

# HOMING AND CELLULAR TRAFFIC IN LYMPH NODES

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Lymph nodes (LNs) are the organs where innate immune responses lead to acquired immunity, where some of our most devastating pathogens evade immunity, and where autoreactive lymphocytes first encounter tissue-specific self-antigens and are either tolerized or activated. The many roles of LNs depend on the coordinated migration of its cellular constituents. This article covers new insights into the organization and microvascular specialization of LNs, the guidance mechanisms that allow lymphocytes and antigen-presenting cells to find their correct place in the nodal parenchyma; and the role of afferent lymph flow in LN function.

**FOLLICULAR DENDRITIC CELLS (FDCs).** Stromal cells of non-haemopoietic origin in B-cell follicles that present intact antigen to B cells and function in the selection of memory B cells during germinal-centre reactions.

**HIGH ENDOTHELIAL VENULES (HEVs).** A specialized type of postcapillary venule that is lined by cuboid or high endothelial cells. HEVs are only found in secondary lymphoid organs, except the spleen. They are the main site of lymphocyte entry from the blood.

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Lymph nodes (LNs) are strategically positioned collecting stations for antigens that are present in peripheral tissues of higher animals. Naive lymphocytes search for their cognate antigen during frequent visits to these local 'antigen libraries'<sup>1</sup>. To trigger an immune response, the rare antigen-specific T and B cells must first interact with professional antigen-presenting cells (APCs), dendritic cells (DCs) and FOLLICULAR DENDRITIC CELLS (FDCs), respectively, and then with each other. Free antigen and/or antigen-loaded DCs are transported to the LNs from distal tissues through afferent lymph vessels, whereas naive lymphocytes enter these organs in HIGH ENDOTHELIAL VENULES (HEVs)<sup>2,3</sup>. If lymphocytes fail to recognize specific antigen within a few hours to days, they return to the circulation through efferent lymph vessels and the thoracic duct<sup>1</sup>. On average, naive lymphocytes spend less than a half hour in the circulation before homing to another lymphoid organ<sup>4</sup>. Experimental animals can be almost completely depleted of lymphocytes when lymph is continuously removed through a thoracic duct fistula, indicating that most of the body's lymphocytes are recirculating<sup>5</sup>.

When a naive T cell is exposed to cognate antigen, it must decide whether to mount an effector response that is aimed at eliminating the source of the antigen or to become tolerant and/or die to avoid autoimmunity. The LNs have a crucial role in collecting the prerequisite information that allows lymphocytes to make this fateful decision<sup>6</sup>. In the context of appropriate co-stimulation,

T cells undergo clonal expansion and, after several days, acquire effector functions<sup>7,8</sup>. Unlike naive T cells, effector cells can produce cytokines, kill antigen-expressing cells (in the case of cytotoxic T lymphocytes; CTLs) or modify the behaviour of other leukocytes (in the case of T helper (T<sub>H</sub>) cells), and express homing molecules, which allow them to migrate to the source of their antigen in peripheral tissues. So, effector cells that home to the skin are preferentially generated in the skin-draining LNs, whereas mesenteric LNs or Peyer's patches produce mucosa-homing subsets<sup>9-13</sup>. Most effector cells die when the antigen has been eliminated, but a small fraction remains as long-lived memory cells. Memory cells can be divided into two subsets known as effector and central memory cells<sup>14</sup>. Whereas effector memory cells home to peripheral tissues, central memory cells continue to recirculate through LNs in which they mount strong recall responses whenever the antigen returns<sup>14-16</sup>.

So, the LNs have several important functions in the immune system: to recruit large numbers of naive lymphocytes from the blood; to collect antigen and DCs from peripheral tissues; to provide the environment for antigen-specific tolerance or productive primary and secondary effector responses; to modulate the homing characteristics of effector or memory T cells, targeting them to tissues that contain their cognate antigen; and finally, to provide a 'look-out' for central memory cells. To carry out these diverse functions the cellular

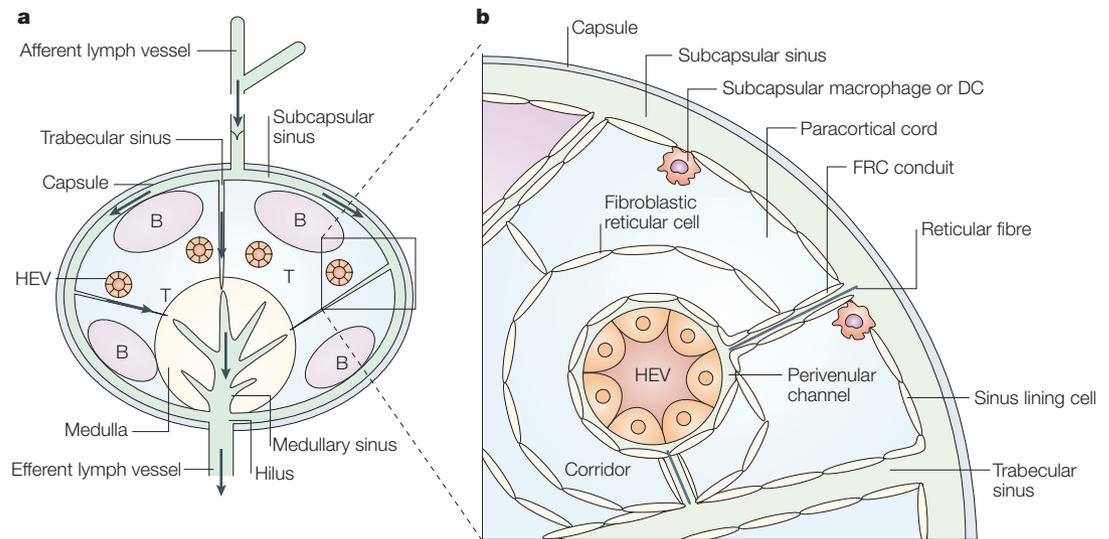


Figure 1 | **Lymph-node architecture. a** | Schematic diagram showing the major structural components of a lymph node. The main routes of lymph flow into and within lymph nodes are indicated by arrows. Blind-ending afferent lymph vessels collect and channel interstitial fluid into the subcapsular sinus. From here, the lymph is drained towards the hilus through the fibroblastic reticular cell (FRC) conduit and trabecular sinuses that connect to medullary sinuses. **b** | Schematic depiction of a paracortical cord (modified according to REFS 21,22). The T-cell-rich cord (light blue) is shown adjacent to a B-cell follicle (pink) and demarcated by lymph-filled sinuses (green). The cord is penetrated by reticular fibres consisting of type 1 and type 3 collagen that are contained within the sleeves of the FRCs forming a conduit. At the centre of each cord is a high endothelial venule (HEV) that is surrounded by concentric layers of FRCs. The FRC conduit drains lymph into the perivenular channel.

participants must migrate into the LNs and find their correct place within them. This article discusses our present understanding of the role of LNs in the immune system, with particular emphasis on the trafficking signals that make these organs a crucial interface between the innate and adaptive cellular components of the immune system.

### Lymph-node architecture

Two main regions can be distinguished histologically in LNs — the cortex and the medulla (FIG. 1a). The cortex is further divided into the paracortex (the T-cell area), and the more superficial B-cell area that consists of primary follicles and (after antigen challenge) germinal centres. B-cell follicles are the main site of humoral responses, whereas the paracortex is the site where circulating lymphocytes enter the LNs<sup>2</sup> and where T cells interact with DCs<sup>17</sup>. The medulla is a labyrinth of lymph-draining sinuses that are separated by medullary cords, which contain many plasma cells, and some macrophages and memory T cells. The function of the medulla is still poorly understood.

