OPINION

Lymphatic and interstitial flow in the tumour microenvironment: linking mechanobiology with immunity

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Abstract | Tumours often engage the lymphatic system in order to invade and metastasize. The tumour-draining lymph node may be an immune-privileged site that protects the tumour from host immunity, and lymph flow that drains tumours is often increased, enhancing communication between the tumour and the sentinel node. In addition to increasing the transport of tumour antigens and regulatory cytokines to the lymph node, increased lymph flow in the tumour margin causes mechanical stress-induced changes in stromal cells that stiffen the matrix and alter the immune microenvironment of the tumour. We propose that synergies between lymphatic drainage and flow-induced mechanotransduction in the stroma promote tumour immune escape by appropriating lymphatic mechanisms of peripheral tolerance.

The spread of tumour cells to sentinel, tumour-draining lymph nodes (TDLNs) has been documented for centuries. Most carcinomas first metastasize to the TDLNs, and the presence of cancer cells in TDLNs is a major prognostic indicator in skin, breast, colon and other cancers. Tumour cells are thought to gain access to a TDLN by recruiting and entering local lymphatic vessels, which provide a physical connection between the tumour and its TDLN. As such, lymphatic vessels in the tumour microenvironment have an important role as a route for dissemination. However, the lymphatic vessels, and the interstitial and lymphatic flows that they enable, have other roles that may be equally or even more important to the survival and metastasis of the tumour.

Importantly, lymph nodes are major sites of immune regulation where antigen-specific immune responses are directed against both foreign or pathogenic antigens and self-antigens that are drained from the upstream peripheral tissue. This results in the establishment of productive immunity against pathogens or the maintenance of immunological tolerance to self-antigens. The TDLN is constantly bathed in cytokines and antigens deriving from the tumour, which are carried there by the tumour-associated lymphatic vessels, as well as antigen-presenting cells (APCs) from the tumour microenvironment. Lymph flow from tumours has been reported to be elevated compared with that from normal tissue[1–3], and increased lymph drainage has been positively correlated with metastasis[4]. Thus, lymphatic drainage from the tumour along with tumour recruitment of surrounding lymphatic vessels are likely to have important roles in modulating the host anti-tumour immune responses.

Increased fluid drainage from the tumour microenvironment, which is enabled by the tumour-draining lymphatics, along with heightened pressure gradients (described below), implies that interstitial flow is increased at the tumour margin[5,6]. This heightened interstitial flow through the tumour stroma, in turn, induces mechanical stress on the extracellular matrix (ECM) and stromal cells[7], which can increase transforming growth factor-β (TGFβ) expression and activation, myofibroblast differentiation and stromal stiffening. Such stromal changes might have important implications for suppressing anti-tumour immune responses in the tumour stroma through multiple mechanisms.

We suggest that increased interstitial and lymphatic flow in the tumour microenvironment, and the resulting mechanical changes to the tumour stroma, may strongly alter host immunity to the tumour. We review the intersection between three traditionally unrelated topics: first, tumour and TDLN lymphangiogenesis, lymphatic drainage and the changes in interstitial flow in the tumour microenvironment that they cause; second, interstitial flow and its mechanobiology on the tumour stroma; and third, the inflammatory microenvironment of the tumour and its draining lymph node, which suppress anti-tumour immunity. Therefore, we propose that lymphatic drainage links tumour mechanobiology with tumour immunology (FIG. 1).

Flow in the tumour microenvironment

Pressure gradients in the tumour margin drive interstitial flow. Lymphatic flow is an important component of the circulation. In nearly all tissues, plasma leaks out of blood capillaries, flows through the interstitium and drains into lymphatic vessels, where it passes through lymph nodes before being returned to the venous blood. Interstitial fluid is a plasma filtrate, and fluid flow between the blood, interstitial and lymphatic compartments at the microcirculatory level is considered to be governed by Starling forces (the net differences between hydrostatic pressure and osmotic pressure). Newly formed lymph has essentially the same composition as interstitial fluid, as there is very little filtration of interstitial fluid when traversing the lymphatic endothelium. This afferent lymph enters the lymph node where it is filtered owing to protein and particulate uptake by the immune cells there; the efferent lymph exiting the lymph node thus has a different protein composition from that of the afferent lymph[8,9].

Since the 1970s, it has been recognized that the balance of fluid between the blood, interstitium and lymph is altered in solid tumours[10,11] (FIG. 1). The hypoxia that is generated by a rapidly growing tumour mass drives aberrant tumour angiogenesis, which is one of the most well-studied hallmarks of cancer[12]. Tumour angiogenesis is rapid and

