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Interstitial leukocyte migration *in vivo*

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Abstract

Rapid leukocyte motility is essential for immunity and host defense. There has been progress in understanding the molecular signals that regulate leukocyte motility both *in vitro* and *in vivo*. However, a gap remains in understanding how complex signals are prioritized to result in directed migration, which is critical for both adaptive and innate immune function. Here we focus on interstitial migration and how external cues are translated into intracellular signaling pathways that regulate leukocyte polarity, directional sensing and motility in three-dimensional spaces.

Introduction

The trafficking of leukocytes into peripheral tissues and lymphoid organs is critical for both innate and adaptive immune function and has been extensively reviewed [1–3]. A key step in this process is the interstitial motility of leukocytes within three-dimensional (3D) spaces, which can be either random or directed. Interstitial motility involves cycles of motility and arrest that is orchestrated by a complex hierarchy of external cues that are translated into changes in intracellular signaling. This function is evolutionarily conserved as demonstrated by the chemotactic interstitial migration of *Drosophila* hemocytes (immunosurveillance cells) to sites of tissue damage and infection (reviewed in [4]).

The rapid single-cell migration of leukocytes is classically known as “amoeboid” migration. The term “amoeboid” is based on a changing cell morphology, but can encompass different modes of locomotion (reviewed in [5,6]). Pseudopod driven gliding motility is the major mode of locomotion. During this mode of motility, actin polymerization below the leading plasma membrane generates forces for membrane extension. This is coupled with actomyosin contraction in the cell’s rear, which detaches the cell and propels the cell body forward. Leukocytes can also display a blebbing type of migration under some conditions where actomyosin contraction generates hydrostatic pressure to form bleb-based protrusions.

Migration in 3D

Efficient leukocyte migration through interstitial tissues is a key component of the normal function of the cells of the innate and adaptive immune system. The patrolling function of

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leukocytes requires cells to maneuver rapidly through complex matrix environments at speeds of up to 20–30 microns per minute. The type of protrusions formed by different leukocytes can be quite distinct, as demonstrated by neutrophils and macrophages migrating *in vivo* (Figure 1A and B). Neutrophils project small pseudopodia that often bifurcate, while macrophages frequently extend long, thin filopodia-like protrusions in many directions as they maneuver through the interstitium toward damaged tissues within zebrafish embryos (Figure 1C, D and E). T cells, on the other hand, often migrate randomly in their surveillance of lymph nodes in mice, with extension of small pseudopodia at the leading edge [7]. The mechanisms that regulate the generation and maintenance of amoeboid pseudopodia and biased selection are under intense investigation both *in vitro* and *in vivo* (reviewed in [8,9]).

Leukocyte amoeboid motility, in general, occurs in the absence of strong adhesive interactions with surrounding cells or tissues (reviewed in [10]), although leukocyte arrest often requires integrin-mediated adhesion, in particular during transendothelial migration (reviewed in [11]). Integrin-mediated adhesion is essential for migration on two-dimensional surfaces. However, integrins can be dispensable for leukocyte migration in interstitial tissues [12] or in 3D collagen lattices [13]. The idea that movement within confined spaces can be integrin-independent was paradigm shifting and highlights the key role for adhesion in limiting leukocyte migration speed and mediating leukocyte arrest rather than an essential role in motility under many *in vivo* conditions.

Interstitial environments contain both soluble and tissue-bound cues that help guide leukocytes to migrate or mediate cell arrest. Here, we review the key steps in interstitial leukocyte migration including sensing of environmental cues and the intracellular signaling mechanisms that orchestrate leukocyte motility and cell arrest (Figure 2A and B).