The fine architecture of the LN cortex is complex and variable<sup>18–21</sup>. Nevertheless, common structural features have been identified<sup>21,22</sup> (FIG. 1b). On the basis of electron-microscopy studies, it has been proposed that the paracortex is arranged in paracortical cords that originate between or below the B-cell follicles and extend towards the medulla where they merge into medullary cords<sup>22</sup>. The cords are bordered by lymph-filled cortical sinuses and permeated by reticular fibres. At the centre of each paracortical cord is an HEV that is surrounded by concentric layers of pericytes known as fibroblastic

reticular cells (FRCs). A narrow space between the basement membrane of the HEV and the pericytes is known as the perivenular channel. It has been proposed that this channel receives an ultrafiltrate of lymph from the FRC conduit (see later). Networks of FRCs that are often arranged in spiral layers around the HEVs enclose 10–15  $\mu\text{m}$  wide corridors along which lymphocytes are thought to migrate<sup>21</sup>.

The microvascular anatomy and haemodynamics have been characterized in mouse inguinal LNs<sup>23</sup> (FIG. 2). Venous blood flows through HEVs along a venular ‘tree’, the ‘trunk’ of which is formed by a large collecting venule in the medulla. This venule drains into a large vein at the hilus — a discrete region where the capsule is penetrated by efferent lymph and blood vessels. Adhesive interactions between leukocytes and endothelial cells are absent in LN arterioles and capillaries, but they occur frequently in venules. Recent work indicates that the medulla-associated segments of the venular tree express unique adhesion molecules that are distinct from those expressed by HEVs<sup>24</sup>.

HEVs are normally only found in secondary lymphoid tissues (except for the spleen)<sup>3</sup>. The composition and distribution of lymphocyte traffic molecules on HEVs differs between lymphoid organs, and the presence and function of HEVs is regulated throughout life. For example, HEVs in the LNs of newborn mice express mucosal addressin cell-adhesion molecule 1 (MADCAM1), but not peripheral node addressin (PNAD). The adult LN-specific (that is, PNAD<sup>+</sup>MADCAM<sup>-</sup>) HEV phenotype is only gradually acquired post-natally<sup>25</sup>. The maintenance of HEVs and the ability of LNs to recruit lymphocytes from the blood depends on intact lymphatics<sup>26–28</sup>.

Although the lymph-borne factor(s) that provide this maintenance signal have not been identified, some evidence indicates that macrophages are involved in its generation<sup>29,30</sup>. Cytokines also regulate the development of HEVs — for example, ectopic HEVs can be induced by transgenic expression of lymphoid chemokines or lymphotoxin (LT)<sup>31–34</sup>, or by local injections of LT $\alpha$ –immunoglobulin chimeric fusion protein<sup>35</sup>. Indeed, LT and its receptors are required for the formation of HEVs as well as of the LNs themselves<sup>36,37</sup>. HEVs are often induced at non-lymphoid sites of chronic inflammation, presumably because flat-walled venules are exposed to differentiation signals from infiltrating inflammatory cells<sup>3</sup>.

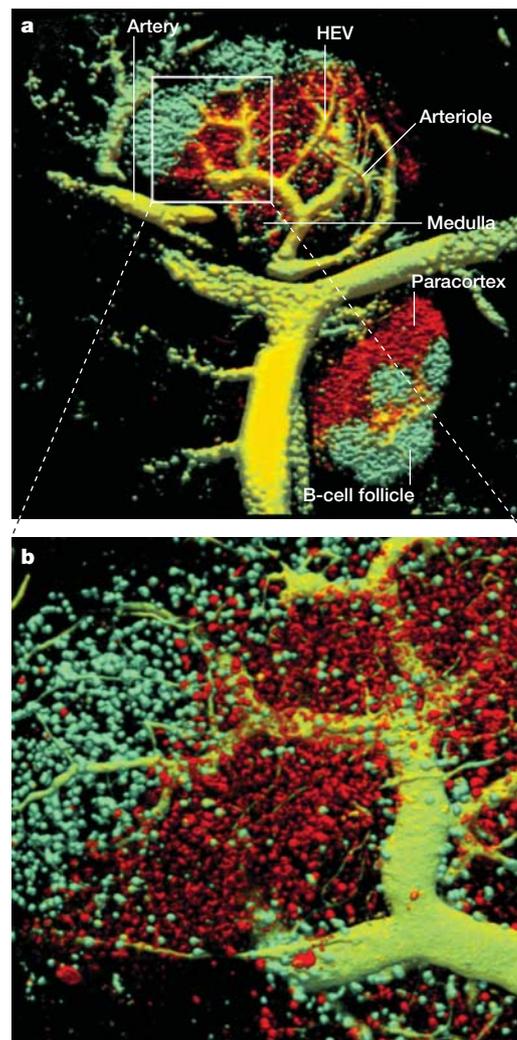
### Lymphatics: the information superhighway

Lymph fluid (BOX 1) and any cells or particles that are contained within it must run through one or a chain of several LNs. The lymph enters from afferent lymph vessels into the subcapsular sinus — a hollow space below the fibrous capsule that covers the LN (FIG. 3). From here most of the lymph is channelled around and, through trabecular sinuses, across the LN parenchyma towards a medullary network of sinuses and from there into an efferent lymph vessel that leaves the LN (FIG. 1a). The intranodal lymph channels are enclosed by FRCs<sup>21</sup>, mannose receptor-expressing sinus-lining endothelial cells<sup>38,39</sup> and, especially in the subcapsular sinus, macrophages and CD11b<sup>+</sup> DCs<sup>40</sup>, which sample the lymph and remove microorganisms and debris that are in it. These cells also transport and/or process antigenic material for presentation to B and T cells.

Lymph-borne molecules do not have free access to the lymphocyte compartment<sup>41</sup>. Only small molecules with a molecular radius less than ~4 nm can reach the T-cell area through the FRC conduit. FRCs wrap around collagen fibres, forming channels that project from the subcapsular sinus into the T-cell area<sup>21,42</sup> (FIG. 1b). The FRC conduit is thought to act as a sizing channel, allowing water and low-to-moderate molecular weight molecules, such as chemokines, to reach the perivenular channels<sup>21,41,43–45</sup>. By contrast, a lymph-borne antibody specific for a cell-surface marker on DCs was shown to accumulate on DCs throughout the T-cell area<sup>46</sup>. So, the FRC imposed size barrier to lymph-borne molecules might not be absolute, but select macromolecules, such as antibodies, can gain access to the nodal parenchyma by mechanisms that remain to be identified.

Lymph drainage ensures that antigenic material from peripheral sites is efficiently transported to draining LNs and presented to lymphocytes that home to LNs from the blood. However, microorganisms that breach the body's surface barriers can use the lymphatics to reach the systemic circulation and to disseminate. A good example is *Yersinia pestis* — arguably the most devastating bacterial pathogen in human history<sup>47</sup>. Bubonic plague is often caused by infected fleas, which deposit *Y. pestis* in the skin. The pathogen then travels through the lymphatics to the LNs where it proliferates. *Y. pestis*-infected LNs typically contain few myeloid phagocytes, but contain

large masses of bacteria that look like ground glass in histological sections. By contrast, LNs infected by less virulent *Yersinia* strains recruit numerous inflammatory cells. So, it has been speculated that the success of *Y. pestis* might be due to its ability to block the recruitment of phagocytes to the LNs<sup>48</sup>. Many other viral and bacterial pathogens can also propagate through the LNs, including HIV, mycobacteria and anthrax<sup>49,50</sup>. Therefore, LNs must also function as sites of innate recognition of lymph-borne pathogens.