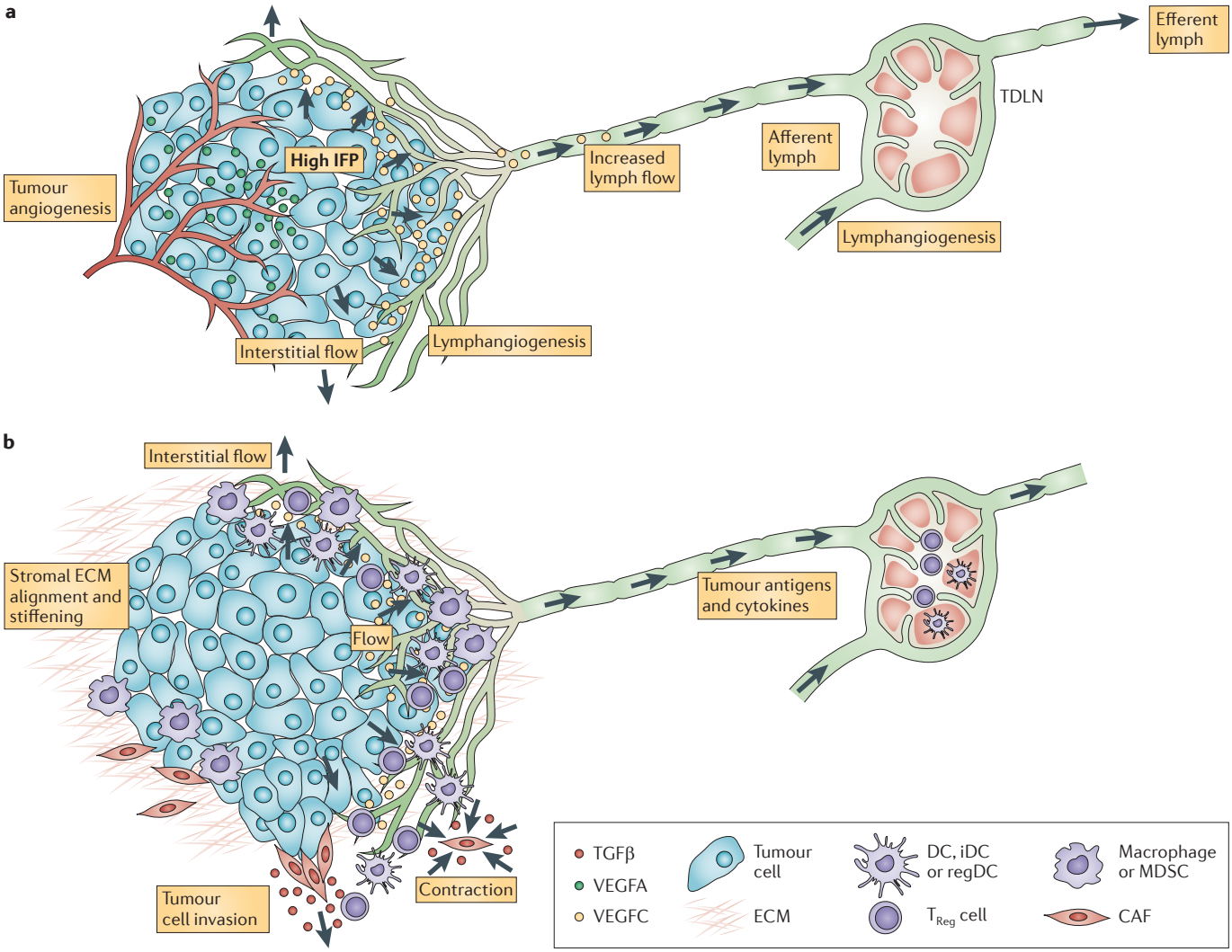

Figure 1 | **Linking mechanobiology, lymphangiogenesis and anti-tumour immunity.** Lymphangiogenesis, mechanobiology and immunity in the tumour microenvironment contain many interdependent features. **a** | Fluid pathways in the tumour microenvironment are shown. Angiogenic, immature tumour vessels (shown in red) are hyperpermeable, driving heightened interstitial fluid pressure (IFP) in the tumour. This creates steep pressure gradients at the tumour margin that drive interstitial flow through the stroma and into peritumoral lymphatic vessels (green), which are often expanded and hyperplastic. These lymphatic vessels carry tumour interstitial fluid to the sentinel or tumour-draining lymph node (TDLN), where lymphangiogenesis is also seen. **b** | The heightened interstitial and lymphatic flows (green) in the tumour microenvironment coincide with biomechanical (red) and immunological (purple) changes in the tumour stroma. Biomechanical changes include stiffening and alignment of the extracellular matrix (ECM) of the tumour stroma, owing to cancer-associated fibroblast (CAF) contraction and remodelling that is dependent on transforming growth factor-β (TGFβ). Matrix stiffening promotes tumour cell invasion through CAF-led collective migration and activation of mechanically sensitive stromal components. Interestingly, stromal features of the tumour can mimic those of the TDLN. Immunological changes include biasing of the tumour-infiltrating lymphocyte populations by local factors. Tumour-promoting cytokines promote alternatively activated (M2) macrophages and myeloid-derived suppressor cells (MDSCs), hinder the maturation of immature dendritic cells (iDCs) and stimulate regulatory DCs (regDCs), which in turn promote regulatory T (T_{Reg}) cells and suppress local cytotoxic T cell activity. Similar cell populations are biased in the TDLN. VEGF, vascular endothelial growth factor.

haphazard, leading to leaky tumour vessels and the accumulation of macromolecules, such as albumin, from the plasma into the tumour tissue. At the same time, ECM production and remodelling at the tumour margin generates mechanical stress[13,14], which, together with the leaky vessels, leads to increased interstitial fluid pressure (IFP) within the tumour[11,15]. Whereas normal tissue pressures range from -2 to 0 mm Hg[16], tumour IFP can be as high as capillary pressure[17], with values reported in humans in the range of 10–40 mm Hg[11,15]. Importantly, vessel normalization by anti-angiogenic agents can partially decrease tumour IFP[15,18]. The heightened IFP in the tumour leads to pressure gradients at the tumour margin[5,6], which in turn may drive heightened interstitial flow in the tumour stroma and into surrounding lymphatic vessels.

Interstitial flow in the tumour microenvironment is heterogeneous and difficult to measure directly. Butler *et al.*[10] first measured IFP in chambers implanted in mammary tumours in rats, and reported bulk fluid transfer out of the tumour by comparing red blood cell concentrations in the afferent and the efferent blood vasculature of the tumour[10]. In mice, magnetic resonance imaging has been used to demonstrate

increased fluid convection in the peritumoral tissue[1] or increased lymphatic drainage to the sentinel lymph node[2,3,19]. Thus, although few direct measurements of interstitial flow in the tumour microenvironment have been reported, increased lymph flow to the TDLN along with high pressure gradients at the tumour margin should physically reflect increased interstitial flow in the tumour stroma.

Tumour lymphangiogenesis and increased flow. In addition to inducing angiogenesis, tumours can also drive lymphangiogenesis or lymphatic hyperplasia in their microenvironment; in fact, lymphangiogenesis and lymphatic expansion is seen in many types of chronic inflammation[20–24]. The molecular regulation of lymphangiogenesis is well-described elsewhere[25–27] and is not discussed here. Importantly, however, in many human and murine tumours, increased lymphatic density and expression of the lymphangiogenic vascular endothelial growth factor C (VEGFC) and VEGFD are correlated with poor prognosis, invasion and metastasis[25,28,29]. VEGFC secretion by tumours also increases lymph flow from the tumour to the TDLN[4,30]. Even when tumours do not explicitly express lymphangiogenic factors or induce lymphangiogenesis, their draining lymph nodes undergo lymphangiogenesis before metastatic cells are seen in the TDLN[2,3,25,29]. Pathologists have described tumour-associated lymph node lymphangiogenesis as sinusoidal hyperplasia, which is a phenotype that correlates with metastasis[31]. Taken together, these observations suggest that lymph node lymphangiogenesis plays an important part in 'preparing the soil' for metastatic dissemination. The specific functional roles of this expanded lymphatic network and the mechanisms by which it might promote metastasis, however, remain unclear.

Lymphangiogenesis in the tumour margin and TDLN may also be affected by the increased interstitial flow in the tumour stroma that drains to the TDLN. In dermal tissue regeneration, interstitial flow was found to be an important contributor to lymphangiogenesis[32]. Furthermore, interstitial flow helps to organize lymphatic capillaries *in vitro*[33] partly by guiding local gradients of proteases and growth factors[34], as well as by direct interactions between the ECM and lymphatic endothelium[35]. Fluid flow in the tumour margin may also cause channelling and may increase mechanical heterogeneity in the tumour microenvironment, altering the trafficking of macrophages that help to guide lymphangiogenesis[21,28,36]. In support of this, Raju *et al.*[37] reported a striking correlation between IFP and lymphatic vessel density in rat squamous cell carcinoma, which also correlated with cancer progression[37]. However, direct studies of the effects of flow on tumour lymphangiogenesis are lacking, as it is not experimentally feasible to alter tumour interstitial flow in a selective way.

