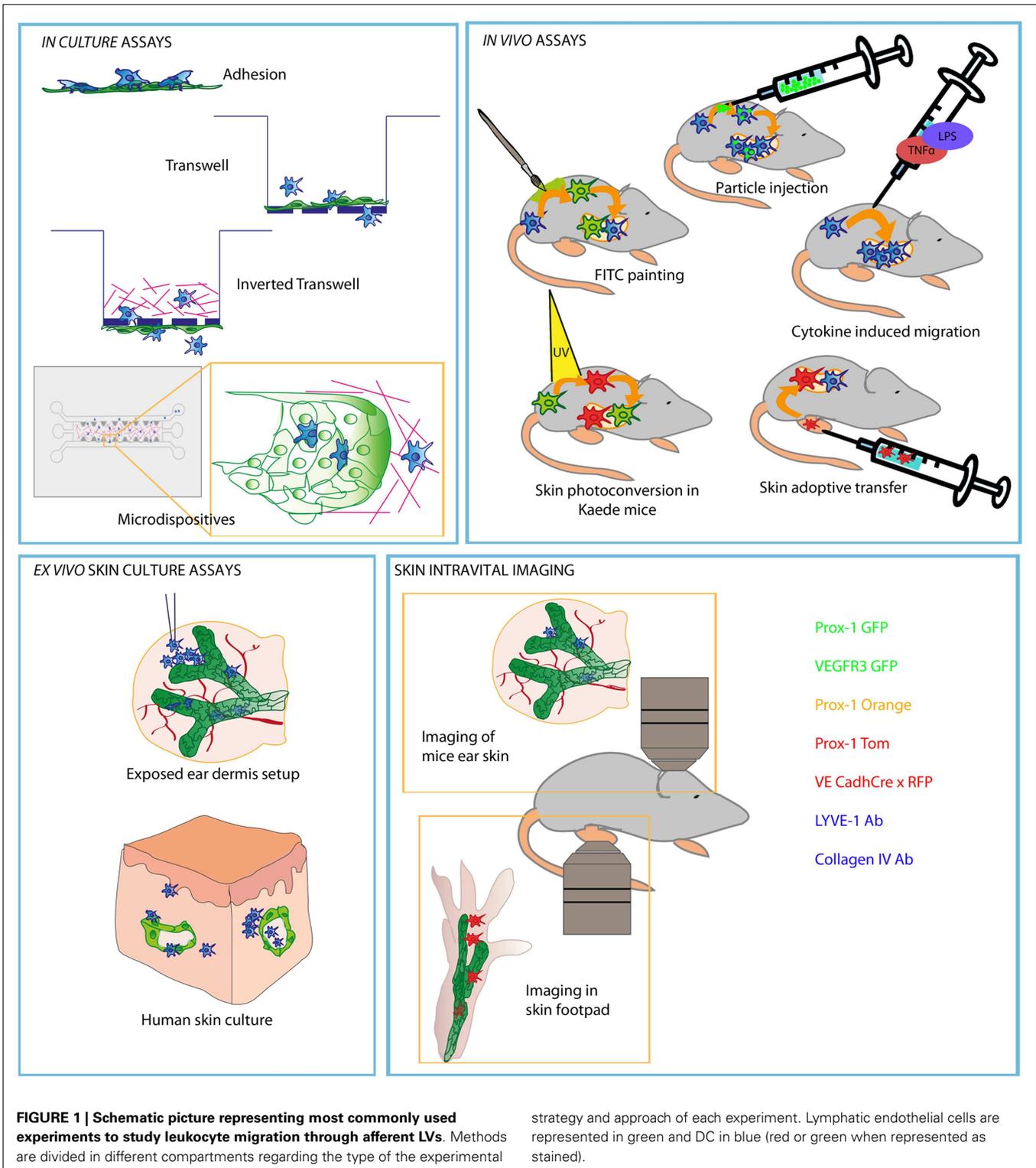
In addition to APC and effector T lymphocytes, T cells that infiltrate healthy or inflamed tissues may differentiate into a memory subset that expresses CCR7 and recirculates to LN (40–42). Similarly to DC, the arrival of antigen experienced T lymphocytes to LN is critical to regulate the intensity of immune responses. It is clear that there is much more detailed information regarding the mechanisms that drive the entrance of leukocytes to tissue to form inflammatory infiltrates than those that govern egress from tissue via afferent LVs. The most actively studied lymph-traveler leukocytes are DCs. However, memory T cell traffic for recirculation is also quite important and its understudied molecular mechanisms deserve much future attention, as is also the case with polymorphonuclear leukocytes (PMN).

Polymorphonuclear leukocytes upon acute inflammation are routed to LN via the lymph (43, 44) with potential to ferry antigens and pathogen-associated patterns (45) and regulate antigen presentation at the LN (43). Besides, DCs which have engulfed PMN also migrate via afferent LVs and may deliver PMN associated antigenic material to LN resident DC (45).

The experimental methodology used so far for the assessment of the interplay between leukocytes and LVs is quite similar to those in use for many years to study cell transit across blood capillaries and are being described next.

CURRENT EXPERIMENTAL MODELS TO STUDY LEUKOCYTE TRANSIT ACROSS LYMPHATIC VESSELS

Much of our current view on leukocyte transit across different lymphatic vascular beads depends on the experimental setting used. A graphical summary of such approaches is shown in **Figure 1**.