**Figure 2 | Lymph-node microcirculation. a** | The microcirculation of the lymph node has been visualized by intravenous injection of a mixture of green and red fluorescent dextrans. The plasma markers fill all microvessels, which are yellow. The preparation is partly covered by fatty tissue, which diffracts fluorescent light emitted from the superficial epigastric vein resulting in a 'bubbly' appearance. To identify the different lymphoid compartments, purified fluorescent B cells (green) and T cells (red) were injected intravenously 20 hours before. After their entry into the lymph node through high endothelial venules (HEVs), the homed cells segregate. B cells populate the more distal B-cell follicles, whereas T cells remain in the paracortex. **b** | A higher magnification of the boxed region in panel a illustrates the relationship of B and T cells and the microcirculation (see movie online).

## Box 1 | What is lymph?

The capillaries in most tissues are normally leaky. In a healthy adult, this results in daily leakage of ~20 litres of protein-poor fluid into the extravascular space. About 90% of this aqueous fluid is locally resorbed, whereas the remaining ~2 litres per day is returned to the circulation through lymph vessels. These vessels have valves that only allow unidirectional flow through the lymph nodes (LNs) towards the body's core. Lymph vessels channel the constant stream of interstitial fluid (the lymph) through successively larger branches into the thoracic duct. Rhythmic changes in intrathoracic pressure during respiration and peripheral increases in interstitial pressure or compression of lymph vessels, for example, due to muscle work, propel the lymph through the thoracic duct into the left subclavian vein (lymph from above the diaphragm returns to the circulation through the bronchomediastinal duct or the right lymph trunk).

**Determinants of lymphocyte homing**

For their roles in both innate and adaptive host defence, LNs must attract myeloid and lymphoid leukocytes from the blood and peripheral tissues. There are two main ports of entry into LNs: by way of HEVs or through the afferent lymph in the subcapsular sinus. The different cellular components of LNs prefer either one or the other access route. For example, some memory cells reach LNs from peripheral tissues through the afferent lymph vessels<sup>51</sup>, but most T and B cells enter LNs through HEVs<sup>2</sup>. This route of lymphocyte traffic is highly efficient<sup>5,52</sup>. A single inguinal mouse LN recruits ~2% of the recirculating pool per day<sup>23</sup>. During inflammation, lymphocyte accumulation in draining LNs is markedly increased<sup>45,52–54</sup>, whereas their exit into the efferent lymphatics is transiently blocked<sup>52</sup>. These two inflammation-induced changes in lymphocyte traffic rapidly increase lymphocyte numbers in the draining LNs, which might enhance the probability for antigen encounter by the rare T cells that express a relevant T-cell receptor (TCR).

It is widely accepted that the trafficking of intravascular leukocytes is controlled by a sequence of at least three molecularly distinct adhesion and signalling events<sup>7,8,55</sup>. These adhesion cascades are initiated by a tethering step that allows leukocytes to bind loosely to endothelial cells (step 1a). The marginated cells are then pushed forwards by the blood stream resulting in slow rolling along the vessel (step 1b). Subsequently, rolling cells encounter chemotactic stimuli on the endothelium that engage specific leukocyte receptors (step 2). Chemoattractant binding, in turn, induces intracellular signals triggering activation-dependent adhesion steps that allow leukocytes to stick firmly (step 3) and to emigrate through the vessel wall.

Intravital microscopy analyses have defined the adhesion cascades that mediate T- and B-cell homing to LNs<sup>15,43,56,57</sup> (TABLE 1). First, tethering and rolling of both subsets is mediated by L-selectin (CD62L). The endothelial L-selectin ligand is PNAD — an O-linked carbohydrate moiety, the main components of which are recognized in humans and mice by the monoclonal antibody MECA-79 (REFS 58,59). In addition, some LN venules express structurally and antigenically distinct L-selectin ligands that are not detected by MECA-79 (REFS 24,60). The structure of L-selectin ligands in HEVs and the role of glycosyl- and sulphotransferases in the

biosynthesis of these ligands is a topic of intense study<sup>61,62</sup>. Next, firm arrest of rolling T cells in the LN HEVs is mediated by the integrin leukocyte function-associated antigen 1 (LFA1; CD11a–CD18), which interacts with intercellular adhesion molecule 1 (ICAM1) and probably ICAM2 on HEVs<sup>56,63,64</sup>. Lymphocytes failed to home to LNs in one strain of LFA1-deficient mice<sup>64</sup>, whereas some residual homing was reported in a second strain, which was mediated by  $\alpha_4\beta_7$  integrin–vascular cell-adhesion molecule 1 (VCAM1) interactions<sup>65</sup>. Whatever integrins might be involved, they must be activated to mediate arrest. INTEGRIN ACTIVATION on naive and central memory T cells is mediated by the CC-chemokine ligand 21 (CCL21; also known as SLC, TCA4, exodus 2 or 6Ckine)<sup>15,43</sup>, which is constitutively expressed by high endothelial cells and binds to CC-chemokine receptor 7 (CCR7)<sup>66–68</sup>. A second CCR7 agonist, CCL19 (also known as Epstein–Barr virus-induced gene 1 ligand chemokine; ELC or macrophage inflammatory protein 3 $\beta$ ; MIP3 $\beta$ ), is expressed by the lymphatic endothelium and interstitial cells in LNs, but not by HEVs. Perivascular CCL19 can be transported to the luminal surface of HEVs and induce integrin activation on rolling T cells<sup>44</sup>. The relative contribution by CCL19 versus CCL21 to T-cell homing remains to be determined.

Mice, unlike humans, have two isoforms of CCL21 that differ in one amino acid; CCL21Ser is expressed by HEVs, whereas CCL21Leu is generated in the lymphatic endothelium<sup>69</sup>. A mutant mouse strain known as *plt/plt* (paucity in lymph-node T cells) is deficient for CCL21Ser and CCL19 (REFS 69,70). LNs of *plt/plt* mice and CCR7-deficient mice contain few naive T cells, but the B-cell compartment is less affected and there are marked numbers of memory T cells<sup>71,72</sup>. This indicates that B cells and perhaps also memory T cells respond to different (or additional) integrin-activating signals in LN HEVs that are not recognized by naive T cells. Indeed, rolling B cells can be induced to arrest in HEVs either by CCR7 agonists or by CXCL12 (also known as stromal cell-derived factor 1 $\alpha$ ; SDF1 $\alpha$ ) — the ligand for CXCR4 (REF. 57). Either pathway is sufficient to maintain B-cell homing (albeit at lower levels than in wild-type animals, in which both function simultaneously). Although CXCL12 potentially activates integrins on rolling naive T cells *in vitro*<sup>67,73</sup>, it does so poorly *in vivo*<sup>74</sup>. So, despite low-level expression of CXCL12 by mouse HEVs, naive T cells depend on CCR7 signals, at least in BALB/c and DDD/1 genetic backgrounds<sup>43,57</sup>. By contrast, CXCL12–CXCR4 interactions have a modest, but detectable role in T-cell homing in C57BL/6 mice, indicating that some chemokine pathways are regulated by strain-specific genetic modifiers<sup>57</sup>.

Even though B cells encounter two distinct integrin-activation signals in HEVs, B-cell homing to LNs is less efficient than that of T cells. This is because the level of L-selectin expression by B cells is ~50% lower than the level of expression by T cells, which express ~7–10 x 10<sup>4</sup> molecules per cell<sup>75</sup>. In experiments with stably transfected pre-B-cell clones that express human L-selectin, we observed that at least 50,000 L-selectin molecules per cell were required for efficient rolling in LN HEVs<sup>23,76</sup>

**INTEGRIN ACTIVATION**

The process by which integrins are triggered, often by chemokines or other signals (for example, T-cell receptor stimulation), to switch from a low- to a high-affinity ligand-binding state. This entails a conformational change in the extracellular domain of the integrin heterodimer. In addition, integrins might be redistributed into clusters on the cell surface to enhance binding avidity.