In summary, the strong clinical and experimental correlations observed among peritumoral and lymph node lymphangiogenesis or expansion, pressure gradients at the tumour margin and fluid flow to the draining lymphatics, and tumour progression bolster the idea that lymphatic vessels and the TDLN support the tumour in multiple ways. Although one mechanism is to provide a physical transport route for tumour dissemination, we point out below how lymphatic drainage and tumour-associated lymphangiogenesis may alter the tumour microenvironment in other important ways.

Flow effects on the tumour stroma

As described above, heightened interstitial flow is present in the stroma of most solid tumours. Several recent *in vitro* studies have provided clues about how flow might specifically affect tumour cells and cancer-associated fibroblasts (CAFs) in the tumour stroma using three-dimensional (3D) cell culture insert chambers with imposed flow[38,39] or specially designed microfluidic chambers[40,41](FIG. 2).

Flow activates TGFβ and fibroblasts to stiffen the ECM. Interstitial flow has important effects on fibroblasts and the ECM that are highly relevant to the tumour stroma (FIG. 3). In normal tissues, interstitial flow is thought to be in the order of 0.1–1 μm per second[1,42], and interstitial and lymph flows from the tumour margin are substantially increased[1–3,10,30]. In experimental 3D culture models, it was found that interstitial flow of 3–10 μm per second can cause fibroblasts to align collagen fibres within 12–24 hours[40].

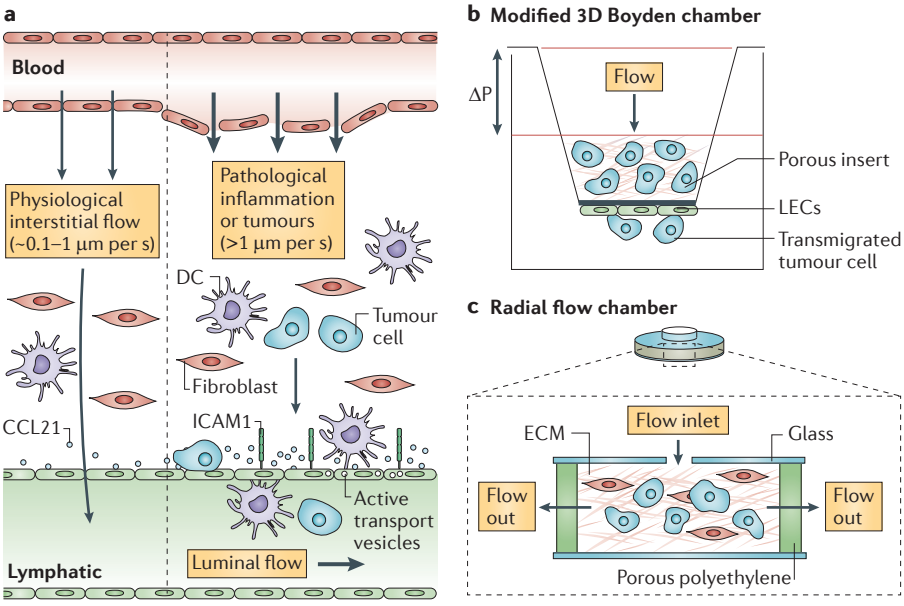

Figure 2 | **Modelling the biomechanical aspects of the tumour microenvironment *in vitro*.** **a** | Normally (left), Starling forces are thought to drive low levels of interstitial flow between blood and lymphatic vessels. At the onset of inflammation, vascular permeability is rapidly upregulated, increasing interstitial flow by up to an order of magnitude. Lymphatic endothelial cells (LECs) respond to these changes by increasing permeability and active transport mechanisms, and by upregulating their expression of the chemokine CCL21 and intercellular adhesion molecule 1 (ICAM1) to enhance dendritic cell (DC) and tumour cell trafficking. **b** | To recapitulate certain features of this three-dimensional (3D) interstitial flow microenvironment *in vitro*, a modified 3D Boyden chamber can be used. LECs are seeded on the underside of the porous insert, and an extracellular matrix (ECM) such as type I collagen, containing tumour cells or DCs, is added to the insert. Interstitial flow is controlled by maintaining a fixed pressure head (ΔP), and transmigrated cells can be counted. **c** | The radial flow chamber[33,40,129], along with more recent microfluidic devices that capture similar features[41], allow the direct observation of cells in 3D matrices under interstitial flow. In the radial flow chamber, the cell-containing ECM is sandwiched between two glass coverslips and anchored with porous polyethylene boundaries; ECM remodelling can also be visualized in real time using confocal reflectance microscopy.

Matrix alignment is caused by fibroblast contraction, which can alter the matrix up to several cell lengths away, and such contraction-mediated alignment causes matrix stiffening both locally and globally[43]. One mechanism for flow-induced fibroblast contraction and matrix remodelling is flow-induced upregulation of TGFβ by fibroblasts[40], which causes myofibroblast differentiation, triggering further contraction and matrix stiffening, as well as TGFβ production. When TGFβ signalling was blocked by TGFβ receptor-neutralizing antibodies, the interstitial flow-induced changes were abrogated. In addition to upregulating TGFβ production by fibroblasts, flow-induced matrix contraction and shear stress can activate the stores of latent TGFβ in the ECM[44,45]. Therefore, flow activates myofibroblast differentiation, as well as alignment and stiffening of the ECM via TGFβ-dependent mechanisms.

Stromal stiffening promotes tumour progression and invasion. How can stiffening the stroma affect tumour progression? The work of several groups has demonstrated that stromal stiffening promotes tumour initiation, progression and invasion, at least in breast cancer, where it has been the most extensively studied[46–49]. Increased mammary tissue density correlates with the poor prognosis of patients with breast cancer, and collagen alignment at the invasive edge of breast tumours is negatively correlated with survival[50]. The expression of lysyl oxidase, which crosslinks and stiffens collagen, is increased in malignant lesions in humans in response to intratumoral hypoxia and reactive oxygen species[51,52], and lysyl oxidase expression is conversely downregulated by pro-inflammatory cytokines such as tumour necrosis factor (TNF)[53]. Additionally, transgenic expression of lysyl oxidase in mice could induce the invasion of otherwise non-invasive cancer cells[54]. CAFs often exhibit myofibroblastic features[55] and express caveolin 1, which drives RHOA and force-dependent contraction, matrix alignment and stromal stiffening; these collectively promote directional migration[56]. Migrating CAFs, in turn, use proteases and generate forces to tunnel through the matrix, thereby leading collective tumour cell invasion[57,58] either by physical interactions or by the secretion of chemokines such as CXCL12 (REF. 59). Fibroblasts and tumour cells can also migrate along bundled collagen fibres that radiate outwards from the tumour[60,61]. Thus, stromal stiffening and remodelling is associated with tumour progression and invasion. As fluid flow upregulates TGFβ expression and activation from latent stores in the matrix, which in turn activates fibroblasts to be more invasive and biosynthetic, fluid flow can promote tumour cell invasion via indirect actions on fibroblasts.