Cell signaling underlying leukocyte motility

Chemoattractants transmit signals through heterotrimeric G-protein-coupled receptors (GPCRs) [14], which activate a plethora of effectors [15,16]. One of the key effectors is the class Ib phosphatydylinositol-3-kinase (PI(3)K) (Figure 2C). Although the role of PI(3)K during *in vitro* chemotaxis is controversial, PI(3)Kγ is required for cell polarity and motility *in vivo* [17–19]. PI(3)K promotes Rac-mediated actin polymerization at the leading edge and generates F-actin anteroposterior polarity (dynamic F-actin at the leading edge and stable F-actin at the rear; Figure 2C and F). Inhibition of PI(3)K results in impaired F-actin polarity and neutrophil recruitment to both wounds and infection in zebrafish [17,20]. The importance of PI(3)K signaling is further illustrated by studies showing that the modulation of PI(3)K signaling can result in an inflammation phenotype. The PI(3)K products PI(3,4,5)P3-PI(3,4)P2 are localized to the leading edge of zebrafish neutrophils *in vivo* [17,21]. PI(3,4,5)P3 can be hydrolyzed to PI(3,4)P2 by SH2-domain-containing inositol 5-phosphatases (SHIP). SHIP limits myeloid cell motility through the modulation of PI(3)K signaling *in vitro* [22] and *in vivo* in zebrafish [21]. In addition, SHIP knockout mice show increased myeloid infiltration into vital organs [23,24]. Taken together, the modulation of PI(3,4,5)P3 levels through SHIP can negatively regulate cell motility.

Small GTPases are another class of key effectors downstream of GPCR signaling (reviewed in [25]). They function as molecular switches and play a critical role in the generation and maintenance of cell polarity and motility [25,26]). We will focus on some recent *in vivo* findings highlighting the role of different small GTPases (i.e., Ras and Rho subfamily) in leukocyte interstitial motility. The Ras subfamily Rap1 GTPase is important for leukocyte migration *in vivo* (Figure 2D). Interstitial motility of B and T cells in the mouse lymph node is dependent on a Rap1 effector molecule RAPL (Rap1-GTP binding protein) [27]. The

complex of RAPL and GTP-bound Rap1 activate the serine-threonine kinase Mst1 (Ste20-like kinase) [28] and Mst1 is required for T-cell and B-cell motility within lymph nodes [29].

The Rho subfamily GTPases including Rac, Cdc42 and Rho are also critical for leukocyte interstitial migration (Figure 2E and F). In agreement with *in vitro* studies [30,31], Rac is required for neutrophil polarity and motility in zebrafish [32]. Rac2 depletion results in impaired neutrophil motility to wounds. In addition, dominant negative Rac2 expression in neutrophils impairs PI(3,4,5)P3-PI(3,4)P2 polarization and pseudopod formation [32]. Local activation of Rac can mediate protrusion, induce the polarity of F-actin dynamics, activate PI(3)K signaling and is sufficient to direct neutrophil migration in zebrafish [17]. In mature dendritic cells (DCs), Rac1 and Rac2 deletion in mice also impairs migration to lymph nodes [33]. It is becoming increasingly clear that both Rac and PI(3)K are required for leukocyte interstitial motility through a positive feedback loop.

Cdc42 regulates pseudopod stability [30] and controls neutrophil polarity and migration *in vitro* via crosstalk between WASp, CD11b and microtubules [34]. Although Cdc42 deficient DCs retain the ability to sense chemotactic cues and form protrusions, these protrusion are temporally and spatially dysregulated resulting in impaired interstitial migration in mice [35] (Figure 2C). The Cdc42 GEF, DOCK8, regulates interstitial DC migration in mice at least in part by controlling Cdc42 activity. DOCK8 deficiency impairs Cdc42 activation at the leading edge and results in defective migration [36]. The accumulating evidence suggests that Cdc42 activity maintains pseudopod protrusion.

Rho regulates cell polarity and helps propel the cell forward (Figure 2F). Expression of constitutively active Rho or dominant-negative RhoA impairs neutrophil F-actin polarity in zebrafish [17]. Inhibition of the Rho effector, Rho-associated protein kinase (ROCK), impairs neutrophil migration in zebrafish [17] and DC migration within the interstitium in mice [37]. Overall, Rho GTPases play an important role in leukocyte motility through the regulation of cell protrusion and retraction.

Mitogen-activated protein kinases (MAPKs) are other downstream effectors of GPCR signaling [38,39] that regulate leukocyte interstitial migration *in vivo* (Figure 2D). Understanding the role of MAPKs has been difficult, in part, due to some conflicting results. An *in vivo* study using mice reports that p38 positively regulates neutrophil interstitial chemotaxis to a keratinocyte-derived cytokine [40]. However, in zebrafish, p38 has been reported to negatively regulate myeloid cell migration, or may be dispensable [41,42]. In zebrafish, ERK has been shown to be required for neutrophil recruitment [43] but is dispensable for macrophage recruitment to wounds [42]. The requirement of JNK in leukocyte migration *in vivo* is also controversial [41,42]. These discrepancies may be due to different regulatory roles played by MAPKs depending on the *in vivo* conditions, and will be questions for future investigation.

