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Review Article

Bioengineered *in vitro* models of leukocyte–vascular interactions

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Leukocytes continuously circulate our body through the blood and lymphatic vessels. To survey invaders or abnormalities and defend our body against them, blood-circulating leukocytes migrate from the blood vessels into the interstitial tissue space (leukocyte extravasation) and exit the interstitial tissue space through draining lymphatic vessels (leukocyte intravasation). In the process of leukocyte trafficking, leukocytes recognize and respond to multiple biophysical and biochemical cues in these vascular microenvironments to determine adequate migration and adhesion pathways. As leukocyte trafficking is an essential part of the immune system and is involved in numerous immune diseases and related immunotherapies, researchers have attempted to identify the key biophysical and biochemical factors that might be responsible for leukocyte migration, adhesion, and trafficking. Although intravital live imaging of *in vivo* animal models has been remarkably advanced and utilized, bioengineered *in vitro* models that recapitulate complicated *in vivo* vascular structure and microenvironments are needed to better understand leukocyte trafficking since these *in vitro* models better allow for spatiotemporal analyses of leukocyte behaviors, decoupling of interdependent biological factors, better controlling of experimental parameters, reproducible experiments, and quantitative cellular analyses. This review discusses bioengineered *in vitro* model systems that are developed to study leukocyte interactions with complex microenvironments of blood and lymphatic vessels. This review focuses on the emerging concepts and methods in generating relevant biophysical and biochemical cues. Finally, the review concludes with expert perspectives on the future research directions for investigating leukocyte and vascular biology using the *in vitro* models.

Introduction

Leukocytes are types of blood cells that are made in the bone marrow and found in the blood and lymph tissues in mammals. When blood-circulating leukocytes encounter signals of infection or inflammation from blood endothelial cells (BECs), they start to extravasate and penetrate deep into the interstitial tissue and survey invaders in the tissue [1–3]. After performing immune functions to protect the tissue from the invaders, leukocytes leave the interstitium through draining lymphatic vessels guided by the signals from lymphatic endothelial cells (LECs). Then, leukocytes pass through lymph nodes and eventually merge into blood circulation through the lympho-venous conjunction, where lymphatic ducts meet subclavian veins [4–7].

Until now, many efforts have been made to study the process of leukocyte trafficking through blood vessels [1–3] and lymphatic vessels [4–7]. Recent advances in microscopy techniques (e.g. fast scanning confocal microscopy and multiphoton microscopy) have enabled the visualization of leukocyte dynamics in inflamed regions with enhanced spatiotemporal resolution in live animals [8–12]. Nevertheless, it is challenging to precisely and independently control inherently coupled biological factors in live animal models, reproduce specific experimental conditions, and quantitatively analyze

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leukocyte behaviors in complicated *in vivo* microenvironments. Thus, *in vitro* models have been developed and used as complementary approaches to the conventional *in vivo* models. Recently, three-dimensional (3D) *in vitro* models operated with microsystems (e.g. microfabrication or microfluidics) have become powerful tools as they surpass the limit of two-dimensional *in vitro* models (e.g. transwell assays) by providing more *in vivo*-like microenvironments and vascular structure [13–16]. Here, we introduce bioengineered 3D *in vitro* models for studies of the mechanisms in leukocyte trafficking through the blood and lymphatic vessels. We introduce microsystem-based 3D *in vitro* models for leukocyte extravasation through blood vessels and 2D, conventional *in vitro* models for leukocyte intravasation through lymphatic vessels, since 3D *in vitro* models for leukocyte trafficking through lymphatic vessels have not been well developed as much as *in vitro* models for leukocyte trafficking through blood vessels [17]. However, we discuss 3D lymphatic vessel models currently available as potential base models. Lastly, we discuss future research directions for investigating leukocyte trafficking through these vessels using *in vitro* models.

Leukocyte trafficking through blood and lymphatic vessels

Leukocyte extravasation from blood vessels

Leukocyte infiltration into inflamed tissue occurs in postcapillary venules or small venules (diameter up to 50 μm) rather than in arteries, arterioles, capillaries, or veins, where luminal flow shear stress is lower (~2 dyne/cm^2) than in other blood vessels [18]. Postcapillary venules consist of two cell types, BECs and pericytes, and basement membrane (BM) [2,19]. BECs form a confluent cell layer with tight junctions and adherens junctions, while pericytes form a discontinuous cell layer on top of BEC monolayer. Both BECs and pericytes secrete BM in postcapillary venules, and it surrounds the interface of BECs and pericytes. Compared with a lack of BM in lymphatic capillaries, BM in postcapillary venules is dense with a pore size of ~50 nm [20]. BECs interact with pericytes via N-cadherin-mediated cell–cell adhesions and BM via integrin-mediated focal adhesions. Both interactions are essential for blood vessel maintenance as well as formation [2,19].