Cells can sense matrix stiffness either through endogenous force generation (contraction) or through exogenous forces such as interstitial flow. Stress is transmitted across the cell–matrix interface through focal adhesions, activating ERK and RHOA–Rho-associated protein kinase (ROCK), in turn increasing myosin activity and actin assembly[14,46,47,62]. Softer matrices generate less tension following cell contraction, and similarly, smaller exogenous forces generate less tension on the focal adhesions. Thus, the effects of interstitial flow on the stromal matrix may be self-reinforcing: flow drives matrix tension and pulls on cell–matrix contacts, leading the cells to respond by aligning and stiffening the ECM, in turn generating more tension on the cells upon contraction, which makes them more sensitive to interstitial flow (FIG. 3).

Direct flow effects on cell migration. As for direct effects on tumour cells, flow has been shown to increase their invasiveness, at least *in vitro*. One suggested mechanism has been autologous chemotaxis[39,41,63]. For example, the secretion of the chemokine CCL21 by tumour cells that also express its receptor CCR7 leads to the formation of local (pericellular) gradients of CCL21 under fluid flow that drive chemotaxis and invasion in the flow direction. At the same time, fluid flow can also cause matrix tension behind the cell that promotes upstream migration; these two mechanisms may compete in a uniformly distributed tumour cell population *in vitro*[41]. Interstitial flow can also upregulate matrix metalloproteinases to further enhance tumour cell invasion[64].

However, because tumour cells are often found following fibroblasts in invasive tumour margins[57,58,61], flow effects on CAF migration may be more relevant than direct flow effects on tumour cells. First, interstitial flow can increase overall fibroblast motility[65]. Second,

Glossary

Cancer-associated fibroblasts
(CAFs). Heterogeneous population of fibroblasts found in the tumour stroma that often exhibit myofibroblast features. They are responsible for stromal stiffening and can lead collective tumour cell invasion.

Dendritic cells
(DCs). The most potent antigen-presenting cells that can activate T cells and thereby induce antigen-specific immune responses.

Fibroblastic reticular cells
(FRCs). Lymph node stromal cells of the paracortical reticular meshwork, a specialized structure that directs the interactions between dendritic cells and T lymphocytes. FRCs express podoplanin (GP38), which is a key component of the reticular meshwork, and secrete cytokines such as CCL21 and CCL19 to attract lymphocytes and maintain their homeostasis. Importantly, FRC features can be exhibited by CAFs, and such lymphoid-like stromal components have been correlated with tumour invasion and metastasis.

Interstitial flow
Fluid flow within the interstitium, driven by pressure gradients between the blood, interstitial and lymphatic compartments. Elevated tumour interstitial fluid pressure or increased lymphatic drainage can cause increased interstitial flow in the tumour stroma.

Interstitial fluid pressure
(IFP). Hydrostatic pressure in the interstitium; it is usually subatmospheric, meaning that excised tissue imbibes water when placed in saline. IFP in solid tumours is often elevated owing to leaky tumour vessels.

Mechanical stress
An applied force per unit area. In tumours, stresses include IFP gradients, shear stresses owing to fluid flow, matrix tension caused by fluid flow or matrix contraction by CAFs, and compressive stresses from a growing tumour pushing on surrounding tissue. Mechanical stress in the extracellular matrix induces mechanical strain according to stiffness.

Myofibroblast
A fibroblast subtype expressing α-smooth muscle actin that displays a contractile, synthetic and pro-fibrotic phenotype. Transforming growth factor-β (TGFβ) both activates, and is activated by, myofibroblasts.

Regulatory T (T_{Reg}) cells
$FoxP3^+$ $CD4^+$ T cells that suppress effector T cells and are important for maintaining peripheral tolerance to autoantigens, thereby preventing autoimmunity. Natural T_{Reg} cells are educated in the thymus, and inducible T_{Reg} cell activation in the periphery requires TGFβ and interleukin-10.

Stromal stiffening
A material property of the tumour stromal extracellular matrix that describes its resistance to deformation under mechanical stress. It can be altered by matrix protein synthesis, collagen crosslinking, matrix alignment and proteolysis.

Tolerance
The process that ensures that B and T cell repertoires are biased against self-reactivity, reducing the likelihood of autoimmunity.

Tumour-associated macrophages
(TAMs). A heterogeneous population of generally immune suppressive, alternatively activated or M2-type macrophages derived from peripheral blood monocytes that are recruited into the tumour mass and that constitute a major component of the immune infiltrate.

interstitial flow can drive autologous chemotaxis of fibroblasts via TGFβ-dependent mechanisms, in turn, guiding tumour cell invasion. A recent *in vitro* study from our laboratory[38] found that fibroblasts enhanced tumour cell migration only when interstitial flow was present, in a TGFβ-dependent manner[38]. Fibroblasts, but not tumour cells, could undergo chemotaxis up a gradient of TGFβ, and we hypothesized that slow interstitial flow may create pericellular gradients of TGFβ that increase downstream from the cell, driving chemotaxis. Thus, autologous chemotaxis coupled with the collective migration mechanisms described above may also provide a mechanism of flow-enhanced tumour invasion.

In summary, CAFs are highly sensitive to mechanical stress and shift their behaviour in ways that promote tumour progression and invasion under increased mechanical tension. Heightened interstitial flow in the tumour stroma imposes tension on the matrix fibres to which CAFs respond with myofibroblast-like differentiation, TGFβ production, matrix synthesis and alignment, and the generation of more tension. In this way, fluid flow can reinforce a positive feedback loop of matrix remodelling and CAF invasion, guiding the collective invasion of tumour cells. As discussed below, flow-induced matrix stiffening and TGFβ activation also have important implications for the tumour manipulation of host immunity.

Lymph flow and tumour immunity

Tumours can escape host adaptive immunity. The host immune response to a developing solid tumour is complex. The tumour creates a chronically inflamed microenvironment, and the tumour stroma contains numerous types of immune cells[66] that can function collectively to locally suppress host immunity. Tumours can also drive antigen-specific immune tolerance. Because of high mutation rates, tumours can express antigens that are recognizable by the immune system and that can drive anti-tumour cytotoxic T lymphocyte (CTL) responses[67]; however, tumours use multiple mechanisms to suppress such responses. For example, although vaccines could induce substantial numbers of circulating anti-tumour CTLs in patients with melanoma, the CTLs infiltrating the tumour and metastatic lesions were found to be functionally exhausted; that is, producing low levels of cytotoxic cytokines and incapable of lysing their target cells[68]. Activated CTLs can also be suppressed by regulatory T (T_{Reg}) cells infiltrating the tumour (which are attracted by factors such as hypoxia-induced CCL28 (REF. 69) or CCL21 (REFS 39,70)) or in the TDLN. Additionally, naive T cells can be directly activated in a tolerogenic manner by a specialized subset of myeloid dendritic cells (DCs) sometimes referred to as tolerogenic DCs[71]; T cells activated in this manner initially proliferate but lose their effector functions, ignore their antigenic target cells and undergo apoptosis.