The role of microtubules in interstitial motility

Microtubules suppress the polarity and motility of many leukocytes *in vitro* [44,45] and *in vivo* [46,47]. Depolymerization of microtubules using nocodazole enhances neutrophil polarity and motility but impairs directional migration in zebrafish [46]. In macrophages, microtubule depolymerization does not affect migration speed in zebrafish, although directional migration is impaired [47]. It is known that microtubules regulate Rho GTPase activity (Figure 2E). Microtubule depolymerization can activate RhoA *in vitro* in part through the release of microtubule associated Rho GEF [44,48,49]. Both microtubule polymerization and depolymerization activate Rac *in vitro* [46,50]. It is likely that the dysregulation of Rho and Rac activity upon microtubule depolymerization contributes to

impaired directional motility *in vivo*. These observations suggest that the interconnected relationship between microtubules and the Rho GTPases plays a role in regulating leukocyte directional motility. In addition, microtubules may regulate PI(3)K signaling (Figure 2E) which is required for leukocyte polarization and motility [17]. It has been shown that microtubule depolymerization inhibits PI(3)K activation at the leading edge of neutrophils in zebrafish [46]. These observations highlight the essential role for microtubules in leukocyte directional motility *in vivo*.

Directional sensing and interstitial migration

Leukocyte gradient sensing and subsequent biased migration are necessary steps in directed migration. Directional migration can be guided by soluble or tissue-bound chemoattractant signals (Table 1). These directional cues can work separately or, in some cases, in concert.

Injured epithelial cells generate hydrogen peroxide (H_2O_2) through an NADPH oxidase (DUOX), which mediates rapid neutrophil wound detection in zebrafish [51]. Similarly, H_2O_2 is generated by oncogene-transformed cells, and this also mediates neutrophil and macrophage recruitment in zebrafish [52]. These observations suggest that H_2O_2 serves as an important signal for leukocyte detection of tissue damage. Recently, a Src family kinase (Lyn), was identified as a redox sensor that neutrophils use to detect the H_2O_2 gradients in injured tissues in zebrafish [43]. H_2O_2 directly oxidizes and activates Lyn. Activation of Lyn, in turn, leads to the phosphorylation and activation of the MAPK Erk but not p38 or JNK [43]. Interestingly, H_2O_2 is not required for neutrophils to detect localized bacterial infections in zebrafish, since inhibition of H_2O_2 production does not inhibit neutrophil recruitment to localized *Pseudomonas aeruginosa* or *Streptococcus iniae* infection [20].

Other cytokines are up-regulated with wounding or infection, including Tumor Necrosis Factor (TNF) expression in zebrafish [53,54]. The adaptor protein FAN (Factor associated with neutral sphingomyelinase activity) binds to the TNF receptor and plays a role in leukocyte interstitial directed migration in response to wounds and bacterial infections in zebrafish. FAN knockdown results in disorganized pseudopod generation in leukocytes, leading to impaired navigational capacity [55]. FAN mediates TNF-induced Cdc42 activation and actin reorganization *in vitro* [56]. WASP is one of the effectors of Cdc42, and WASP depletion impairs neutrophil and macrophage wound responses in zebrafish, similar to FAN knockdown [57].

In a zebrafish infection model, zCxcl8 (also known as IL-8) expression is induced upon local *Escherichia coli* infection. A zCxcl8 tissue-bound gradient is established by binding to heparan sulfate proteoglycans on the tissue surface. This zCxcl8 gradient mediates neutrophil directional bias by altering the relationship between cell speed and directionality [58]. DCs have been shown to use a similar strategy for interstitial migration in mice. The chemokine CCL21 generated on the lymphatic endothelium forms a tissue-bound gradient, which guides DCs to migrate from dermal tissue into afferent lymphatic vessels [59]. Antigen-engaged B cells in mice also sense the increasing CCL21 gradients from the follicle zone to the B-zone-T-zone boundary for directional migration, where early B-cell-T-cell interactions occur. This CCL21 gradient and its G-protein coupled receptor CCR7 are required for B cell directional migration [60].