(U.H.v.A., unpublished observations). So, it is important to keep in mind that the presence of L-selectin on a leukocyte does not necessarily predict its potential to home to LNs.

The fact that a sequence of three distinct molecular steps must be successfully engaged in HEVs explains why some leukocytes home to LNs, whereas others don't (TABLE 1). For example, granulocytes express L-selectin and LFA1, but not CCR7 or CXCR4; mature myeloid DCs express CCR7 and LFA1, but not L-selectin; and effector CTLs lose expression of both L-selectin and CCR7. Consequently, granulocytes roll, but fail to arrest, whereas DCs and effector cells fail to roll in LN HEVs<sup>15,56,77</sup>. The inability of mature DCs to home to LNs from the blood is important in the context of ongoing clinical efforts to use antigen-pulsed DCs as vaccines (BOX 2).

The homing mechanisms discussed earlier were mainly characterized in cutaneous LNs. HEVs in mucosa-associated LNs, such as the mesenteric LNs, express not only PNAD, but also MADCAM1 — a ligand for  $\alpha_4\beta_7$  integrin<sup>78,79</sup>. As the  $\alpha_4\beta_7$ -MADCAM1 pathway can mediate selectin-independent rolling<sup>80</sup>, L-selectin

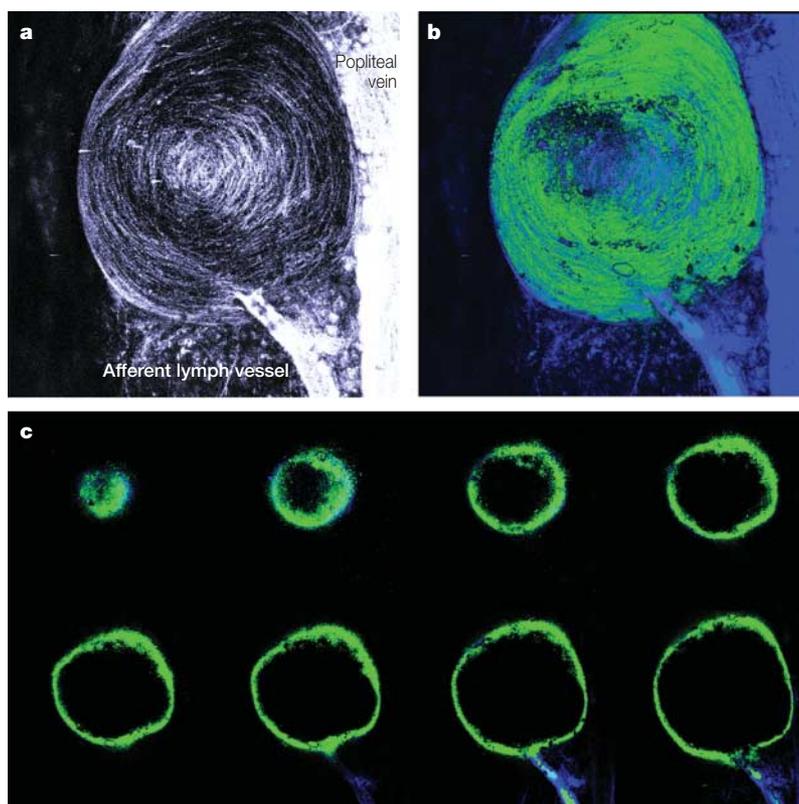
deficiency compromises lymphocyte homing to peripheral LNs more markedly than to mesenteric LNs<sup>81</sup>, whereas  $\beta_7$  integrin deficiency has no effect in peripheral LNs, but causes reduced homing to mesenteric LNs and loss of homing to Peyer's patches where HEVs are PNAD<sup>-</sup>MADCAM<sup>+</sup> (REF. 82). It should also be noted that there are several other adhesion pathways that have modulating effects on lymphocyte homing. These molecules include P-selectin, which is expressed by high endothelial cells<sup>83</sup> and circulating activated platelets<sup>84</sup>, vascular adhesion protein 1 (VAP1)<sup>85</sup>, CD43 (REFS 86,87) and CD44 (REFS 88,89). Their impact on physiological homing to LNs is relatively subtle, and there are conflicting reports on the role of CD43 and CD44.

#### Remote control of leukocyte recruitment to lymph nodes.

The FRC conduit ensures that low molecular weight lymph-borne signals, particularly chemokines, are rapidly transported from the subcapsular sinus to the HEVs. Initial evidence that this conduit can influence leukocyte recruitment came from experiments in *pl1pl1* mice, in which defective T-cell homing was restored by intracutaneous injection of CCL21 or CCL19, but not CXCL12 (REFS 43,44). The exogenously applied chemokines were rapidly transported to HEVs and presented to rolling T cells in the lumen of the vessel. Chemokine transcytosis in abluminal-to-luminal direction was first described in dermal venules where it involves the caveoli network<sup>90</sup>. Similarly, chemokines traverse HEVs in intracellular vesicles rather than across intercellular junctions<sup>44</sup>, but these transport vesicles are still incompletely characterized.

So far, the list of chemokines that were shown to be transported from a non-endothelial source to LN HEVs includes both homeostatic (CCL19, CCL21 and CXCL12) and inflammatory chemokines<sup>41,44,45</sup>. One chemokine that is particularly highly expressed during inflammation is CCL2 (also known as monocyte chemoattractant protein 1; MCP1) — a potent monocyte chemoattractant<sup>91</sup>. CCL2 from inflamed skin gets released into the lymph, transported to draining LNs, and translocated to the luminal surface of HEVs. Here, CCL2 activates CCR2 on rolling monocytes, triggering integrin-mediated arrest<sup>45</sup> (FIG. 4). The interaction of CXCL9 (also known as monokine induced by interferon- $\gamma$ ; MIG) with CXCR3 has been identified as a second mechanism for monocyte recruitment to LNs that drain inflamed tissues<sup>92</sup>. It is not known whether CXCL9 is produced by the inflamed HEVs or by other intra- (or extra-) nodal cells from where the chemokine might have been translocated.

The ability to modulate multi-step adhesion cascades in HEVs remotely by secreting chemokines into the lymph enables peripheral tissues to control the composition and function of leukocytes in draining LNs. It is conceivable that this mechanism opens a gate to LNs not only for monocytes, which might then give rise to macrophages and/or DCs, but also for other circulating leukocytes, such as granulocytes, which express L-selectin and LFA1 and several receptors for inflammatory chemokines<sup>55</sup>. However, recent work indicates that there



**Figure 3 | Lymph conduits and exclusion of large molecules from the lymph-node parenchyma.** **a** | Two-photon microscopy of a mouse popliteal lymph node (LN) allows the visualization of circularly arranged collagen fibres in the lymph node capsule. Other collagen rich structures include a large afferent lymph vessel and the adjacent popliteal vein. The efferent lymph vessel is located below the LN and is not visible here. **b** | A few minutes after injection of fluorescent dextran into the footpad, the fluorescent marker (green) fills the afferent lymph vessel and accumulates below the LN capsule (blue). The image represents a three-dimensional reconstruction of serial optical sections through a 135  $\mu\text{m}$  deep volume of tissue below the ventral pole of the popliteal LN (see movie online). **c** | Individual optical section (10  $\mu\text{m}$  step intervals) show that the fluorescent lymph remains confined to the subcapsular sinus and does not penetrate the LN parenchyma. (see movie online).

DIAPYEDESIS

The passage of cells across a cellular barrier, such as the monolayer of tightly apposed endothelial cells in postcapillary venules.