Although a normal lymph node is responsible for responding to the antigens in the local tissue that it drains, the TDLN seems to promote tolerance. For example, when tumour cells were injected into the lymph nodes of naive mice, the tumour cells were rapidly killed by immune cells, but when the cells were injected into a lymph node that also drained a primary tumour that was derived from the same cell line, the injected cells proliferated[72]. In patients, the TDLN has been found to contain a tolerogenic milieu, particularly when metastases were present, both in melanoma[73] and in early stage cervical carcinoma[74]. Notably, plasmacytoid DCs were enriched in these TDLNs along with $CD4^+$ and $CD8^+$ T_{Reg} cell subsets[74]. In fact, dysfunctional $CD8^+$ T cells are often found in the tumour and TDLN even when high-affinity effector cells are present in the systemic circulation, implying that tolerance mechanisms occur in the tumour and TDLN[68,71,75]. Therefore, the TDLN becomes a key component of the tumour microenvironment, and may undergo modifications by the primary tumour in order to promote immune tolerance and to provide a permissive environment for metastatic growth.

TGFβ links tumour mechanobiology with immune tolerance. How might interstitial and lymphatic flow affect the immune environment of the tumour stroma and the TDLN? Here, we turn our attention again to TGFβ. We detailed above how TGFβ

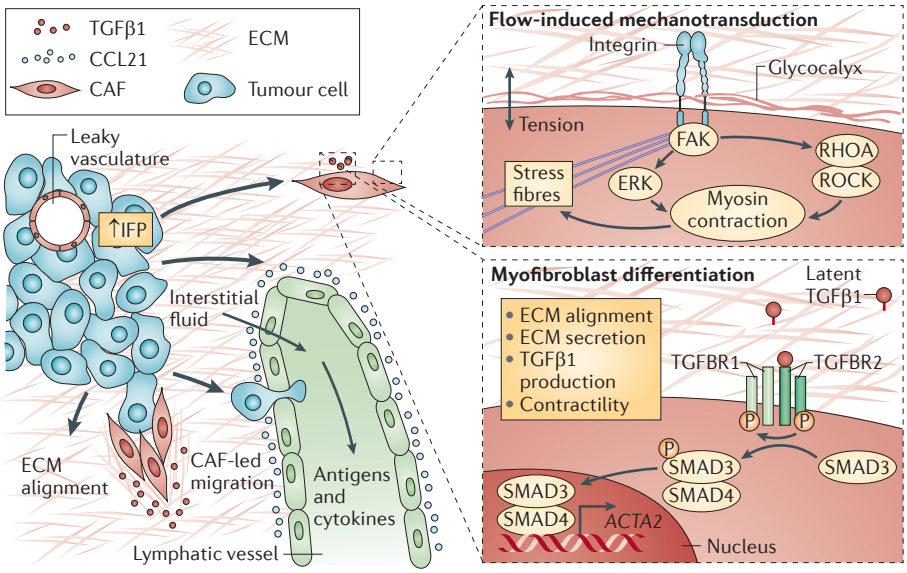

Figure 3 | **Interstitial flow and matrix mechanics manipulate the tumour stroma to promote invasion.** Interstitial flow can act directly on the tumour stroma to promote a dynamic feedback loop that drives the activation of cancer-associated fibroblasts (CAFs), which are a heterogenous fibroblast population that includes myofibroblasts; extracellular matrix (ECM) remodelling; and tumour progression. Elevated levels of interstitial flow may bias autologous chemokine gradients, promoting directional homing of tumour cells and CAFs towards the draining lymphatic network (left panel). Interstitial flow also drives matrix alignment and stiffening, and activates latent transforming growth factor-β1 (TGFβ1), which leads to myofibroblast differentiation and further ECM remodelling. Mechanical forces (top right panel) are propagated through all cells via integrins and the mechanically sensitive glycocalyx. Interstitial flow directly imparts stress on the ECM, inducing strain that transmits signals to the cell through integrin receptors, in turn activating focal adhesion kinase (FAK), RHOA and ERK to upregulate myosin, which is required for stress fibre formation via Rho-associated protein kinase (ROCK). Interstitial flow and contractile forces on the surrounding ECM can activate latent TGFβ1 stored in the ECM (bottom right panel). TGFβ1 binds the receptor TGFBR2, which, together with TGFBR1, drives the phosphorylation (P) of SMAD3 and complex formation with SMAD4 to activate the transcription of α-smooth muscle actin (*ACTA2*), also required for stress fibre formation and the differentiation of fibroblasts into myofibroblasts. In turn, myofibroblasts secrete more TGFβ1 and apply contractile forces that align and stiffen the ECM, driving a positive feedback loop. IFP, interstitial fluid pressure.

can be activated in the tumour stroma by interstitial flow-activated CAFs[38] and flow-induced mechanical stress on the ECM[40,44], but TGFβ also has crucial and multiple roles in immune suppression. Although it should be emphasized that TGFβ stimulates both tumour-promoting and tumour-suppressing processes[76,77], as well as epithelial-to-mesenchymal transition[78], it is among the most important regulators of effector T cell suppression and T_{Reg} cell activation. Importantly, TGFβ has a major role in defining the suppressive environment of the TDLN, where it functions synergistically with programmed cell death protein 1 (PD1) signalling for T cell suppression[79]. Thus, the overall effects of tumour TGFβ on host immunity are tolerogenic, and its activation in the tumour stroma by interstitial flow is a potentially important mechanism by which flow may promote immune tolerance (FIG. 4). Myeloid-derived suppressor cells (MDSCs), which are important for immune suppression, as well as for tumour angiogenesis and invasion[80], produce TGFβ, although, paradoxically, their infiltration into tumours is increased when TGFβ signalling is ablated[81]. TGFβ attracts natural killer cells, neutrophils and macrophages to the tumour, but neutralizes their anti-tumour effector functions[76]. TGFβ also strongly suppresses anti-tumour CTL responses in the tumour stroma, and blocking its signalling enhances anti-tumour immunity[77].TGFβ is required for immature and tolerogenic DCs to selectively promote the differentiation and proliferation of T_{Reg} cells[82], which are found localized to the tumour stroma[83]. In turn, activated T_{Reg} cells secrete more TGFβ. Therefore, abundant levels of TGFβ are required for inducing immune tolerance in the tumour and TDLN, and TGFβ activation is one mechanism by which interstitial flow could alter tumour immunity and promote tolerance.