In response to pro-inflammatory signals under infection (e.g. tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), IL-6) secreted by the inflamed tissue, BECs loosen interactions with pericytes and the BM becoming leaky, up-regulate adhesion molecules and produce chemokines to recruit leukocytes [1–3]. Leukocytes recognize the inflamed BECs and start to extravasate from postcapillary venules in a process that is known as the leukocyte adhesion cascade (Figure 1). The leukocyte adhesion cascade includes successive and prerequisite steps; (i) a short moment of rolling, (ii) firm adhesion, (iii) crawling with the polarized shape on the apical surface of BECs, (iv) transendothelial migration (B-TEM) from the apical surface to the basolateral surface of BEC layer, (v) subendothelial crawling between BECs and pericytes, and (vi) penetration of the BM. After extravasation from postcapillary venules, (vii) leukocytes navigate the interstitial matrix and reach the inflamed target site to perform their defensive immune function (Figure 1). Specific molecules and mechanisms are well addressed in previous reviews [1–3], and some of them are summarized in Figure 2.

Leukocyte intravasation into lymphatic vessels

After resolving the infection/inflammation, the leukocytes need to leave the target legion through lymphatic vessels. Initial lymphatic vessels or lymphatic capillaries (diameters up to 70 μm), where leukocytes initially intravasate, are morphologically different from postcapillary venules due to their primary function of draining interstitial fluid and cells [4,7,21]. Lymphatic capillaries are blind-ended, lack mural cells (e.g. lymphatic muscle cells or pericytes) [22], and have highly porous and infrequent BMs with a pore size of 2–4 μm [20]. LECs in lymphatic capillaries are oak leaf-shaped, forming discontinuous tight/adherens junctions, so-called 'button-like junctions,' and interdigitating flaps with free-openings of ~0.5–1 μm. LECs in lymphatic capillaries are exposed to interstitial fluid pressure (IFP) that is required to open the lymphatic junctions. On the contrary, collecting lymphatic vessels, the downstream of lymphatic capillaries, are bigger lymphatic vessels surrounded by lymphatic muscle cells and contain luminal valves, generating unidirectional lymph flow. They form continuous tight/adherens junctions, so-called 'zipper-like junctions' instead of 'button-like junctions' to transport fluid and immune cells to lymph nodes without leaking.

The main subset of leukocytes that intravasates into lymphatic vessels are T cells (80–90%) and dendritic cells (DCs) (5–15%) [7,23,24]. Other leukocytes rarely intravasate into lymphatic vessels during homeostasis, while the frequency of intravasation by granulocytes like neutrophils, eosinophils, and basophils is increased

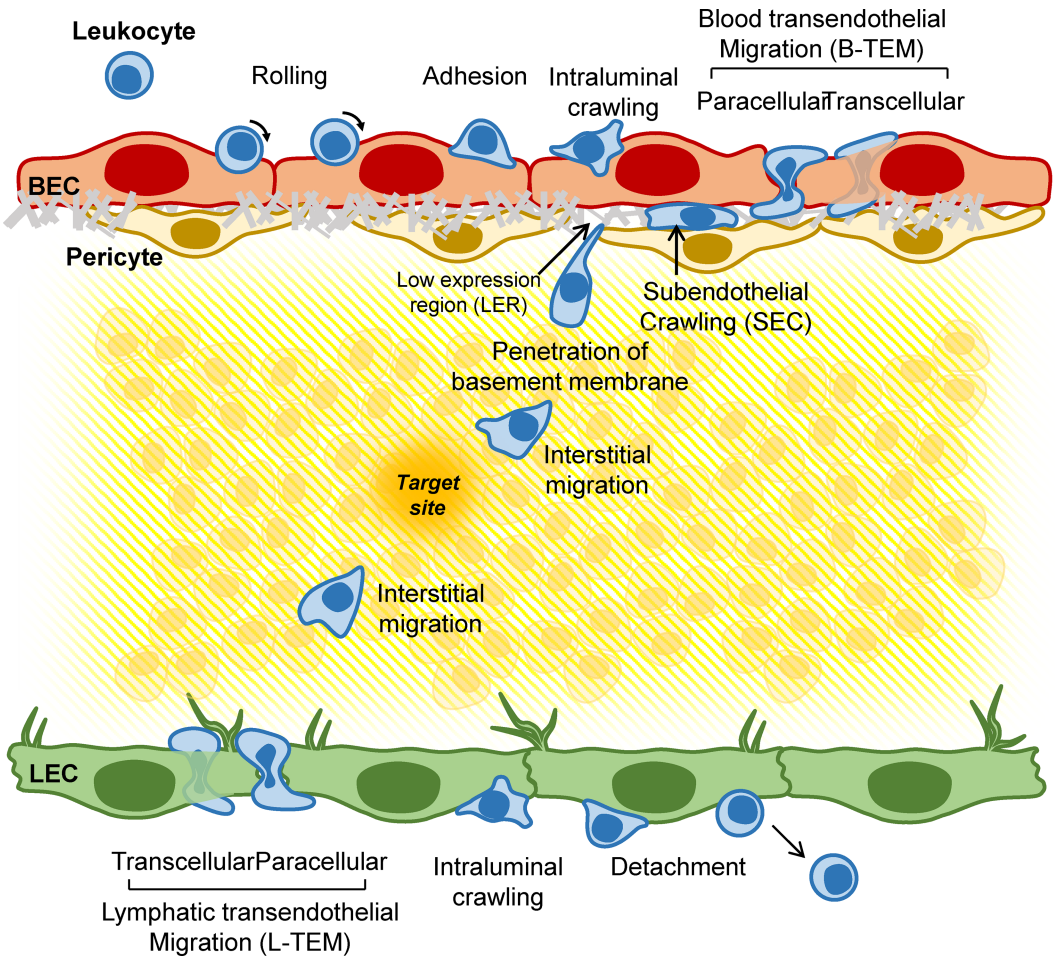

Figure 1. Sequential steps in leukocyte migration from postcapillary venules to the interstitial space and lymphatic capillaries.