ENDOTHELIAL CELL CULTURES

Human LECs can be purified based on the expression of specific markers (46) and are also commercially available as primary cells purified from human tissues such as dermis or lung.

In this line, it is worth mentioning that LEC present different biological properties depending on their original tissue niche (47). From them, immortalized mouse LEC cell lines have been derived. The cell line most frequently used was purified from

transgenic mice strains (SV-LEC) in which the expression of temperature sensitive SV40T antigen immortalized the lymphatic endothelial cells (48). This cell line is grown in special culture media and needs to be kept in culture at 33°C to maintain their immortalized status, making their use as routine experimental tool rather cumbersome. Other lymphatic endothelial cell lines derived from murine lymphoangiomas such as MELC cells have also been reported (49). This cell line retains most of the characteristics of primary lymphatic endothelial cells, but do not retain other features such as LYVE-1 expression. Still, they are a valuable source for research on lymphatic endothelial cell function since they can be *in vitro* propagated and assayed for transcriptional and functional assays. Primary isolated or immortalized lymphatic endothelial cells can also be transfected by liposome-based methodology and more powerful transfection techniques such as nucleofection or retrovirus mediated gene transfer (50, 51). Reliable methods to culture primary mouse LECs are not in place at the moment although it would offer definitive advantages.

The *in vitro* study of leukocyte adhesion to LEC monolayers provides quantitative data and permits experiments with blocking monoclonal antibodies to assess the role of different adhesion molecules (29, 52, 53) as a preliminary tool to describe cell transit across these vessels. Besides, this set up allows DC imaging on fixed and live specimens of lymphatic endothelial cells (33, 54, 55). In contrast, one of the mayor limitations of this experimental approach derives from the fact that leukocyte binding to monolayers of LEC adhered to plastic or ECM coated wells ignores the apical to basal polarization of the endothelial cells. Even though a lack of polarization has been reported for these cells *in culture* (29), in those experiments, DC used the same molecules to adhere to either the apical or the basal face of the endothelium (56). However, more research is needed about the importance of polarity for cell transit across LVs.

A second feasible and reproducible *in vitro* assay relies on the use of transwell systems in which the endothelial cells are seeded on the bottom of the transmigration filter between chambers. Migratory cells are then added on the upper well of the chamber and allowed to cross the endothelium following a chemotactic gradient originated in the bottom well of the dispositive. This methodology also provides the researcher with a quantitative system to assess chemokine-driven active migration across monolayers. By using blocking monoclonal antibodies and decoy receptors the contribution of each of the different receptors can also be tested. An advantage of the use of transwell assays is the possibility to analyze basal to apical migration, but attention should be paid to pore size and the type of ECM proteins used to coat the transmigration membranes between chambers (33, 54).

Novel approaches in cell culture have been developed to grow endothelial structures in 3D micro-devices that allow both physicochemical and confocal microscope-based analysis of cell transmigration across endothelial tubules (57–59). These devices have been mainly applied to study tumor cell biology and metastasis but are promising in the context of cell migration across LEC as an attractive system for live imaging of leukocyte-LEC interaction.

EX VIVO ASSAYS

This experimental approach is based on the isolation and culture *in vitro* of skin samples obtained from animals or surgery samples, under sterile conditions. It has been used for the study of DCs emigration from tissue (60). This technical approach simulates more accurately the physiological context than the previously mentioned *in vitro* assays. In this *ex vivo* set up, the visualization of tissue-resident DC in their way across LVs by confocal microscopy is challenging due to the scarce number of endogenous DC in the tissue sample and their lack of motility once explanted from tissue. Therefore, it is more feasible to directly add *in vitro* differentiated DC onto the explanted skin samples or inject traceable DC to the animals before sacrifice. Using this methodology leukocyte migration toward LVs has been observed by *in situ* imaging (61) and quantitative assays in this respect have been reported (29, 62). Though offering evident advantages, tissue explants also present some drawbacks because: (i) DC may abandon the tissue using other routes different from LVs; (ii) explanted tissue offers limited viability in culture (3 days, the longest in our hands); (iii) the experiments are performed in the absence of lymph flow.

IN VIVO MODELS

The main *in vivo* approximations for the study of DC migration across LVs have been reviewed in depth elsewhere (63). Briefly, current functional assays for the study of leukocyte migration toward the LNs are based largely in two different experimental approaches: the application of different inflammatory stimuli to induce endogenous DC migration, and the adoptive transfer of *in vitro* differentiated DC. The first type of experiments include FITC painting (16, 64), injection of fluorochrome-loaded particles (65), or the application of pro-inflammatory agents to the skin (66). FITC painting allows easy tracing of those leukocytes that have migrated out from skin since they are fluorescent. A pitfall of this methodology is that FITC molecule works as hapten and produces inflammation *per se*, hence being unsuitable for the investigation of steady-state cell migration. Injection of labeled microparticles is used to follow phagocytic DC populations and presents the advantage of allowing the study of DC migration under steady-state conditions. In addition, since these microparticles can be labeled with different fluorochromes, multiple DC populations can be traced in one single experiment. Direct injection or application of pro-inflammatory agents on the skin makes possible the study of the changes in endogenous DC populations both in the skin and in the draining LN. This approximation is useful to assess differential immunological responses to diverse inflammatory stimuli. However, with this technique it is not possible to directly follow DC migratory events. To overcome this problem, mice engineered to express the photoconvertible fluorescence protein Kaede, which changes from green to red when exposed to violet light have been used in experiments to trace the destiny of different leukocyte populations. In this line, Tomura and co-workers trace skin lymphocytes routed to draining LNs after skin exposure to violet light (5). Again, it is likely that photoconversion might act as an inflammatory stimulus.