Flow drives lymphoid features in the tumour stroma. In addition to regulating TGFβ, interstitial flow may promote lymphoid-like features to develop in the tumour stroma (FIG. 4). First, flow can modulate expression of the cytokine CCL21 in the tumour microenvironment. Physiologically, CCL21 is expressed by lymphatic endothelial cells (LECs) and — along with CCL19 — lymph node fibroblastic reticular cells (FRCs); CCL21 normally functions as a lymphoid-homing chemokine, binding CCR7 on APCs and naive T cells to direct them to the lymph node and position them for efficient interactions[84]. CCL21 expression by LECs and FRCs is regulated by interstitial and lymph

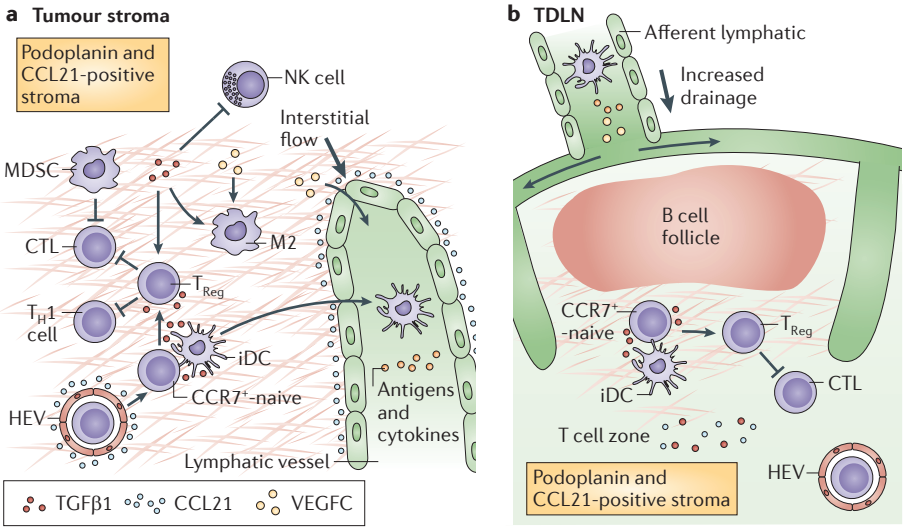

Figure 4 | **Interstitial flow influences the immune microenvironment in cancer. a** | The immune microenvironment of the tumour consists of multiple cell types, cytokines and stromal components that can further attract immune cells and guide their fate. Transforming growth factor-β1 (TGFβ1), following its release from the extracellular matrix (ECM) by interstitial flow-induced matrix tension and myofibroblast differentiation (FIG. 3), promotes tumour-associated regulatory T (T_{Reg}) cells and myeloid-derived suppressor cells (MDSCs), which suppress cytotoxic T lymphocyte (CTL) function to facilitate tumour immune escape. Interstitial flow also drives the production of the cytokine CCL21 by both the tumour-associated stromal cells and the lymphatic endothelium. The overexpression of CCL21 can recruit naive chemokine receptor 7 (CCR7)$^+$ T cells into the tumour stroma through high endothelial venule (HEV)-like structures, where they can be educated in the presence of a tolerogenic cytokine milieu. High levels of CCL21 may also alter patterns of antigen presentation and lymphatic homing of CCR7$^+$ dendritic cells (DCs), which are largely maintained in an immature state (iDCs). **b** | Lymphatic drainage promotes the delivery of tumour antigen and regulatory cytokines into the immune microenvironment of the tumour-draining lymph node (TDLN). This cytokine milieu can promote the tolerance-maintaining functions of the lymph node, including the induction of T_{Reg} cells and the suppression of local CTLs. In this way, lymphatic drainage of tumour fluid to the TDLN may help to mediate immune escape. NK, natural killer; T_H1, T helper 1; VEGFC, vascular endothelial growth factor C.

flow[85,86], as well as by VEGFC[87]. We and others have reported its expression by some tumours[39,70,88,89], and we have recently correlated its secretion with invasiveness and immune tolerance in murine melanoma[70]. Tumours that secreted CCL21 at physiologically relevant levels (similar to levels in the lymph node) recruited CCR7$^+$ lymphoid tissue-inducer cells. The recruitment of these cells correlated with the development of FRC-like features in the local CAF population — namely, expressing the reticular matrix component ER-TR7, secreting more CCL19 and CCL21, and upregulating podoplanin, which is a type-1 transmembrane sialomucin-like glycoprotein that is expressed by FRCs in the lymph node T cell zone. Podoplanin is also expressed by LECs, and has been found on some tumour cells and tumour-associated stromal cells, particularly in more aggressive tumours[90]. Although its direct role in tumour immunity is unclear, positive correlations between podoplanin expression and cancer stage, lymphatic invasion, lymph node metastasis, and peritumoral lymphatic vessel density have been reported[90–94]. Therefore, from the perspective of circulating lymphocytes, the flow-activated tumour stroma mimics features of the lymph node T cell zone that are crucial for attracting, recruiting and educating T cells in the tumour microenvironment.

How might such tumour mimicry of lymph node stroma promote tolerance? In a normal lymph node, the cytokines and co-stimulatory molecules expressed by APCs determine how T cells are educated: danger signals such as Toll-like receptor activation promote effector responses, and signals from apoptotic cells or complement regulation after tissue injury, for example, can promote tolerance[84]. In contrast to a normal lymph node, the tumour stroma is steeped in suppressive cytokines, suppressor cells and factors that keep APCs in a functionally immature state. Furthermore, in the normal lymph node, CCL21 and CCL19 have key roles in modulating the balance between immunity and tolerance[84,95]. In addition to positioning DCs and naive T cells for adaptive immune responses, these CCR7 ligands are required for T_{Reg} cell function[96]; they

can also directly inhibit CTL proliferation[97], and mice that lack CCR7 signalling cannot maintain tolerance to peripheral antigens[98]. By attracting naive and T_{Reg} cells to the tumour environment where they can interact with immature APCs and MDSCs while under the influence of regulatory cytokines, the tumour and its TDLN may encourage tolerogenic T cell education[70]. In this way, the lymphoid-like changes that may be initiated or enhanced by increased flow and VEGFC levels in the tumour microenvironment are likely to support immune tolerance.