Leukocytes respond to the luminal (apical) side of blood endothelial cells (BECs) in postcapillary venules and initiate a leukocyte adhesion cascade. Leukocyte rolling, adhesion, and intraluminal crawling enable optimal scanning for transmigration from apical to basal side of BECs. After transmigration, leukocytes encounter a subendothelial area formed between BECs and pericytes. Leukocytes crawl along the pericyte discontinuous monolayer in the subendothelial area, finding a low expression region (LER) of the basement membrane (BM). Once leukocytes penetrate BM via LER, leukocytes eventually leave the postcapillary venules (extravasation) and start interstitial migration. After proper immune response in the interstitial area, leukocytes leave the interstitial space through the lymphatic capillaries. After additional interstitial migration, leukocytes encounter lymphatic capillaries guided by the chemotactic gradient (e.g. CCL21). Contrary to postcapillary venules, lymphatic capillaries have highly porous BMs and no mural cells. Leukocytes proceed transmigration from basal to apical side of lymphatic endothelial cells (LECs) (intravasation). Once transmigrate, leukocytes remain attached to the intraluminal side of LECs. Due to the lower expression of adhesion molecules of LECs and lymph flow, leukocytes detach from LECs and are passively transported to draining lymph nodes.

during inflammation. In response to inflammatory signals and increased IFP caused by leaky postcapillary venules, LECs in lymphatic capillaries up-regulate adhesion molecules and produce chemokines (e.g. CCL21) to recruit CCR7-positive leukocytes (Figure 1).

Leukocyte intravasation to lymphatic capillaries occurs in reverse, compared with leukocyte extravasation from postcapillary venules cascade (Figure 1); (i) transit from interstitium to lymphatic capillaries (ii) engagement with the basolateral surface of LECs, (iii) TEM from the basolateral surface to the apical surface of LEC layer (L-TEM), (iv) active intralymphatic crawling on the apical surface of LECs, (v) leukocyte detachment

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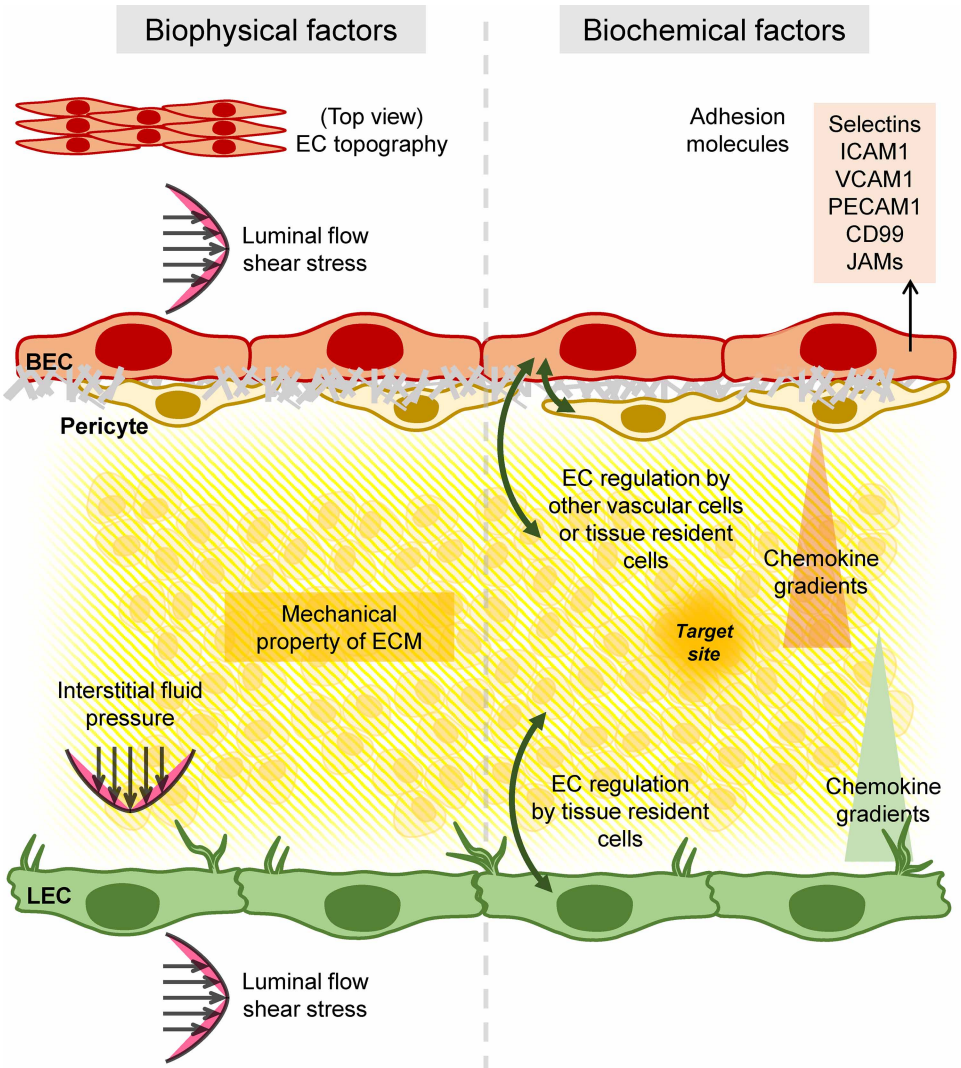