More accessible methods are based on the adoptive transfer of pre-stained or genetically tagged DCs or T cells. This approach facilitates the study of the migration of leukocytes obtained from

different transgenic models, while keeping a wild type background in the receptor mice (53, 67). The main limitations of this technique derive from the fact that experiments are usually performed with DC differentiated in culture and not with actual tissue-resident populations. It should be noted that DC are phenotypically diverse and may respond differently to environmental stimuli. Besides, the injection of the cells directly into the tissue may produce minor inflammation and undesired leukocyte activation.

Recently, intravital imaging has become a powerful tool to dissect the biology of leukocyte intravasation into the LVs. This approach captures images of cells as they migrate toward and traverse the lymphatic endothelium in live animals in a non-invasive fashion. For this purpose, increasingly sophisticated two-photon fluorescent microscopy technology leads our progress. Indeed, this methodology has provided crucial *in vivo* information about DC migration across LVs in steady-state conditions (34, 55). Besides, many mouse models have been developed to visualize LVs by *in vivo* microscopy based on the selective expression of fluorescent proteins under the regulation of different promoters (55, 68–72) or by the use of fluorescence-labeled antibodies (34, 73). Non-invasiveness and performance of the experiments in intact animals allows continuous lymph flow and makes this approach very suitable, since the main shortcomings from the previously explained methodologies are overcome. Yet, there are limitations to *in vivo* microscopy such as paucity of relevant transgenic animals expressing fluorescent protein under suitable promoters to study leukocyte entrance into lymphatics, and the fact that experimentation with human cells in this setting is impossible.

ADHESION MOLECULES, CYTOKINES, AND CHEMOKINES THAT CONTROL LEUKOCYTE TRANSIT INTO LYMPHATIC VESSELS

CHEMOKINES INVOLVED IN LEUKOCYTE TRAFFICKING INTO LVs

Dendritic cells and T cells follow chemotactic gradients that lead them toward the LVs and facilitate their transmigration and crawl on the luminal-side of the lymphatic capillaries until they reach the wider collector vessels from where lymph flow drifts them toward LNs (34). The role of the chemokine receptors and adhesive molecules and the models in which they have been investigated are summarized in **Table 1**.

The main chemokine-chemokine receptor system that controls leukocyte migration to LNs is CCL21/CCL19-CCR7 axis. The CCR7 chemokine receptor is expressed on DC under steady-state conditions but is strongly up-regulated upon maturation (74). This chemokine receptor has been described as instrumental for DC migration to LNs in experiments using adoptively transferred or autochthonous dermal DCs both under inflammation and steady-state conditions (11, 67). The same function applies for T lymphocyte (40, 41) and neutrophil (44) migration into the LVs.

The ligands of the CCR7 receptor are CCL21 and CCL19 chemokines. CCL21 is mainly produced by LEC in peripheral tissues and it is adsorbed onto heparan sulfate residues present in the ECM through its positive charged C-terminal end (75, 76). A single aminoacid variant of CCL21 (CCL21-Ser) is expressed

by high endothelial venules (HEVs) and fibroblast reticular cells (FRCs) present in secondary lymphoid organs to guide T lymphocytes and DC into the T zone. CCL21 is better sensed by CCR7⁺ leukocytes when it is adsorbed onto surfaces and enhances cell mobility by a phenomenon called haptotaxis (77, 78). Besides, CCL21 can be proteolyzed by activated DC generating soluble gradients that attract DC by chemotaxis as well. Following inflammation, CCL21 biosynthesis by LEC is up-regulated and accounts for the enhanced leukocyte chemotaxis toward the LVs observed in animal models (30, 31, 79). In fact, *in vivo* confocal-based evidences show how DCs directly interact with the CCL21 patches deposited on the areas where lymphatic endothelium is deprived of basal membrane (34). From these points of entry DC subsequently crawl inside LVs (**Figure 2**). Interestingly, it has been demonstrated how CCL21 is needed for integrin dependent transmigration across LEC and promotes DC integrin activation through the erection from their low affinity bent conformation to the extended high affinity form (31, 80, 81). As expected, DCs lacking CCR7 do not dock successfully to CCL21 depots on LVs.

CCL19/MIP3 β is a soluble CC chemokine abundantly expressed in the thymus and in FRC from the LNs but it is not expressed by LVs. Contrarily to what happens with CCL21, CCL19 lacks a negatively charged C-terminus and therefore does not bind to the glycosaminoglycans present in the ECM. In tissue, CCL19 is mainly produced by activated DCs and diffuses as soluble gradients for subsequent DC, and maybe T cells, to follow behind (82). All in all, the conjunctive activity of CCL21 and CCL19 cytokines, accounts for all CCR7-dependent DC migration toward LVs (77). Still, the specific role of each one of these chemokines in DC migration is still under discussion, although the majority of the experimental evidence shows that CCL21 is the predominant chemokine in the guidance of DC migration and intravasation (34, 78).