Table 1 | Multi-step adhesion cascades in lymph-node HEVs determine homing specificity

	Step 1 Rolling	Step 2 Activation	Step 3 Adhesion	Step 4 Transmigration
	L-selection: PNAD	B cells CCL21: CCR7 CXCL12: CXCR4 T cells CCL21: CCR7 (CXCL12: CXCR4)	LFA1: ICAM1	
Naive B cells	Yes	Yes	Yes	Yes
Naive or central memory T cells	Yes	Yes	Yes	Yes
Monocytes/Neutrophils	Yes	No	No	No
Effector memory or effector T cells	No	No	No	No

Naive and central memory T cells, and naive B cells initiate rolling through L-selectin binding to peripheral node addressin (PNAD). High endothelial venules (HEVs) present CC-chemokine ligand 21 (CCL21) and, to a lesser degree, CCL19 and CXCL12 (CXCL12) to rolling lymphocytes. CCL21 and CCL19 activate CC-chemokine receptor 7 (CCR7), which induces rapid G $\alpha$ i-signalling and leukocyte function-associated antigen 1 (LFA1)-mediated arrest on endothelial intercellular adhesion molecule 1 (ICAM1) or ICAM2. B cells activate LFA1 also in response to CXCR4 stimulation by CXCL12. Following extravasation, B cells migrate to B-cell follicles — an event that depends on CXCR5–CXCL13 interactions. The restricted pattern of traffic molecules in HEVs ensures recruitment selectivity. Most myeloid cells express L-selectin and LFA1, but not CCR7 or CXCR4. Consequently, these cell types can roll, but do not arrest. Conversely, activated effector T cells or effector memory cells are L-selectin negative and fail to initiate the prerequisite rolling step.

are also counter-regulatory mechanisms. The lymphatic endothelium expresses the non-signalling serpentine receptor D6, which binds several chemokines, including CCL2 (REF 93). After ligation, D6 triggers internalization and degradation of its cargo and might therefore control the leakage of chemokines into the lymph<sup>94</sup>. HEVs express a similar non-signalling chemokine receptor — the Duffy antigen-related receptor for chemokines (DARC)<sup>95</sup>, which might also function in the transport, presentation or turnover of chemokines. However, the events that control the expression and function of either D6 or DARC are still poorly understood.

**Transendothelial migration.** Early electron-microscopy studies indicated that homing lymphocytes migrate into and across individual high endothelial cells before entering either the basolateral interendothelial junction or the subendothelial space<sup>2,96</sup>. However, others observed that DIAPYEDESIS occurs through interendothelial junctions<sup>97</sup>. There is also uncertainty about the adhesion-molecule requirements for lymphocyte passage across HEVs and is it not known whether chemoattractants are required. Exposure to hydrodynamic shear flow is sufficient *in vitro* to induce lymphocyte migration through endothelial monolayers without a chemotactic gradient<sup>73</sup>. However, in intravital microscopy experiments, T cells rarely emigrate into surgically exposed LNs even though they roll and arrest at a high frequency, and numerous T cells emigrate into undisturbed LNs of the same animal<sup>43,56</sup> (U. H. v. A., unpublished observations). This indicates that a crucial factor is lost during surgical tissue preparation that is required for lymphocytes to undergo diapedesis. As blood flow is always intact, fluid shear flow is probably not sufficient for lymphocyte diapedesis in HEVs.

Another interesting issue is whether, and to what extent, transendothelial migration occurs in an abluminal-to-luminal direction<sup>20</sup>. For example, homed lymphocytes in pig LNs leave through HEVs rather than through efferent lymphatics<sup>19</sup>. Electron-microscopy studies of rat LNs found that 92% of lymphocytes in HEVs migrated towards the nodal parenchyma, whereas the remainder were oriented towards the lumen<sup>97</sup>. So, reverse migration across HEVs is probably not unique for pigs, even though its frequency varies between species. The phenotype and immunological function of cells migrating in reverse and the traffic signals that control this unorthodox behaviour are unknown.

Box 2 | Targeting dendritic-cell vaccines to lymph nodes

Mature dendritic cells (DCs) express low levels or no L-selectin on their cell surface. If such DCs are injected intravenously, they accumulate in the spleen and non-lymphoid tissues, but not in the lymph nodes (LNs)<sup>77</sup>. The inability of mature DCs to home to LNs from the blood is an important obstacle to the use of antigen-pulsed DCs as vaccines. Most DC vaccination protocols require the injection of DCs into the skin or directly into the LNs. Inevitably, this approach will restrict DC traffic and, therefore, confine the vaccine effect to one or a few regional LNs. We have recently explored the hypothesis that DC vaccination might be improved if DCs could be manipulated to home to LNs throughout the body after intravenous injection. For this, DCs were retrovirally transformed with a chimeric E-selectin–L-selectin molecule — a substitute for L-selectin — that recognizes peripheral node addressin (PNAD)<sup>76</sup>. This molecule was chosen because expression of L-selectin does not change when DCs are retrovirally transfected with the full-length molecule, presumably because the ectodomain is proteolytically removed<sup>77</sup>. Importantly, L-selectin is clustered on the tips of microvilli on leukocytes, which greatly enhances its ability to form tethers under high shear flow<sup>147</sup>. Because E-selectin–L-selectin fusion protein contains the transmembrane and intracellular domain of L-selectin, it is also targeted to tips of microvilli<sup>76</sup>. The ectodomain derived from E-selectin is not cleaved, mediates slow rolling of DCs through PNAD interactions, and enables DCs to extravasate directly from blood through LN high endothelial venules<sup>77</sup>. So, by reconstituting a missing component of the LN-homing cascade, it is possible to target circulating leukocytes to the LNs.

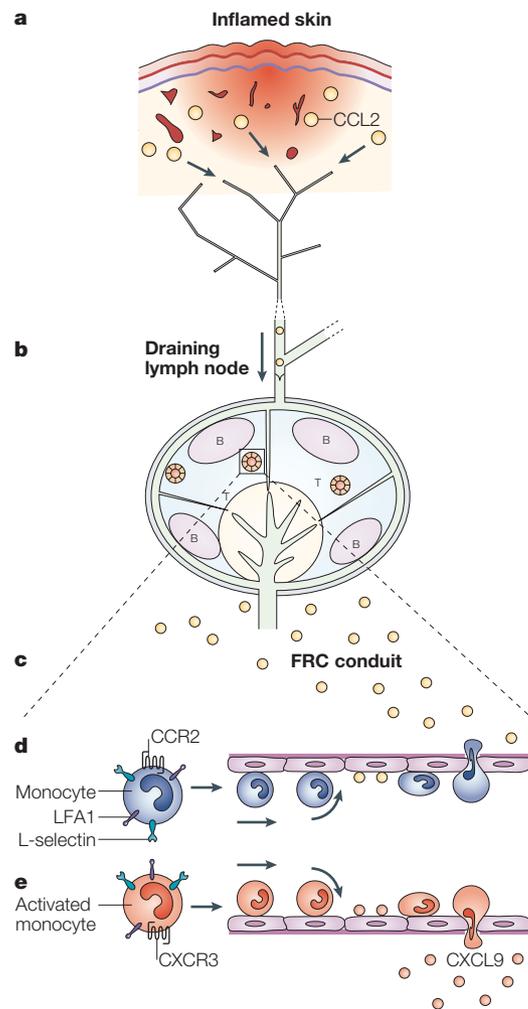
### Dendritic-cell migration through afferent lymph

Lymphocyte homing remains inconsequential unless lymphocytes are presented with antigen by DCs<sup>98</sup>. These cells capture and present antigen to naive T cells more efficiently than any other APC does, and so have a large impact on immune reactivity or tolerance to antigen<sup>99</sup>. In particular, DCs uniquely elicit CTL responses to exogenous-cell-associated antigens such as intracellular pathogens<sup>100</sup>. The nature of the DC–T-cell interaction also influences the functional programming of T<sub>H</sub>1 or T<sub>H</sub>2 cells. Moreover, T-cell activation by DCs from mesenteric LNs and Peyer's patches, but not from the spleen or cutaneous LNs, induces the expression of gut-homing receptors by effector cells, indicating that DCs assume a lymphoid-tissue-specific phenotype, which instructs T cells about where to find their antigen in the periphery<sup>12,13</sup>.