Figure 2. Biophysical and biochemical factors in complicated microenvironments affecting leukocyte migration.
Representative biophysical factors affecting leukocyte migration are endothelial cell (EC) topography, luminal shear stress, interstitial flow, and mechanical property of extracellular matrix (ECM). ECs are aligned with the blood vessel due to directionality of luminal flow. BECs are exposed to luminal flow-induced shear stress, while LECs are mostly exposed both to the luminal flow-induced shear stress and interstitial fluid pressure. The mechanical property of ECM is different depending on tissues (organ-specificity) or conditions (diseases, ages). Representative biochemical factors affecting leukocyte migration are the expression of adhesion molecules, chemokine gradient, and EC-other cell interactions. ECs constitutively express few adhesion molecules but, upon activation by cytokines, up-regulate the expression of adhesion molecules. Chemokine gradients from the target site or LECs determine the overall direction of leukocytes. EC interaction with other types of cells — for example, BEC interaction with pericytes or tissue-resident macrophage — adjust the subtle degree of EC activation, which affects leukocyte migration.

from LECs by decreased expression of specific adhesion molecules and chemokines in collecting lymphatic vessels. Then, the detached leukocytes are passively transported with the lymph flow generated by lymphatic muscle contraction and luminal valve function. Leukocytes, arriving at the subcapsular sinus of draining lymph nodes, extravasate across the LECs to initiate or modify immune responses by interacting with lymph node residing lymphocytes. The involved molecules and mechanisms are beyond this review's scope and well addressed in previous reviews in detail [4–7].

Leukocyte migration in complicated microenvironments

Considering the fact that leukocytes in the volumetric size of 50–500 femtoliters wander around the body's roughly 10^{12-15}-fold greater volume, leukocytes must have strategies to reach and leave out the target site in tissue within a few hours to days of initial signaling [25]. Indeed, leukocytes are specialized for sensing and responding to spatiotemporal heterogeneity of biochemical and biophysical cues in complicated microenvironments and exhibit diverse motility modes depending on the microenvironments (Figure 2) [20,26]. Biochemical factors leukocytes respond to include soluble chemokines (that are mediating chemotaxis), sequestered chemokines on the extracellular matrix (known to regulate haptotaxis), and adhesion receptors (known to mediate haptokinesis). Biophysical factors include body fluid-induced luminal shear stress, interstitial fluid flow, topography of endothelial cells (ECs) and mural cells, and variable stiffness/confinement of the extracellular matrix in the microenvironment (Figure 2). These biochemical and biophysical factors determine the direction, speed, and retention of leukocyte migration. Leukocytes exhibit adhesion-dependent haptokinetic movement (primarily integrin-mediated interactions) in crawling on ECs, while leukocytes exhibit adhesion-independent amoeboid movement in tissue interstitium. In general, leukocytes move (up to 30 μm/min) much faster than mesenchymal cells that are using focal adhesion-dependent locomotion (<1 μm/min) by avidly gathering information from the microenvironment [26].

Bioengineered blood vessel models *in vitro*

In the process of leukocyte migration through blood vessels, leukocytes encounter numerous biochemical or biophysical factors in the microenvironment and respond to them by constantly and rapidly changing their shape and motility mode [20,26,27]. Due to the early characterization and isolation of BECs from tissues [28], BEC-leukocyte interactions using *in vitro* models have been broadly investigated since the 1990s [29,30]. This section does not describe 2D, conventional *in vitro* models consisting of BECs cultured on cover-glasses or transwells in static conditions. Instead, we introduce recent microfabrication or microfluidic-based 3D *in vitro* models used to study leukocyte extravasation from blood vessels and the role of the microenvironment in inflammation and diseases.

Bioengineered models mimicking inflammation

Inflammation is a fundamental feature of a protective immune response in the body and one of the primary reasons for leukocyte migration [1,3]. The most common way of activating BECs *in vitro* is to treat BECs with soluble biochemical factors, such as pro-inflammatory cytokines (e.g. TNF-α, IL-1β, IL-6) dissolved in media without gradient. However, in an *in vivo* microenvironment, activated macrophages produce and secret pro-inflammatory cytokines generating a precise chemokine gradient from the interstitial space to the postcapillary [31]. To reproduce a constant chemokine gradient *in vitro*, Han et al. [32] developed a microfluidic device that contains one central channel for a BEC monolayer cultured on extracellular matrix (ECM) and perfusion of neutrophils, and two side channels for chemoattractant solution of N-formyl-methionyl-leucyl-phenylalanine (fMLP) or human IL-8 and blank medium (Table 1). In the system, neutrophils transmigrated across BECs and successfully migrated towards the chemokine gradient, allowing neutrophil dynamics to be quantitatively analyzed [32]. Wu et al. [33] developed another microfluidic device with a similar concept and tested synergistic effects between different chemokines on neutrophil TEM (Table 1).