Immunological roles of lymphangiogenesis. As mentioned above, lymphangiogenesis in the tumour and TDLN is correlated with invasion, metastasis and poor prognosis[25,29,99]. In addition to cancer, lymphangiogenesis occurs during chronic inflammation and in the lymph nodes that drain the inflamed regions[23,24,100–102]. Currently, very little is known about how inflammatory lymphangiogenesis affects immunity, and recent reports have raised puzzling questions. On the one hand, as detailed above, cancer progression is positively correlated with both lymphangiogenesis and immune suppression and tolerance. Furthermore, in mouse models of acute inflammation, lymphangiogenesis was important in resolving the inflammation[100]. On the other hand, lymphangiogenesis is also frequently seen in autoimmunity-related chronic inflammatory disorders[23,101] and in transplant rejection[103], raising the possibility that lymphangiogenesis may contribute to immune rejection. Blocking lymphangiogenesis before experimental corneal or islet transplantation could reduce rates of graft rejection[102,104]; however, in 1-year follow-ups of patients who had undergone renal transplant, transplant function was positively correlated with lymphangiogenesis[105]. Such findings raise questions regarding how inflammatory lymphangiogenesis affects the immune response in different settings.

In considering the potential roles of lymphangiogenesis in inflammation, the context is likely to be important — that is, the cytokines, stromal changes and immune cells present — as this can be vastly different in acute inflammation, chronic inflammatory diseases and cancer. Lymph node lymphangiogenesis following acute inflammatory stimulation may be driven by B cells[106,107] and inhibited by CTLs and interferon-γ (IFNγ)[108]. Consistent with this idea, autoimmunity-associated tertiary lymphoid structures are rich in B cell germinal centres that produce autoantibodies, and in renal interstitial injury, for example, B cell recruitment promoted lymphangiogenesis around the B cell infiltrates[109]. T cells may be directly recruited to these newly formed structures to effectively bypass the local draining lymph node[110]. Additionally, local lymphatic vessels may carry fluid directly to the tertiary lymphoid structures rather than to the lymph node[111]; in other words, lymph-borne antigen may be constantly delivered to these ectopic B cell follicles to further promote autoantibody formation, and the tolerance-maintaining functions of the lymph node would not be present to temper these responses.

By contrast, lymphangiogenesis in the tumour microenvironment and the TDLN is clearly correlated with poor prognosis, as is tumour immune escape. VEGFC recruits $VEGFR3^+$ tumour-associated macrophages (TAMs)[28] that further promote immune suppression[66] and that secrete VEGFC when stimulated by tumour-derived factors such as TNF[36]. Ectopic germinal centres are not generally associated with cancer, and lymphangiogenic tumours show increased, not decreased, lymph flow to the TDLN[2,3,19,30]. Furthermore, LECs can directly inhibit dendritic cell maturation and function[112], and can suppress autoreactive T cells in the lymph node for peripheral tolerance[113]. Thus, although the roles of lymphangiogenesis in modulating immunity remain to be clarified in different inflammatory conditions, lymphangiogenesis in the tumour and the TDLN does not seem to correlate with anti-tumour immunity. Instead, positive feedback loops exist in the tumour stroma between VEGFC expression, interstitial fluid flow, TGFβ upregulation, development of lymphoid-like stromal features that attract naive and regulatory T cells, infiltration of $VEGFR3^+$ and VEGFC-secreting TAMs, and T cell tolerance in the tumour stroma (FIG. 4).

Lymph flow helps to regulate the immune functions of the lymph node. Lymph flow communicates information from the periphery to the local draining lymph node. It delivers both APCs carrying antigen captured in the periphery, and soluble antigens, pathogens and cytokines for uptake by lymph node-resident DCs and macrophages. Lymph flow pathways through the various regions of the lymph node seem to be highly organized and act as a system of filters that direct different types of molecules or particles to different regions of the lymph node, in turn helping to regulate appropriate immune responses to pathogens, immunogenic antigens and autoantigens[8,114,115] (FIG. 5). Prenodal or afferent lymph also contains self-peptides processed by enzymes during tissue catabolism and cell apoptosis, including caspases and matrix metalloproteinases, that differ in epitopes from those processed by endosomal degradation in APCs[116]. In this way, the lymph-borne antigens carry different information to the lymph node than APC-delivered antigens.

Furthermore, the context in which antigen is taken up by APCs — that is, in the periphery versus in the lymph node — and the different kinetics of transport and presentation to T cells can substantially influence the immunological outcome[8,117,118]. For example, on encountering pathogens, DCs that are activated in the periphery may be exposed to higher local concentrations of effector cytokines, and they often undergo maturation en route to the lymph node, upregulating co-stimulatory molecules and cytokines that are necessary for activating potent CTLs. By contrast, lymph node-resident DCs are maintained in an immature state, and, although they can also be stimulated to undergo maturation and activate effector T cells, their interactions with T cells occur on a shorter timescale and in the presence of a different cytokine milieu, leading to faster but weaker T cell activation[118].

Importantly, immature DCs residing in the lymph node have important roles in maintaining peripheral tolerance. In addition to constitutively expressing endogenous autoantigens from the local draining tissue[119], they constantly sample lymph-borne antigens while continuously interacting with circulating T cells. In 1972, studies using dermal-contact hypersensitivity raised the hypothesis that lymph flow is required for inducing new peripheral tolerance, as tolerance to the applied chemicals could only be achieved when local lymphatic drainage from the skin was intact, but not when lymphatic drainage was blocked in transplanted skin islands[120].

In the context of tumours, as mentioned above, immature DCs resident in the TDLN are bathed in the suppressive cytokine milieu draining from the tumour, which prevents their maturation and thus biases them to tolerogenic antigen presentation. This cytokine milieu is facilitated at least in part by the expression of indoleamine 2,3-dioxygenase by DCs in the tumour and the TDLN, which can both specifically and indirectly suppress anti-tumour immune responses and induce T_{Reg} cells[121]. Additionally, tumour-secreted exosomes, which are lipid vesicles containing cell membrane and cytoplasmic proteins, are found in abundance in tumour-draining lymphatic