Afferent lymph is the main route by which antigens reach LNs. Two mechanisms of antigen delivery can be distinguished<sup>101</sup>. First, antigenic material becomes lymph borne and is taken up by DCs in a LN. Second, antigen is acquired by DCs in peripheral tissues and the DCs can then carry the processed antigen to draining LNs<sup>98</sup>. The former scenario requires that the LNs are continuously stocked with immature DCs that can uptake and process antigen (FIG. 5a). DENDRITIC-CELL MATURATION is required for potent T-cell priming, but mature DCs can no longer acquire exogenous antigens and are typically short lived<sup>98</sup>. Mouse LNs have at least six different DC subsets that are distinguished by differential expression of CD4, CD8 $\alpha$ , CD11b, CD11c, CD205 (DEC205), B220, MHC class II and Gr1 (Ly6C/G)<sup>102,103</sup>. CD11b-positive (myeloid) DCs are concentrated in the superficial paracortex, well positioned to capture lymph-borne proteins from the subcapsular sinus<sup>40</sup>. The progeny of Langerhans cells — an epidermal DC population — as well as CD8 $\alpha^{\text{hi}}$  (lymphoid) DCs and plasmacytoid DCs are distributed throughout the T-cell area in LNs<sup>40,103,104</sup>. The route(s) by which LNs are supplied with these resident subsets are probably diverse. Langerhans cells give rise to CD8 $\alpha^{\text{low}}$  or CD8 $\alpha^+$  DCs in LNs<sup>105</sup>, indicating that this population enters the LNs through the lymph. Plasmacytoid DCs express L-selectin, and it has been proposed that they migrate to LNs through HEVs<sup>103,106</sup>. Antibody specific for L-selectin blocks the marked increase of both CD8 $\alpha^+$  and CD8 $\alpha^-$  DCs in LNs of virus-infected mice, but conclusive proof for L-selectin-dependent DC homing through HEVs awaits careful homing experiments.

The dynamics and mechanisms of peripheral DC traffic have been studied extensively. Unlike other tissue-resident DCs, which must be replenished by bone-marrow-derived precursors that differentiate into immature DCs, Langerhans cells can self renew in the skin<sup>107</sup>. However, tissue inflammation elicits the recruitment of circulating DCs and of monocytes, which can locally differentiate into DCs<sup>108,109</sup>. Immature DCs patrol tissues and engulf microorganisms, dead cells and cellular debris, and break them down into peptides that are loaded onto MHC complexes. After exposure to inflammatory stimuli, tissue DCs undergo maturation

whereby the expression of receptors for inflammatory chemokines is lost, whereas the expression of those for lymphoid chemokines, particularly CCR7, CXCR4 and CCR4 is upregulated<sup>110</sup>. Maturation allows DCs to enter lymph vessels (FIG. 5b) and to access the T-cell area in draining LNs<sup>72</sup> (FIG. 5c). While in transit, DCs upregulate the expression of apparatus for antigen presentation and T-cell stimulation, and begin to generate chemokines that allow them to attract T cells<sup>110–114</sup> (FIG. 5d). They also



**Figure 4 | Remote control of monocyte recruitment to lymph nodes in inflammation.**

**a** | Inflammation at a peripheral site such as the skin induces the production of inflammatory chemokines, particularly CC-chemokine ligand 2 (CCL2). **b** | Inflammation induces increased microvascular permeability leading to local oedema and enhanced lymph flow. Lymph-borne CCL2 and other inflammation-induced chemokines are transported to the subcapsular sinus in draining lymph nodes. **c** | The fibroblastic reticular cell (FRC) conduit channels CCL2 from the subcapsular sinus to perivascular spaces adjacent to high endothelial venules (HEVs). **d** | After transendothelial vesicular transport to the HEV lumen, CCL2 is presented to rolling monocytes and triggers CC-chemokine receptor 2 (CCR2)-dependent integrin activation. **e** | A subset of activated monocytes, which express CXCR3, can be recruited by a second pathway involving CXCL9. The cellular source of CXCL9 is unknown.

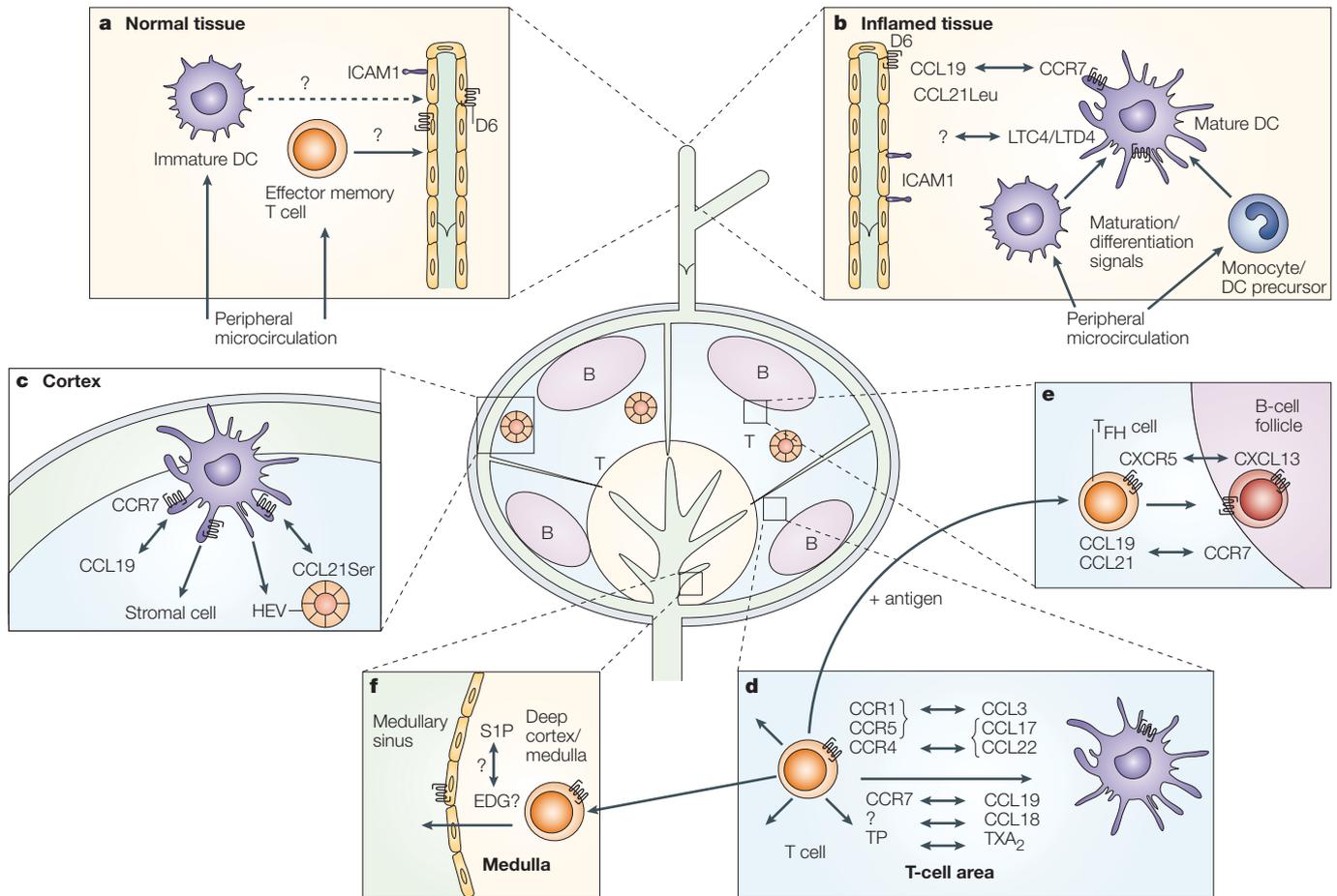
#### DENDRITIC-CELL MATURATION

The process by which dendritic cells (DCs) are reprogrammed from a mainly antigen collecting to an antigen presenting, immunostimulatory mode. Maturation can be induced by signals associated with tissue damage, inflammation or infection, resulting in enhanced cell-surface presentation of MHC complexes and co-stimulatory molecules and altered trafficking, enabling them to enter the T-cell area of secondary lymphoid tissues.

secrete thromboxane A<sub>2</sub> (TXA<sub>2</sub>), which might increase chance encounters with T cells by enhancing their random motility<sup>15</sup>.