Luminal flow-induced shear stress is a major biophysical factor that blood vessels are exposed to *in vivo*. Parallel-plate flow chambers, consisting of a fluidic chamber along a BEC monolayer, are commonly used to study leukocyte extravasation under shear stress conditions [34]. The shear stress can easily be controlled by connecting the fluidic chamber to syringe pumps or peristaltic pumps with pulsation dampers. The shear stress affects not only leukocyte dynamics but also BECs. In *in vivo* microenvironment, BECs are aligned with the vessel axis due to the directionality of the luminal flow (Figure 3) [35,36]. BECs can be aligned *in vitro* by applying high shear stress (>10 dyne/cm^2) of flow for extended periods (>40 h) [10]. Song et al. controlled BEC alignment and fluidic direction (either parallel or perpendicular to BEC alignment) by culturing them on nanogroove surfaces assembled into parallel-place flow chambers (Figure 3 and Table 1) [37]. They demonstrated that T cells underwent TEM faster and more frequently at tri- or multi-junctions where the BECs were oriented randomly [37]. Other biophysical factors affecting leukocyte dynamics are micron-scale topography and stiffness. From the same research group, Song et al. demonstrated that T cells sensed topographical landscapes of the BEC via lamellipodia and nuclei of BECs via filopodia as they crawled on BECs to optimize pathways for

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Table 1 In vitro models for leukocyte–vascular interactions

	Mimicking *in vivo* microenvironment		
Cells	**Biochemical factors**	**Biophysical factors**	**Ref.**
• hMVECs (Human microvascular endothelial cells) • Neutrophil differentiated from HL-60 (human leukemia cell line)	• Chemoattractant gradient in collagen gel in microfluidic devices		[32]
• HY926 (human endothelial cell line) • Human primary neutrophils	• Chemoattractant gradient in collagen gel in microfluidic devices		[33]
• bEnd.3 (mouse brain endothelial cell line)		• BEC alignment on nanogroove surface.	
• Murine primary T cell blasts		• Laminar shear stress by using parallel-place flow chambers	[37–39]
• bEnd.3 • Murine neutrophils differentiated from immortalized myeloid progenitors	• Chemoattractant gradient in collagen gel by placing agarose chemokine reservoir under the collagen gel	• Laminar shear stress by using parallel-place flow chambers	[41]
• bEnd.3 • Murine primary T cell blasts	• Co-culture of BECs and macrophages on different sides of nanofiber membrane.	• BEC alignment by a highly aligned and free-standing nanofiber membrane.	
• RAW264.7 (murine macrophage cell line) • HUVECs (Human umbilical vein endothelial cells)		• Laminar shear stress by using parallel-place flow chambers	[46]
• Human neutrophils, Primary peripheral blood lymphocytes (PBLs), CD14+ monocytes • Human umbilical cord SMCs	• Suspension of smooth muscle cells (SMCs) in collagen gel cast within microfluidic chips	• Disturbed flow by using modified parallel-place flow chambers.	[42]
• HUVECs (Human umbilical vein endothelial cells) • THP-1 (human monocytes cell line)	• Chemoattractant gradient in collagen gel in microfluidic devices	• Disturbed flow by using modified parallel-place flow chambers.	[44]
• BAOECs (Bovine aortic endothelial cells) • Mouse skin primary lymphatic endothelial cells (LECs)	• Chemoattractant gradient in collagen gel in microfluidic devices		[48][1]
• Human skin primary LECs • Primary murine CD4 T cells, CD8 T cells, DCs • Human effector T cells		• Interstitial pressure by using transwell assay (hydrostatic pressure mediated)	[55]
• imlEC (immortalized murine lymphatic endothelial cells) • Murine bone marrow-derived dendritic cells (BMDCs)		• Laminar (luminal) shear stress by using parallel-place flow chambers.	[57]
• Human skin primary LECs • MDA-MB-231 (human mammary adenocarcinoma cells)		• Interstitial pressure by using a modified transwell flow chamber.	[64][1]

[1]Indicates that the experiments did not include leukocytes but had featured methods that can be extended to leukocyte trafficking studies.