An interesting and unresolved paradox is that while CCR7 expression is the trademark of central memory T lymphocytes (83, 84) it is not expressed by T effector memory counterparts. The afferent lymph contains abundant CD4⁺ and CD8⁺ T cells which are CCR7⁺ as well (40, 84). It is then conceivably that some of the T cells infiltrating peripheral tissue regain CCR7 expression to enter afferent LVs in a CCR7-dependent fashion (40, 41). It has been reported that egress of CD4⁺ T cells is more efficient than CD8⁺ T cells or B cells (41) but the underlying mechanism has not been uncovered yet. To complicate things further, it has been described how under chronic inflammation dependency for out-migration on CCR7 is not total and other mechanisms are involved including random migration (42).

CXCR4 is another member of the chemokine receptor family up-regulated on DC upon maturation (74). The implications of its cognate chemokine CXCL12 in DC migration have been demonstrated *in vivo*. It has been reported that hypoxia and inflammation drives up-regulation of this chemokine in LEC (30). CXCR4 inhibition impairs DC migration in response to FITC and CHS (85). Interestingly, CXCL12 has been proposed to enhance survival and promote the maturation of DC (86).

The lipid Sphingosine-1-Phosphate (S1P) and its receptors (87) have been extensively studied in lymphocyte egress from the LN

Table 1 | Summarizing table of the main molecular players described in Leukocyte traffic through LVs.

LEC receptor/ ligand	Leukocyte receptor/ligand	Experimental model	Key observations	Reference
CCL21	CCR7	DC and T adoptive transfer of CCR7 ^{-/-} , LN analysis on CCR7 ^{-/-} mice, IVM of footpad	DC, lymphocytes, and neutrophils fail to migrate into LVs to LNs both under steady-state and inflammatory conditions	(11, 34, 40, 41, 67, 78)
CXCL12	CXCR4	FITC painting in the presence of a chemical inhibitor, transwell assays, inflammation models	Impaired DC migration to LN when treated with inhibitor. Induces transmigration across LEC in transwell assays. Impaired CHS response when inhibited	(62, 85)
S1P	S1PR1-5	FITC painting in the presence of chemical inhibitor FTY720, DC analysis in LN under inflammation and in the presence of inhibitor, adoptive skin transfer of T lymphocytes, and whole mount immunofluorescence after ear injection in FTY720 treated cells	Impaired DC and T cell migration from skin to LNs. Induced by inhibitor. Impaired <i>in vitro</i> trans-endothelial migration in LVs. Induces transmigration across LEC in transwell assays	(54, 91, 92, 97)
D6	Inflammatory chemokines	Immunofluorescence of skin LVs and LNs and study of LN DC populations after TPA induced inflammation in D6 ^{-/-} mice	Accumulated inflammatory cells blocking LV function and other DC migration in D6 ^{-/-} mice	(105)
CX3CL1	CX3CR1	FITC painting in CHS preinflamed skin in the presence of CX3CL1 Abs, adoptive transfer of CX3CL1 ^{-/-} BMDCs, transwell assays	DC migration to LN is delayed. Impaired <i>in vitro</i> migration when the chemokine or its secretion is blocked. Effects only observed under inflammation	(99)
PECAM	PECAM	Transwell assays, <i>ex vivo</i> human skin culture in the presence of blocking Ab, immunofluorescence, and DC count inside LVs	Impaired trans-endothelial migration and intravasation in human skin explants, evidence provided only in human	(62)
ICAM-1	CD11a,b	FITC painting in the presence of blocking antibodies or in ICAM-1 deficient mouse, BMDC adoptive transfer in the presence of blocking Abs or from CD18 ^{-/-} mice, whole mount immunofluorescence of ears after DC injection, transwell assays	In inflammatory and high flow conditions Blockade of ICAM-1 and blockade of $\beta 2$ integrins inhibit trans-endothelial migration and DC migration to LN	(29, 33, 56, 107, 108)
VCAM	VLA-4	BMDC adoptive transfer in the presence of blocking Abs, transwell assays	LN impaired DC migration under inflammation and impaired <i>in vitro</i> trans-endothelial migration	(29)
CD99	PILR	Transwell assays, <i>ex vivo</i> human skin culture in the presence of blocking Ab, immunofluorescence, and DC count inside LVs	Impaired trans-endothelial migration and intravasation in human skin explants, evidence provided only in human	(62)
L1CAM	L1CAM	FITC painting assays in mice deficient in L1CAM under Tie 2 promoter, transwell, and adhesion assays	Impaired adhesion, transmigration in human (moDC) and mice (BMDC), impaired migration to LN	(52)
ALCAM	CD6	Lung injection of FITC microbeads in ALCAM ^{-/-} mice	Impaired DC arrival to LNs in ALCAM ^{-/-} mice	(119)
Podoplanin	CLEC-2	FITC painting in CLEC1b ^{-/-} mice, adoptive transfer of CLEC1b ^{-/-} DC, immunofluorescence of mice ear dermis cultured with BMDC	Impaired arrival of DC to LN, impaired intravasation, importance for protrusion formation	(123)
JAM-A	JAM-A	CHS response and FITC painting in Jam-A ^{-/-} and adoptive transfer of Jam-A deficient BMDCs, transwell assays	Jam-A ablation increases DC migration to LN and CHS responses as well as <i>in vitro</i> TEM	(161)

(Continued)

Table 1 | Continued

LEC receptor/ligand	Leukocyte receptor/ligand	Experimental model	Key observations	Reference
CLEVER-1	–	Adoptive lymphocyte transfer in the presence of blocking Abs in mice and rabbit	Lymph node migration of lymphocytes is blocked	(127)
Mannose receptor	Glycoproteins	Adoptive lymphocyte transfer in footpad of MR ^{-/-} mice and IF of LNs	Lymphocyte migration to LN and adhesion to LEC in LNs is impaired	(130)
Semaphorin 3A	Plexin-A1	Adoptive transfer of Plexin-A1 ^{-/-} BMDCs, or wt DCs in Sema3A ^{-/-} mice, functional studies of T cell responses upon OVA skin sensitization in Plexin-A1 ^{-/-} mice. <i>In vitro</i> videomicroscopy	Both, Plexin-A1 and Sema3A absence impairs DC migration to LNs. Sema3A is able to induce actomyosin contraction in BMDCs	(125)

The table includes ligands expressed on both leukocyte and LEC, the evidence provided in leukocyte traffic and the experimental approach performed.