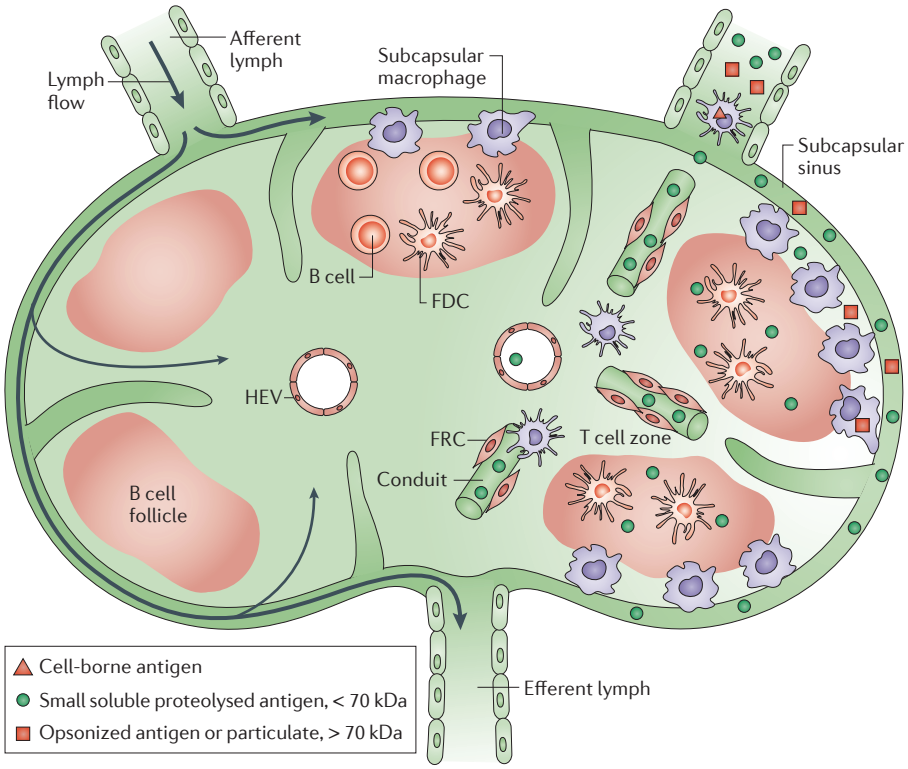

Figure 5 | **Patterns of lymph flow and antigen delivery in the lymph node.** Lymph flow pathways through the lymph node are highly organized and filter different types of molecules or particles to different regions of the lymph node[8,115]. Afferent lymphatics carry lymph from the periphery into the subcapsular sinus, where subcapsular macrophages filter the lymph of large particles, pathogens and molecular aggregates for subsequent presentation to B cells. Lymph is directed from the sinus into the cortex by narrow conduits (200 nm–3 μm), which are lined by fibroblastic reticular cells (FRCs), that only permit the flow of unopsonized small molecules (<70 kDa) for uptake by lymph node-resident dendritic cells (DCs)[9]. Peripherally drained chemokines are also rapidly transported through the conduits to the high endothelial venules (HEVs) to attract lymphocytes to the paracortical region[130]. It is unclear how increased flow in the tumour microenvironment may change these pathways, but the loss of afferent lymph flow leads to HEV collapse and reduced lymphocyte entry[131], suggesting that flow also has a crucial role in the maintenance of the lymph node architecture. FDC, follicular dendritic cell.

vessels; these may also help to promote tolerance in TDLNs[122] and support the TDLN as a tumour stem cell niche[123].

Inflammatory signals modulate lymph flow and lymphangiogenesis. As lymph flow seems to be so important in lymph node function, one might expect it to be regulated by inflammatory signals. Unfortunately, very few studies exist on the modulation of lymph flow by inflammatory cues (as opposed to inflammation-induced lymphangiogenesis (discussed above)); however, emerging evidence suggests that the lymphatic endothelium may actively modulate flow in response to inflammatory cues. Fluid transport across LECs (or lymph formation) is typically considered passive, moving between loosely overlapping cell–cell junctions. However, vesicular-based transendothelial transport may constitute an important and even dominant mechanism of solute transport compared with passive convective transport, at least as shown in recent *in vitro* studies[85]. Such active transport may be differentially regulated by pro-inflammatory or anti-inflammatory cytokines[124] and heightened interstitial flow[85], both of which are altered in the tumour microenvironment. Furthermore, although nitric oxide (NO) mediates the contraction of collecting lymphatic vessels that drive flow, inducible NO synthase (iNOS)-expressing MDSCs have been shown to inhibit lymphatic contraction but to increase lymphatic diameter[125]. Thus, although modulators of lymphatic flow are just beginning to be identified, lymph formation seems to be at least partially actively regulated by local inflammatory cues that limit or enhance the rate of antigen delivery to the lymph node.

In addition to fine-tuning antigen delivery to APCs, flow pathways in the lymph node are important for antigen delivery to the non-haematopoietic stromal cells that develop and maintain the lymph node architecture. In fact, these stromal cells seem to be indispensible for maintaining peripheral tolerance to self-antigens[126]. Lymph node stromal cell subsets, including FRCs and LECs, present peripheral tissue-restricted antigens and can delete autoreactive T cells[113,127]. However, their roles in peripheral tolerance to exogenous lymph-borne antigen are only beginning to be elucidated.

The tolerance-maintaining functions of the lymph node stromal cells bring our discussion back full-circle to the changes in the tumour stroma that are accompanied by VEGFC, increased flow and increased drainage to the lymph node (FIG. 4). Indeed, the expansion of LECs in the tumour microenvironment and the TDLN, together with the transformation of CAFs to mimic lymphoid FRCs[70,92,94], raises fascinating possibilities that tumours appropriate lymphoid stromal mechanisms of tolerance. As discussed above, the activated tumour stroma may express podoplanin and CCL21, and podoplanin-expressing CAFs display other features of FRCs including the secretion of CCL19, CCL21, CXCL12 and TRANCE[94]. If such tumour-associated FRC-like CAFs and LECs can in fact present tumour antigens to T cells, as they do in the lymph node with peripheral antigens, then they might directly modulate T cell education in addition to simply providing the chemoattractive and structural cues necessary to promote DC–T cell interactions within the tumour microenvironment.

Indeed, recent work from our laboratory[128] has shown that VEGFC promotes immune tolerance in the tumour microenvironment and the TDLN in murine melanoma[128]. VEGFC expression increased flow to the TDLN and attracted more T cells. In tumours expressing a non-endogenous antigen (chicken ovalbumin (OVA)), VEGFC could protect against even pre-existing anti-OVA immunity that was induced by vaccination before tumour implantation. Importantly, LECs in the TDLN could take up and cross-present OVA on major histocompatibility complex class I molecules, which caused the deletion of adoptively transferred, OVA-specific $CD8^+$ T cells. Together, these findings support the idea that tumour-associated lymphatic vessels and lymphatic drainage are key components of the immunosuppressive microenvironment of tumours.

Conclusions

In conclusion, we have proposed arguments for how interstitial flow and lymphatic drainage may affect tumour progression and invasion, both by direct mechanomodulation of the tumour stroma and also by altering the

host immune response. More research is needed to elucidate the direct roles of lymphatic drainage in modulating immunity and in promoting immunological tolerance, and as tumours apparently appropriate lymphatics for progression, much can be learned from studying the effects of tumour lymphangiogenesis directly on the immune response. Future research should also better integrate tumour immunology with tumour mechanobiology, focusing on the similar features shared by tumour and lymph node stroma, as well as on the mechanisms of how T cells can be educated or exhausted in the tumour microenvironment and the contributions of stromal cells to this process. A deeper understanding of the relationships between lymph flow, stromal transformation and immunity should not only lead to new immunotherapeutic strategies to block the tolerance-promoting functions of lymphatic drainage in tumours, but may also lead to new tolerogenic therapies for autoimmune diseases and transplantation.

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FURTHER INFORMATION
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