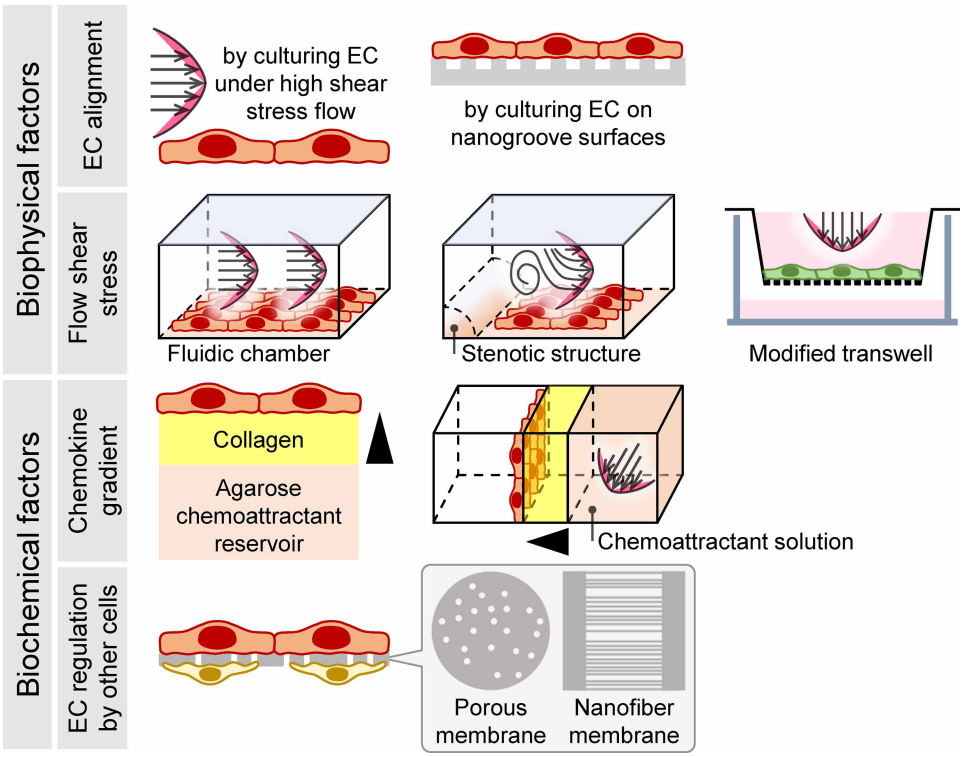

Figure 3. Microsystem-based *in vitro* models for leukocyte–vascular interactions.

ECs can be aligned *in vitro* by applying high shear stress of flow for extended periods or by culturing them on nanogroove surfaces. Fluidic chambers, such as parallel-plate flow chambers, allow us to apply flow on EC layers. Altered flow can be adapted by modifying the flow channel. For example, a stenotic structure mimicking a key feature of atherosclerosis induces disturbed flow in the post-stenotic area. Interstitial pressure can be applied on the EC layer in transwells by filling higher media volume on the upper well than the lower well. Agarose chemoattractant reservoir under collagen gel or perfusing chemoattractant solution continuously via microfluidic next to collagen gel channel generates chemokine gradients. EC-other cell interactions can be induced by culturing them on each side of porous or nanofiber membrane, respectively.

TEM (Table 1) [38,39]. Similarly, Lee et al. [40] demonstrated that T cells sensed tight confinement and focal adhesion of BECs in subendothelial spaces during TEM and subendothelial crawling. Furthermore, to model both luminal flow and chemokine gradients *in vivo* microenvironment, Molteni et al. [41] reconstituted *in vitro* models using a parallel-plate flow chamber with chemokine agarose gel reservoir (Table 1). The perfused neutrophils into the chamber successfully extravasated across the BECs and migrated toward the chemokine reservoir.

Other biological interactions between BECs and other cellular components also affect BEC condition and consequently leukocyte migration [2,3]. Suspension of smooth muscle cells (SMCs) or pericytes in collagen gel within microfluidic chips, followed by culturing BECs on the collagen surface, is a common technique (Table 1) [42–44]. BEC monolayers co-cultured with SMCs have lower permeability than BEC monolayers cultured alone. In many adult tissues, perivascular macrophages (PVMs) contact blood vessels to perform immune functions as tissue homeostasis and pathology [45]. Park et al. [46] developed a BEC-PVM double-layered model with a highly aligned and free-standing nanofiber membrane, enabling real-time visualization of dynamic leukocyte infiltration and subsequent interactions with PVMs (Table 1).

Bioengineered models studying atherosclerosis

Atherosclerosis is a disease characterized by artery stenosis, which significantly changes flow patterns in blood vessels [47]. Post-stenotic blood flow shows localized recirculation, turbulent velocity fluctuations, and blood flow separation. Chen et al. [42] exploited vertical-step flow chambers with two different size gaskets to