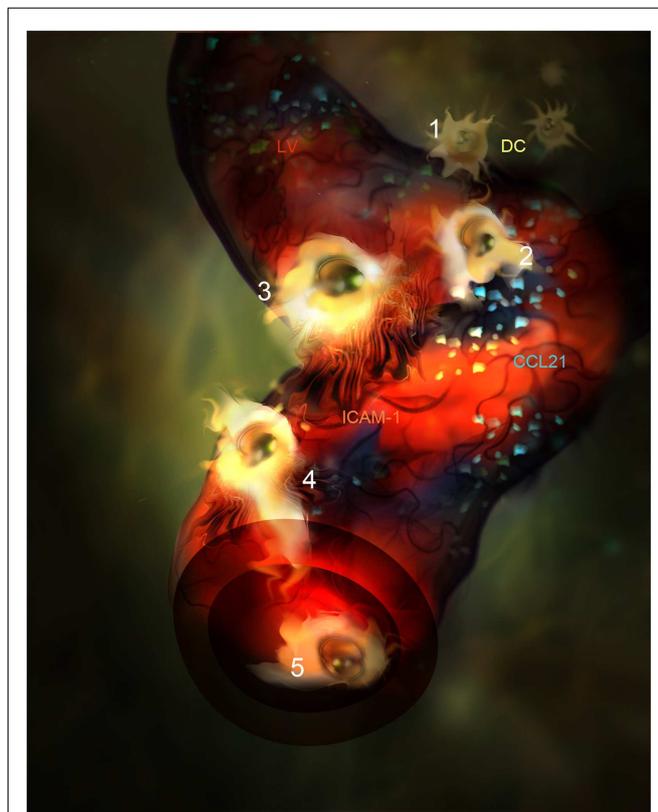


FIGURE 2 | Artistic representation of novel steps in DC migration into LVs under inflammatory conditions.

(1) DC (Yellow) are attracted by CCL21 interstitial gradient and other chemokines toward LVs lined by specialized endothelial cells (red). (2) DCs dock to CCL21 (blue) adsorbed as enriched patches prior to adhesion and intravasation. (3) DCs adhere to LEC surface and get entrapped by ICAM-1 enriched microvilli projections (Orange). (4) DC intravasate in a LV accompanied by ICAM-1 enriched microvilli projections. (5) DC actively crawl inside the LV.

into lymph and blood (88, 89). Besides, the LVs had been reported to express Sphingosine Kinases and secrete S1P.

Although S1P expression is dispensable for homeostatic migration of DCs (90), S1P readily augments upon inflammation and

presents a leading role in guiding T lymphocytes into LVs during acute inflammation (54) but is less important for memory/effector T cell egress from tissues during chronic inflammation (42). In fact, a number of reports show how S1P directs the migration of bone marrow-derived mature DC (91), skin DCs (92), or other DC subsets (93). Treatment of DCs with FTY720, a potent S1P analog that induces internalization of S1P receptors (94), block DC migration into LVs and their arrival to LNs (91). In addition, it has been reported how lack of CD69, that sequesters S1PRs in lymphocytes (95, 96), enhances the effects of S1P-driven migration on T cell and DCs (97). Other inflammatory lipids such as PGD₂ also sensitize memory T cells for egress from tissue to LN in a CCR7-dependent manner (98).

Recently the chemokine CX3CL1/Fractalkine has been involved in DC transit across LVs (99). This cytokine is only expressed upon inflammation and is differently processed in blood and LVs. In blood capillaries CX3CL1 is expressed predominantly as a transmembrane endothelial cytokine with both adhesive and chemoattractant functions while in lymphatic capillaries it is, almost in its totality, shed from the basolateral surface of the lymphatic capillary and released as soluble chemokine. Importantly CX3CL1 lacks acidic residues and hence diffuses freely through the ECM promoting chemotaxis but not haptotactic migration. In fact, it seems to act as a premier inflammation-driven soluble attractor for tissue-resident DC before CCL21 is deposited on the ECM. In addition, CX3CL1 receptor (CX3CR1) impairment partially inhibits DC migration to LN and concomitant blockade of CCL21 does not show additive effects. The study of the specific mechanisms that this chemokine promotes in an *in vivo* setting deserves further research.

Another molecule involved in chemokine-driven DC migration is the chemokine decoy receptor D6. This protein is a non-signaling scavenging receptor (100, 101) that binds some inflammatory chemokines but does not bind CCL21 (102, 103). D6 is expressed on LEC surface after being cultured in the presence of inflammatory cytokines such as IL-6 or IFN γ (104). *In vitro* D6 elimination from primary LECs selectively increases the adhesion of immature DCs but not mature DC (104) and mice knocked-down for D6 showed impaired DC migration to LNs by means of macrophage accumulation around LVs (105). Therefore, it seems

that D6 acts on LVs as scavenger receptor to avoid the adhesion of DC or other leukocytes that fail to express CCR7.