CCR7-deficient DCs fail to migrate from the skin into lymphatics<sup>71</sup>, whereas DCs in *plt/plt* mice still gain access to lymphatics (which express the CCL21Leu isoform), but they fail to penetrate the cortex after they have arrived in the draining LNs<sup>72</sup>. Lymph vessels also express CCL19, but DCs require autocrine production of cysteinylated leukotrienes to respond to this chemokine<sup>116</sup>. Access to lymphatics probably requires (presumably chemokine induced) the activation of β<sub>2</sub> integrin on DCs, because DC entry is compromised in ICAM1-deficient lymphatics<sup>17</sup>.

After a naive T cell has found a DC that expresses its cognate antigen, it must decide whether and in what way to respond. Ideally, foreign pathogens should provoke T-cell activation and effector activity, whereas self-antigen should induce tolerance or anergy. How this distinction is made is one of the most important riddles in immunology. DCs might hold the key to this question. First, different DC populations can have preferential stimulatory or suppressive functions<sup>99,118</sup>. Second, the rate of DC migration into LNs might have a role. Local pathogen-induced inflammation usually attracts large numbers of monocytes and/or immature DCs to tissues<sup>108</sup>. These newly recruited cells differentiate into mature DCs and, together with activated resident DCs,



**Figure 5 | Interstitial traffic signals for T-cell and dendritic-cell migration in lymph nodes.** Directions of cell migration are indicated by arrows; relevant traffic molecules are shown and receptor–ligand pairs are indicated by double-headed arrows. For a description of anatomical features in lymph nodes (LNs; centre) refer to FIG. 1. **a** | Immature dendritic cells (DCs) (or their precursors) and effector memory cells home to peripheral tissues. Occasionally, they enter lymph vessels and migrate to draining LNs. These interstitial leukocytes are thought to be CC-chemokine receptor 7 (CCR7) negative; the chemoattractant(s) for their entry into lymph vessels are unknown. **b** | Inflammation in peripheral tissues causes enhanced recruitment and maturation of DCs, which upregulate the expression of CCR7 and secrete cysteinylated leukotrienes (LTC4 and LTD4) to detect CC-chemokine ligand 21Leu (CCL21Leu) and CCL19 in lymphatics. As a result, large numbers of mature DCs are transported to the subcapsular sinus in draining LNs. **c** | DCs are then attracted into the paracortex by CCR7 agonists produced by high endothelial venules (HEVs) and stromal cells. **d** | T cells that have entered the T-cell area migrate rapidly and in random directions, possibly in response to DC-derived thromboxane A<sub>2</sub> (TXA<sub>2</sub>). Mature DCs also secrete several chemokines that attract T cells. **e** | Activation of CD4<sup>+</sup> T cells by antigen induces differentiation into CXC-chemokine receptor 5 (CXCR5)-positive follicular helper T (T<sub>FH</sub>) cells, which are attracted by CXCL13 in B-cell follicles. **f** | Lymphocytes exit LNs through sinuses in the medulla. Synthetic agonists for sphingosine-1-phosphate (S1P) receptors block this step. Whether endogenous S1P regulates lymphocyte exit from LNs has not been determined. ICAM1, intercellular adhesion molecule 1.

**Box 3 | Multi-photon microscopy**

Multi-photon imaging allows three-dimensional time-lapse imaging of fluorescent signals deep below the surface of living tissues, allowing cell migration and cell–cell interactions to be tracked. The fluorescent images are of slightly lower resolution compared with those obtained by traditional confocal microscopy. However, multi-photon imaging has several notable advantages compared with other fluorescence-based techniques, including: greatly diminished phototoxicity to living cells; reduced bleaching of fluorescent dyes; the ability to excite several different fluorophores with the same excitation beam; the phenomenon of second harmonic generation of ultraviolet photons by collagen fibres, which allows non-invasive imaging of extracellular matrix components<sup>148</sup>; and deeper tissue penetration (in our hands, the maximal imaging depth in mouse lymph nodes is ~100 µm for confocal microscopy versus ~400 µm for multi-photon excitation)<sup>121</sup>.

migrate to the draining LNs, allowing for strong and sustained T-cell stimulation. By contrast, without inflammation DCs with weak stimulatory capacity migrate to LNs in numbers too low to drive T-cell activation. The resulting sub-threshold stimulation of autoreactive T cells could promote and sustain T-cell tolerance to self-antigen<sup>99,118</sup>.

**Beyond HEVs: how T cells find their antigen**

Throughout the twentieth century, the migratory behaviour of lymphocytes and DCs in the LN parenchyma has been inferred from increasingly sophisticated examination of histological sections. These studies indicated that T cells were initially activated in the T-cell area, especially the superficial paracortex, whereas the antigen-specific B cells were scattered throughout the B-cell follicles. Later on, CD4<sup>+</sup> T cells localized to the follicle edges where they provided help to antigen-specific B cells and promoted the formation of germinal centres<sup>40,119</sup>. A limitation of histology-based techniques is that they provide snapshots of cellular localization, but cannot resolve the true dynamics of interstitial cell migration.

Ongoing technological innovations, in particular multi-photon microscopy (BOX 3), are revolutionizing our ability to track individual migrating cells in living tissue<sup>120,121</sup>. Strategies to visualize lymphocyte and DC migration in mouse LNs were recently described<sup>122,123</sup>. In experiments by Stoll *et al.*<sup>123</sup>, fluorescent-labelled TCR-transgenic lymphocytes were injected intravenously and antigen-pulsed DCs labelled with a different fluorophore were injected into the skin. The draining LNs were then removed and maintained in a culture dish in normal air while they were visualized by confocal microscopy at an imaging depth of up to 80 µm below the surface<sup>123</sup>. Within a day, the tagged DCs and lymphocytes had homed to LNs through afferent lymphatics and HEVs, respectively. In this study, naive T cells were virtually stationary and migrated relatively slowly (5–7 µm/min) after prolonged exposure to antigen. In the presence of antigen, T cells and DCs formed stable clusters at a 1:1 ratio for over 15 hours<sup>123</sup>. By contrast, in a parallel study by Miller *et al.*<sup>122</sup>, removed LNs were maintained in a 95% oxygen environment and lymphocytes were visualized up to 350 µm below the surface

using multi-photon microscopy. These authors found that T cells migrate faster, on average, than follicular B cells (10.8 µm/min versus 6.4 µm/min). Both subsets migrated in random directions, a behaviour that is inconsistent with the idea that lymphocyte migration is governed over long distances by chemotactic gradients. Although random migration was also observed by Stoll *et al.*, the T cells observed by Miller *et al.* were more dynamic. Moreover, although DCs were not directly visualized by Miller *et al.* the kinetics and geometry of T-cell migration in response to antigen indicated that each DC might interact with many T cells and that only a minority of T-cell–DC interactions are long lived<sup>122</sup>. This idea was recently substantiated in another multi-photon study of excised LNs by Bousso and Robey<sup>124</sup> who observed that a single DC might contact as many as 500 different T cells per hour and engage more than 10 T cells simultaneously.