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reconstruct the disturbed flow in the post-stenotic area. (Figure 3 and Table 1). They perfused human neutrophils, peripheral blood lymphocytes (PBLs), and CD14+ monocytes isolated from human blood samples over BEC monolayer co-cultured with SMCs. Compared with culturing each cell type alone, the co-culture of BECs and SMCs increased their expressions of adhesion molecules and chemokines required for leukocyte trafficking. Importantly, disturbed flow increased the frequency of adhesion and TEM by neutrophils, PBLs, and monocytes, while the duration of TEM by neutrophils, PBLs, and monocytes was decreased [42]. The flow at the stenotic site has higher shear stress than pre-stenotic/post-stenotic blood flow. Menon et al. [44] developed a multi-layered microfluidic device with a cell culture channel (top) and an orthogonally crossed air channel (bottom) in where they were overlapped but separated by a thin PDMS membrane (10 μm) (Table 1). By pumping air into the channel, the overlapped region had the 3D stenotic structure (Flat, 50%, and 80% constriction). BECs in the cell culture channel increased intercellular adhesion molecule 1 (ICAM-1) expression, and consequently, the adhesion by perfused monocytes onto the 3D stenotic structure significantly increased [44]. Thomas et al. [48] developed a bi-layer device in which two layers were orthogonally crossed and partially overlapped to mimic the early progression of atherosclerosis. The upper layer had BECs cultured on a semi-permeable membrane with fluid flow, while the lower layer had TNF-α suspended in collagen. Only BECs overlapping the lower layer were treated with TNF-α. They divided sections as upstream/downstream of TNF-α-treated BECs and analyzed ICAM-1 and F-actin distribution (Table 1).

Bioengineered models mimicking recapitulating tumor microenvironments

Tumor microenvironments (TMEs) contain tumor cells, stromal cells (e.g. cancer-associated fibroblast), leukocytes, ECM components, blood, and lymphatic vessels. *In vitro* models mimicking complex TMEs have been developed to screen drugs and to investigate tumorigenesis with a focus on immune cell components. For example, Zervantonakis et al. [49] developed a 3D microfluidic model for tumor cell intravasation and revealed that macrophages assisted tumor cell intravasation. To investigate the process of metastasis, Chen et al. [50] developed a device with a microvascular network and observed that LPS-stimulated neutrophils formed heterotypic aggregates with tumor cells. Although many *in vitro* models mimicking complex TMEs have revealed impressive results, leukocyte extravasation through tumor blood vessels is not fully understood. To overcome current immunotherapy obstacles (for example, bioengineered leukocytes show reduced motility on tumor blood vessels), advanced *in vitro* models of tumors are needed to expand our knowledge of leukocyte interactions with tumor blood vessels.

Bioengineered lymphatic vessel models *in vitro*

Compared with the blood vessel *in vitro* models, lymphatic vessel *in vitro* models have undergone much less development. That is because identifying lymphatic endothelial markers (e.g. LYVE-1, Podoplanin, and VEGFR3) was discovered in the 1990s–2000s, and the isolation/maintenance of LEC primary cultures has only been feasible in the past few years. Leukocyte interactions with lymphatic vessels are only starting to be understood, relying on *in vivo* models or conventional *in vitro* models. Thus, *in vitro* models for leukocyte intravasation into lymphatic vessels have not advanced as much as for leukocyte extravasation through blood vessels. This section addresses conventional *in vitro* models used for studies of leukocyte intravasation through lymphatic vessels, which commonly came up with *in vivo* intravital imaging, and provides examples of *in vitro* lymphatic vessel models that can be applied to leukocyte dynamics studies.

Models mimicking inflammation

In most *in vitro* experimental models, LECs have been cultured on 2D cover-glasses or transwells for leukocyte transmigration assays. For transwell assays, it is common to plate LECs on the lower side of transwells and monitor leukocyte transit from the upper to the lower compartment [24,51–53]. By exploiting transwell-based *in vitro* models, Johnson et al. [24] showed that inflammatory cytokine-activated LECs expressed key leukocyte adhesion receptors, including ICAM-1, vascular cell adhesion molecule 1 (VCAM-1), and E-selectin, which improved both DC adhesion and transmigration across the LEC monolayer. From the same research group, Johnson et al. [51] revealed that LECs constitutively expressed intracellular CCL21 in homeostasis while LECs rapidly secreted CCL21 after exposure to TNF-α during inflammation. CCL21 stimulated TEM of DC by a chemotactic mechanism in homeostasis and a β2 integrin-mediated mechanism during inflammation. Johnson et al. [52] also revealed that TNF-α treatment dramatically increased expression of transmembrane chemokine CX3CL1 by LECs, and LECs immediately shed all CX3CL1 at their basolateral surface through matrix

metalloproteinases (MMPs) to generate CX3CL1 soluble forms to promote conventional chemotaxis. Similarly, using transwell-based *in vitro* models, Vaahtomeri et al. [54] found that DC contact-induced calcium signaling in LECs triggered secretion of CCL21-positive vesicles, which promoted DC transmigration.

LECs are exposed to two different types of flow force; IFP and luminal flow-induced shear stress (Figure 2) [21]. To mimic IFP using transwells, Xiong et al. [55] filled the lower well with the minimum volume of fluid possible and the upper well up to the maximum volume of fluid possible, which created a hydrostatic pressure difference of 0.8–0.9 cm H_2O, similar to interstitial pressure *in vivo* (Figure 3 and Table 1). Russo and Teijeria et al. performed *in vitro* experiments by using a parallel flow chamber. They cultured immortalized murine LECs (imlEC [56]) in the absence or presence of low laminar flow (0.015 dyne/cm^2) and high laminar flow (1.5 dyne/cm^2) for 24 h to mimic the luminal flow in lymphatic capillaries and blood postcapillary venules, respectively. (Table 1) [57]. They found that the low laminar flow established a CCL21 gradient along the LEC monolayers, promoting intralymphatic downstream-directed DC trafficking [57].