In our group we have been interested in the role of inflammation induced receptors in the modulation of DC transit across LVs. We have studied the participation of CD137/TNFR9/4-1BB, a receptor of the TNFR family receptors in this process. We demonstrated CD137 up-regulation in inflamed LVs. Besides, CD137 cross-linking with an agonistic mAb resulted in the up-regulation of VCAM, and increased production of CCL21 and DC accumulation close to LVs (81).

ADHESION MOLECULES INVOLVED IN LEUKOCYTE TRAFFICKING INTO LVs

To traverse LVs, leukocytes must find the gaps opened in their basal membrane and situate on top of the open endothelial flaps devoid of intercellular junctions. From there, cells must squeeze their cytoplasm by cytoskeletal contraction and nuclear deformation, a process in which Rho associated protein kinases have a said (106). This transit although originally described to occur in an integrin-independent manner, is now seen as a dynamic process modulated by context dependent-factors. In fact, the participation of adhesion molecules as expressed on LVs during leukocyte transit, particularly those belonging to the family of integrin receptors, is a field under intensive investigation (Table 1). Besides, a number of receptors not belonging to CAMs family have recently been described in this process such as podoplanin, CLEVER-1, Plexin A, and CD137.

The role of ICAM-1 and VCAM in leukocyte intravasation into LVs has been controversial. Seminal studies showed a role of ICAM-1 and $\beta 2$ integrins in DC migration toward LNs (107, 108). However, as mentioned above Lämmerman and colleagues showed the entrance of DCs genetically devoid of every known integrin into LVs in *ex vivo* explanted non-inflamed mouse ears (61). In contrast, TNF α , or TLR agonists induce the integrin ligands ICAM-1 and VCAM expression on LEC (29, 30, 109). These same ligands are expressed at low levels in non-inflamed LVs. Besides, blocking these CAMs or their integrin ligands inhibited the migration of DC to LNs under inflammation as well as *in vitro* assessed trans-endothelial migration (TEM) (29, 31, 53). These apparently opposing results are reconciled in light of the differential distribution of the adhesive structure under inflammatory conditions: McDonald and co-workers showed the transformation of button-like junction structures into zipper-like junctions after chronic inflammation of lung lymphatic capillaries. Thus, it seems reasonable to speculate that under these circumstances the LVs become less permeable to cell transit as a mean to regulate leukocyte transit during the resolution of tissue inflammation. In this regard, we have recently observed that ICAM-1 and VCAM integrin ligands usher DC crawling over LEC and TEM by forming microvilli like projections similar to those previously described in vascular endothelium (56, 110, 111). Such structures were only observed under inflammatory conditions and were not formed if $\beta 1$ and $\beta 2$ integrins were blocked on the leukocyte surface or when the CCL21 chemokine was sequestered by neutralizing monoclonal antibodies. There are no experimental data providing a direct involvement of CCL21 in the activation of $\beta 2$ integrins on DC adhering to LVs *in vivo* but all the indirect evidences point in this direction.

Interestingly, it has also been described how DC crawling within initial lymphatics depends on ICAM-1 only under inflammatory conditions (55). Of note, no role has been reported yet for ICAM-2 and ICAM-3 on LEC.

PECAM (CD31) is a molecule expressed on most endothelial cells and involved in leukocyte extra- and intravasation (112). LVs express less CD31 (113) than their blood counterparts and it is mostly distributed at cell-cell homotypic interactions (17, 29). Studies made with human cells have shown that blocking this molecule as well as CD99 in CXCL12 treated LEC was able to reduce TEM, both *in vitro* and on *ex vivo* tissue cultures (62). PECAM binds to integrin $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins expressed on LVs surface, but care should be taken since PECAM is also expressed by most leukocyte subsets and might mediate homophilic interactions.

L1CAM and ALCAM have been reported to participate in leukocyte transit across LVs, although the experimental evidence provided in this regard is limited. L1CAM is a transmembrane protein widely described in neurons (114). It is also expressed in skin LC and bone marrow-derived DCs (115). L1CAM has been detected on inflamed LVs (52). This integrin ligand mediates both homophilic binding (116) and heterophilic interactions with a number of integrins (i.e., $\beta 3$ and $\beta 5$) (117). Recent studies described L1CAM as a protein that mediates DC adhesion and TEM across EC (52), although the molecular mechanisms involved and the relevance of this adhesion molecule are far from being clear.

ALCAM receptor mediates homophilic (ALCAM-ALCAM) or heterophilic (ALCAM-CD6) intercellular adhesion. This receptor is well established as one of the protagonist of leukocyte extravasation across blood vessels, the stabilization of the immunological synapse, and T cell activation (118) Although ALCAM function seems to be of great importance in LV organogenesis, its participation in DC migration has been described *in vitro*. However the definitive role of DC migration through LVs in lungs was not definitely proved (119).