The discrepancies between the studies by Stoll *et al.*<sup>123</sup> and Miller *et al.*<sup>122</sup> could have several reasons, including the difference in imaging depth or tissue oxygenation<sup>125</sup>. We have observed that cardiovascular arrest is followed within minutes by a marked loss of lymphocyte motility in the LNs of anaesthetized mice (U.H.v.A and T.R.M., unpublished observations). Given that oxygen diffusion through metabolically active tissues is only sufficient to a distance of beyond 100 µm<sup>126</sup>, LNs that are removed and cultured in ambient air will probably have hypoxic regions in which cellular motility might be compromised. Hyperoxic culture conditions might alleviate this problem<sup>122,124</sup>. However, supraphysiological oxygen concentrations enhance oxidative stress in living tissues and by shifting redox balances might alter lymphocyte behaviour<sup>127,128</sup>. So, it remains to be seen whether studies of excised LNs can faithfully reproduce the multi-faceted events that occur *in vivo*. Moreover, as discussed earlier, the LN environment is modulated by afferent lymph flow, and LN homeostasis is also influenced by the autonomous nervous system<sup>129</sup>. Clearly, the definitive experiments will be intravital observations of LNs of live animals.

The first report of multi-photon intravital microscopy in surgically exposed mouse inguinal LNs has been published recently<sup>130</sup>. This study found that naive CD4<sup>+</sup> T cells migrate *in vivo* with high speed and random directionality<sup>130</sup>. So, it was proposed that naive T cells are preprogrammed to scan the T-cell area for the presence of antigen-presenting DCs randomly<sup>130</sup>. The study also visualized T cells immediately after they entered the LNs across HEVs. The homed cells migrated rapidly away from the entry site in random directions and without apparent delay. This is somewhat surprising given that HEVs are surrounded by an elaborate system of channels and corridors<sup>21</sup> (FIG. 1b). It had been proposed that emigrating lymphocytes might be delayed during their passage through perivenular channels and that homed cells would initially move in a spiralling motion around HEVs, following preformed corridors<sup>21</sup>. Such an orchestrated migratory behaviour was not detected *in vivo*<sup>130</sup>.

Observations in explanted LNs indicate that B cells, too, have a similar random walk, at least in follicles<sup>122</sup>. However, it is probable that directed migration occurs in LNs in other situations, for example when newly homed B cells traverse the T-cell area to access follicles. This process is mediated by **CXCL13** (also known as B-cell-homing chemokine, BLC) — a chemokine that is generated in B-cell follicles and stimulates **CXCR5** (REFS 131,132). Most T cells do not express CXCR5, but when CD4<sup>+</sup> T cells encounter antigen they transiently upregulate the expression of this receptor and down-regulate the expression of CCR7, which allows them to disengage from the T-cell area and migrate to the B-cell follicles<sup>133</sup> (FIG. 5e). As CXCR5-expressing CD4<sup>+</sup> T cells express prerequisite cell-surface molecules for B-cell help, the term follicular helper T (T<sub>FH</sub>) cell has been proposed for them<sup>134</sup>.

**Lymphocyte exit from lymph nodes**

When T cells fail to encounter antigen, they exit the LN through efferent lymph vessels (and sometimes through HEVs). We have little knowledge about what determines how long lymphocytes stay in the LNs. A few adhesion molecules have been identified on intranodal lymphatics. These include the pan-endothelial-cell marker **CD31** — the mannose receptor, which interacts with L-selectin<sup>39</sup> — and common lymphatic endothelial and vascular endothelial receptor 1 (CLEVER1), an adhesion molecule with as-yet-unknown ligand specificity that is also expressed by HEVs<sup>135</sup>.

Classic experiments in sheep have shown that antigen challenge precipitates a transient (6–18 hours) reduction in lymphocyte egress into efferent lymph vessels despite a several-fold increase in lymphocyte recruitment through HEVs<sup>52</sup>. The decrease in lymphocyte output is most marked with viral and bacterial challenges and is lower or absent in response to non-infectious stimuli. Over several days after challenge, cell output in efferent lymph rises markedly, reaching a peak at day 3 to 4 (REF. 52).

Although the signals that regulate the magnitude of lymphocyte entry and egress in LNs are still unknown, a hint has come from studies of FTY720 — a synthetic derivative of the antifungal agent myriocin<sup>136</sup>. FTY720 causes immunosuppression in animal models of transplantation and autoimmunity<sup>137</sup>. Initial observations showed that FTY720 causes T-cell apoptosis<sup>138</sup>, but subsequent studies have raised doubt that its pro-apoptotic activity is responsible for immunosuppression<sup>139</sup>. The drug markedly decreases the number of circulating lymphocytes, and increases lymphocyte numbers in the

LNs<sup>140</sup>. FTY720 treatment makes T-cell homing to LNs less dependent on CCR7 (REF. 141). Interestingly, FTY720 also prevents lymphocyte egress from the thymus<sup>142</sup> and from the LNs into efferent lymphatics<sup>143</sup> (FIG. 5f).

Recent studies have shown that FTY720 is phosphorylated by sphingosine kinase to an active metabolite that resembles sphingosine-1-phosphate (S1P) and binds to four of the five known S1P receptors, S1P<sub>1</sub>, S1P<sub>3</sub>, S1P<sub>4</sub> and S1P<sub>5</sub> (also known as endothelial differentiation, sphingolipid G-protein-coupled receptor 1 (EDG1), EDG3, EDG6 and EDG8, respectively)<sup>143,144</sup>. S1P<sub>1</sub> and S1P<sub>4</sub> are found on lymphocytes and can modulate lymphocyte responsiveness to chemokines<sup>145</sup>. Recent findings indicate that FTY720 enhances the efflux of cysteine leukotrienes from T cells, and this effect was required for its activity in T-cell homing<sup>146</sup>. S1P receptors are also expressed by the endothelium. So, some of the effects of FTY720 could be caused by action on vascular or lymphatic endothelial cells. Whatever the precise mechanism(s), together these observations raise the possibility that S1P and its receptors are involved in regulating the overall lymphocyte content in LNs.

**Future questions**

Despite considerable progress in our understanding of LNs, many fundamental questions remain to be answered. For example, what are the signals that induce and maintain the unique phenotype of HEVs in LNs? It is known that functional HEVs require intact lymph flow<sup>27</sup>, but the responsible lymph-borne factor(s) and their mechanism(s) of action remain(s) to be identified. Another unresolved issue is that intraperitoneal injection of a protein antigen can elicit T-cell responses in distant LNs that do not receive lymph from the injection site<sup>11</sup>. How does such antigen enter LNs, how is it processed and presented? In addition, there are many questions concerning DCs. What are the migratory properties and immunological functions of the many different DC subsets in LNs? Do LN DCs in different anatomic contexts provide imprinting signals for tissue specificity of effector and effector memory cells, as has been shown for Peyer’s patches and mesenteric LNs<sup>12,13</sup>? If so, what are the mechanisms of imprinting? With regard to lymphocyte migration in the LN, what stimulates the frantic migratory activity of T and B cells<sup>122,130</sup>? And how is lymphocyte exit controlled? Finally, how might pathogens and metastatic tumours circumvent, exploit or subvert the mechanisms described in this article? Answers to these questions might allow us to design improved vaccination strategies and treatments for the many diseases in which LNs are involved.

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