Examples of advanced *in vitro* models with lymphatic vessels

3D *in vitro* models for leukocyte trafficking through lymphatic vessels have not been well developed. However, we discuss 3D lymphatic vessel models without immune cells currently available. Choi et al. [58] recreated single tube formation of LECs in a collagen type I matrix and applied laminar flow and reported a molecular mechanism of laminar flow-induced lymphatic proliferation. Fathi et al. [59] developed a fluidic device with LECs and pumpless cyclical luminal flow. Giving varying fluid shear stress conditions, including static, 0.92 dyne/cm^2 (normal), and 6.7 dyne/cm^2 (disease), they investigated the effect of flow on proliferation and cytokine secretion of LECs.

Tumors induce lymphangiogenesis by secreting lymphangiogenic growth factors, while tumor lymphatic capillaries secret several chemokines that recruit tumor cells and promote metastasis [60]. Lugo-Cintron et al. [61] made the tube formation of lymphatic capillaries in a collagen type I matrix with two different collagen densities, mimicking the normal and cancerous breast tissue environment, respectively, and investigated the effect of ECM density on morphology, growth, cytokine secretion, and barrier function of LECs. They also co-cultured LECs with breast cancer cells distributed in the collagen matrix to induce lymphatic vessel dysfunction. Similarly, Gong et al. [62] co-cultured LECs with breast cancer-associated fibroblasts (CAFs) distributed in the collagen type I matrix and identified that IL-6 secreted by CAFs impaired the barrier function of LECs. Ayuso et al. [63] made a microfluidic chip with two empty lumens in which LECs were cultured in one lumen, and breast cancer cells were co-cultured in the other lumen. Depending on the type of breast cancer cells (estrogen receptor-positive or triple-negative breast cancer), LECs showed differential alteration of gene expression associated with LEC growth, permeability, metabolism, hypoxia, and apoptosis. Pisano et al. developed a flow chamber in which culture media flowed both through tumor cell-containing gel and across LECs, mimicking the flow *in vivo* microenvironment of the lymphatic capillary interstitial flow pass through the LECs (transmural flow). This model examined tumor cell invasion and transmigration dynamics across LECs under different flow conditions (Table 1) [64].

Outlook

As a complementary tool to *in vivo* animal models, bioengineered *in vitro* models have proven to be versatile, well-organized, and controllable platforms for studying leukocyte interactions with blood and lymphatic vessels. Current advances in *in vitro* models generated with microfabrication and microfluidics provide more *in vivo*-like microenvironments for studies given the ability to build up 3D vascular structure. By exploiting these models, understanding leukocyte interactions with vessels has gradually advanced from physiological immune mechanisms to crucial roles in disease pathogenesis. Nevertheless, there are still several hurdles to be considered for better modeling of the leukocyte–vascular interactions. When designing the reductionist's *in vitro* models, we have to balance between simplification and complexity of *in vivo* microenvironment. An over-simplified model may not represent enough biological functions, while an over-complicated model may interfere with user-friendly handling and controlling the models. Although BEC-LEC co-cultured *in vitro* models were developed [65,66], the whole process of leukocyte migration from BEC to LEC in homeostasis, inflammation, and specific disease conditions has not been addressed yet. Likewise, organ-on-a-chip with a circulatory system and organ-specific cells to study organ-specific leukocyte migration and immune responses are required. Investigators should carefully select cell types and sources and develop methods to isolate and culture highly purified primary cells or to utilize induced pluripotent stem cells (iPSC) derived cells. Artificially immortalized

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leukocytes and ECs or primary cells contaminated by other kinds of cells may not correctly respond to specific conditions. By overcoming these challenges, we will be able to move forward in understanding leukocyte migration in our body and eventually apply these models to the clinic for diagnosing diseases or developing personalized treatments.

Perspectives

- It has been challenging to decouple and control biochemical and biophysical factors; and precisely analyze leukocyte behaviors *in vivo*. Tissue-engineered *in vitro* models may provide alternative approaches to better understand leukocyte–vascular interactions.
- Three-dimensional (3D) *in vitro* models operated with biomimetic microsystems have become unique tools by providing more *in vivo*-like vascular structure and the immune environment. Especially, blood vessel interactions with immune cells have been well developed.
- 3D *in vitro* models for leukocyte trafficking through lymphatic vessels have not been well developed as much as models for leukocyte trafficking through blood vessels. Modeling lymphatic system *in vitro* and deciphering lymphatic interactions with dendritic cells and T/B lymphocytes remain to be further investigated.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

BECs, blood endothelial cells; BM, basement membrane; CAFs, cancer-associated fibroblasts; DCs, dendritic cells; ECM, extracellular matrix; ECs, endothelial cells; ICAM-1, intercellular adhesion molecule 1; IFP, interstitial fluid pressure; LECs, lymphatic endothelial cells; PBLs, peripheral blood lymphocytes; PVMs, perivascular macrophages; SMCs, smooth muscle cells; TMEs, tumor microenvironments.

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