Other adhesion molecules that do not belong to the family of integrin receptors have been related to DC adhesion to LVs. One of such examples is the LV marker podoplanin. Podoplanin has been described to bind CCL21 on LEC surface with high affinity (120), and this interaction has interesting implications for lymphocyte trafficking (121, 122). Recent reports demonstrate how podoplanin expressed on LVs surface sustains DC migration and intravasation via the engagement between lectin CLEC-2 as expressed by DC and podoplanin expressed on LVs. CLEC-2 deficiency in DCs impaired their entry into lymphatics and trafficking to and within LN, thereby reducing T cell priming. Besides, the activation of CLEC-2 by podoplanin induced Rho A-mediated rearrangements of DCs actin cytoskeleton to promote motility along stromal surfaces (123). This finding is of particular interest because podoplanin is also expressed on other stromal cells and may sustain DC migration in the tissue as well as on or across LVs.

Semaphorins and their receptors, plexins and neuropilins, have been for long known as modulators of normal and pathological angiogenesis and lymphangiogenesis (124). Interestingly, the plexin-A1/sema3A axis has also been described to participate in the migration of DCs to LN. Thus, binding of sema3A to its receptor,

the complex formed by plexin-A1 and neuropilin-1 expressed on the surface of the LVs, promotes DC contraction of its actomyosin cytoskeleton and squeezing across small gaps (portals) opened on the lymphatic walls (125).

Other receptors such as CLEVER-1 and the mannose receptor have been described to intervene in leukocyte transit across LVs, but their protagonism in this process is far from being clear. CLEVER-1 is a scavenger receptor expressed on LVs (126) that has also been involved in trafficking of adoptively transferred T lymphocytes from the skin to LNs (127). The Mannose receptor is a C-type lectin carbohydrate binding protein primarily present on the surface of macrophages and DCs that mediates endocytosis (128). The expression of the mannose receptor has also been described on LEC of both afferent and efferent LVs and evidence of impaired migration to LNs of DC lacking its expression has been published (113, 129). Still, its importance *in vivo* has only been shown in lymphatic sinuses inside LNs, where the absence of MR impairs lymphocyte adhesion (130).

THE TRANSIENT SYNAPSES OF LEC AND TRANSMIGRATING LEUKOCYTES: EXCHANGE OF INFORMATION TO SHAPE THE IMMUNE RESPONSE

Beyond the control of leukocyte traffic to LNs, LECs have demonstrated to show interesting immunomodulatory functions both in afferent LVs and Lymphatic sinuses inside LNs. In these sinuses, LEC constitutively express MHC class II molecules (131) and some co-stimulatory molecules such as ICAM-1 and CD58, but do not promote allogenic T-cell proliferation (132).

In contrast, LEC of lymph node sinuses directly promote peripheral tolerance by antigen presentation to CD8 T cells (133, 134). In this work it was demonstrated that LECs are significant albeit suboptimal APC and promote peripheral tolerance by their lack from key co-stimulatory molecules such as CD80 or 4-1BBL (135) and the rapid up-regulation of significant expression levels of the co-inhibitory molecule PD-L1 (B7-H1) (134). In fact, the exploitation of the ability of LN sinuses to induce tolerance has been recently shown as an escape mechanism for B16 melanoma grafted tumors. Interestingly, the blockade of lymphangiogenic cytokine VEGF-C in this model was able to reduce LEC-induced CD8 T cell tolerance (136). The tolerogenic potentials of LEC in LN sinuses have been recently revised into detail (137).

The possibility of this immunosuppressive/tolerogenic process taking place in peripheral afferent LVs has not been investigated yet. Although IFN γ promotes MHC class II expression on dermal LEC without concomitant up-regulation of co-stimulatory molecules (unpublished observations) it is not known whether peripheral LVs can promote tolerance in such conditions. The fact that CD4⁺ T lymphocytes traffic frequently via lymphatics may set up a possibility of peripheral antigen presentation on LVs, upon CD4 T cell/LEC contact with potential consequences for instance in transplanted organs. It remains to be demonstrated whether LEC serves as a key professional antigen presenting cell for tolerance. Suggestive data on the likeliness of these phenomena has been reported in allogenic transplantation settings, where lymphocyte nodular infiltrations that resemble a tertiary lymphoid organs have been described in grafted organs, around CCL21-podoplanin complexes expressed by the LVs (120). Even more, recent findings

demonstrated by intravital imaging approaches how DC crawl rather than roll once inside lymphatic capillaries (34, 55) thus favoring extensive and durable contacts between DC and LEC that may be also true for CD4 T cells and LEC. As early mentioned, tight contacts between CD4 T cells or DC and LEC are formed precisely on ICAM-1 enriched microvilli projections (56) that may facilitate a sort of “immune synapse” between leukocytes and LVs. It should not be forgotten that LFA-1 itself is an important co-stimulatory molecule for T cells (138).

In this line, immunomodulatory roles of LEC over DC have already been demonstrated. It has been reported that inflammation induced ICAM-1 is able to decrease the co-stimulation capabilities in immature and TNF- α matured (but not LPS matured) DC. This phenomenon seems to involve Mac-1 integrin ligation on DC (139). These findings raise an intriguing issue since contacts between LEC and DC or T cells do occur both under inflammation and steady-state conditions, it seems that the set of molecules that mediate such intercellular interaction are peculiar for each condition (55, 56) and most probably trigger different phenotypes in the LEC-interacting leukocyte subpopulation. For instance, the integrin ligand ICAM-1 is only engaged under inflammation. In this line, we have observed strong phospho-Tyr staining in the areas of contact between LEC Microvilli and DC (unpublished results) supporting that ICAM-1 in this specific context facilitates bidirectional crosstalk between both cell types that triggers intercellular signals.

Another extracellular receptor involved in LEC-shaping of the immune response is CD137/4-1BB molecule. As already mentioned, we have recently identified CD137 (4-1BB) expression on inflamed LVs. The ligation of this molecule on LEC promotes CCL21 up-regulation (81) and increased DC transmigration. CD137-Ligand reverse signaling would in turn promote the increased expression of co-stimulatory molecules and chemokine receptors on migrating APCs (140). This system may be a first example of molecules denoting inflammation and subsequently fine-tuning lymphocyte activation and migration via afferent LVs. It is tempting to speculate that transit of an activated leukocyte sensitizes for subsequent transit events by a variety of immunologically relevant ligand receptor pairs, including other members of the TNF and TNFR families.

OPPORTUNITIES FOR THERAPEUTIC INTERVENTION AT THE INTERFACE BETWEEN LEC AND IMMUNE SYSTEM CELLS ENHANCING LEUKOCYTE MIGRATION TO INCREASE VACCINE EFFICIENCY

Dendritic cell based vaccines often given subcutaneously constitute an interesting approach for the treatment of cancer (141). Immunization is attempted toward defined antigens shared by tumors or against individual neoantigens product of the altered genome of individual malignancies. The latter is very attractive and encompasses strategies varying from DC loading with mRNA, tumor lysates to intratumoral DC injections.

The intradermal route of administration seems to be more effective in eliciting immune responses (142), while one of the key limitations for its efficacy seems to be DC arrival to LNs (143, 144). Since intravasation in LVs is a key step in the migration of DCs

to LN, intervention in this particular step may provide increased efficiency in DC therapies. It has been reported that preconditioning the area of injection by promoting for example, acute inflammation promotes migration and maturation on DC vaccination when TLR agonist poly I:C is used as an adjuvant (145, 146). The TLR7 agonist imiquimod has also been applied to the skin at the vaccination site with a similar purpose (147). Pro-inflammatory cytokines have proved to be good local adjuvants (148).

Other methods may provide also good preconditioning for injection sites, as local irradiation (149) or laser illumination (150) which additionally enhances migratory possibilities by increasing the degradation of the basal membrane of the vessels. The preparation of DC is also particularly relevant in the migration and efficacy of DC vaccines (141). By including different cytokines in DC maturation cocktails, their migration to LNs can also be enhanced. For instance IFN- α (53, 151) induces increments in the expression of chemokine receptors one LEC and integrin activation on DC surface. The strongest stimulus for CCR7 expression and functionality is the lipid mediator PGE2 (152, 153). However, many immunosuppressive functions of PGE2 on DC discourage the use of this prostaglandin in DC maturation cocktails.

THE ROLE OF LYMPHATICS ON INFLAMMATORY DISEASES

In the last few years it has been shown how the plasticity of LV in response to inflammation can also contribute to the progression of diseases whose mechanisms involve chronic inflammation, as some autoimmune diseases (154).

Increased LV density has been observed in psoriatic skin (155) and rheumatoid arthritis lesions in mice joints (156). As previously mentioned, there is increased LV presence in kidney transplants and their secretion of CCL21 promotes DC migration and alloantigen response and rejection (120). Diminishing (157) or normalizing lymphangiogenesis by VEGFR stimulation (27) is considered a promising treatment to control chronic inflammation.

There is already some evidence for pharmacological treatments directed toward leukocyte intravasation on LVs that may help in the treatment of these diseases. For instance, the blockade of DC traffic with anti-VEGF antibodies reduces inflammation (158) and rejection of mice corneas. Interestingly, in a heart transplantation model, blockade of VEGF with antibodies has proved to decrease inflammation by a mechanism directly dependent on CCL21 production by LVs (159).

While the role of blood vessels in leukocyte traffic is known in great molecular detail, that of LV is less well understood. Regulation of adhesiveness and chemotaxis in blood vessels can be interfered for the sake of suppressing inflammation in multiple sclerosis or transplantation with anti-VLA-4 mAb. In fact, it is quite possible that the S1P antagonist sphingolipid exerts an important effect on memory lymphocyte egress from the inflamed territory suffering autoimmunity toward LN.

In our view there is also much potential in pharmacological manipulation of the CCR7 axis (160). From a drug development perspective is not an easy target but it certainly would provide a tool to disorient recirculation of pathogenic T lymphocytes and limit the arrival of immunogenic autoantigens to lymphoid tissue.

CONCLUDING REMARKS

Research performed mainly in the last 10 years regarding leukocyte migration via afferent LVs has unraveled that the process is highly regulated and more complex than originally expected. Importantly, new incisive experimental procedures including *in vivo* imaging have provided detailed knowledge of the process. Some interesting and unexpected molecular players including chemokines and adhesion molecules have been identified as gatekeepers of LV intravasation and intriguing data about a tight relationship between LEC and leukocytes have been reported. Knowledge on the regulation of DC migration out of peripheral tissue is beginning to be exploited for vaccination, but we are only starting to learn the pathological and therapeutic implications that leukocyte-LVs contact may have. Especially, the field of the tissue-egressing mechanisms of T Lymphocytes remains neglected. We believe that a more in depth knowledge of these leukocyte-LVs interactions may provide interesting cues or potential targets for chronic inflammation. Migration through LVs must not be observed as a passive drain but as process that is highly regulated by changes in the tissue homeostasis, and that may help to shape immune responses both under steady-state and inflammatory conditions.

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