The hierarchy and integration of signaling that govern leukocyte chemotaxis *in vivo* is further illustrated in several sterile inflammation models in which inflammation occurs in the absence of any microorganisms. In a sterile skin inflammation model in mice, a three-step cascade of neutrophil responses can occur with 1) initial scouting neutrophils followed by 2) waves of arriving neutrophils (amplification phase) and subsequently 3) stabilization of neutrophils at injured tissues. The involvement of GPCRs in initial recruitment is

followed by a role for cyclic ADP ribose in the amplification phase [61]. In another mouse sterile skin inflammation model, pericytes that wrap around the capillaries regulate leukocyte interstitial migration through their effects on leukocyte responsiveness to interstitial migratory cues and their effector functions [62]. Upon local sterile inflammation, proteoglycan $NG2^+$ pericytes increase ICAM-1 expression and secrete the chemoattractant macrophage migration-inhibitory factor (MIF) that increases myeloid cell migration. It is suggested that the $NG2^+$ pericytes form a 'highway' for the rapid migration of extravasted leukocytes. By contrast, in a hepatic sterile inflammation mouse model, leukocyte chemotaxis is guided within an intravascular 'highway' instead of the extravascular space mediated by pericytes [63]. In this model, a multi-step process of directional cues guide neutrophils to sites of sterile inflammation with neutrophil chemotaxis in the vasculature directed by macrophage inflammatory protein 2 (MIP-2) gradients. Closer to the tissue injury, formyl peptides released from necrotic cells serve as end-target chemoattractants. Different insults, therefore, can trigger distinct signaling cascades that mediate leukocyte recruitment. It appears that leukocytes can utilize a variety of chemotactic signaling pathways for directional migration in response to tissue damage that is context dependent.

The requirement for proteases in interstitial leukocyte motility is less clear. In 3D collagen matrices, the tyrosine-kinase collagen receptor discoidin domain receptor 2 regulates neutrophil chemotaxis through the induction of metalloproteinase secretion and the subsequent generation of collagen-derived chemotactic peptides [64]. It has been shown that knockdown of Mmp13 metalloproteinase decreases macrophage wound recruitment in zebrafish, indicating that protease activity is required for proper leukocyte interstitial migration [42]. In addition, migrating neutrophils in zebrafish can deform the collagen matrix, suggesting the involvement of proteases in interstitial migration [65].

Interstitial Leukocyte arrest

Localized leukocyte arrest is also a key component of interstitial leukocyte function. It limits leukocyte motility to regions where the effector immune function is needed. This is perhaps best demonstrated in the context of sequestered zones of leukocyte function in lymph nodes that are guided by specific chemokine signatures (reviewed in [66]). Leukocyte arrest/immobilization appears to be an active process that is mediated by specific chemokines and downstream effector pathways (Figure 2A). In support of this idea, there has been extensive work showing an essential role for calcium signaling in T cell arrest, including developing T cells in 3D mouse thymic tissues [67] through intracellular Ca^{2+} flux mediated by calcium release-activated calcium (CRAC) channels, such as Orai (reviewed in [68]). Evidence suggests that intracellular calcium influx through CRAC channels also mediates neutrophil arrest (reviewed in [69]), likely downstream of chemokine signaling. During tissue injury in zebrafish, the chemokine Cxcl8 has been implicated in mediating neutrophil retention [58]. Deficiencies in Cxcl8 result in decreased neutrophil retention at sites of infection. CXCR4-Rac2 signaling is also required for neutrophil retention in hematopoietic tissues in zebrafish [32]. Lack of Rac2 function results in decreased neutrophil motility but increased neutrophil release into the circulation, suggesting that retention is an active process mediated by Rac signaling. Stromal cell-derived factor 1 (SDF1, CXCL12) has also been implicated in the retention of leukocytes in hematopoietic tissue in zebrafish [70]. These observations suggest that leukocyte arrest in interstitial tissues, including wounds and hematopoietic tissues, is an active process. The regulation of leukocyte mobility versus arrest allows leukocytes to perform proper immune functions at the right place and at the right time, and is a fundamental part of leukocyte directional decision making during leukocyte interstitial motility.

Conclusion

Advances in multi-photon vital imaging technology and new model systems such as the zebrafish, have made it possible to study interstitial migration with higher resolution *in vivo*. What is becoming increasingly clear, however, is that although much of the knowledge acquired from *in vitro* studies can apply to the *in vivo* environment, not all mechanisms are similar. Therefore, future challenges will be to apply and combine both *in vitro* and *in vivo* analyses to increase our understanding of leukocyte interstitial migration and how responses are prioritized to competing extracellular cues.

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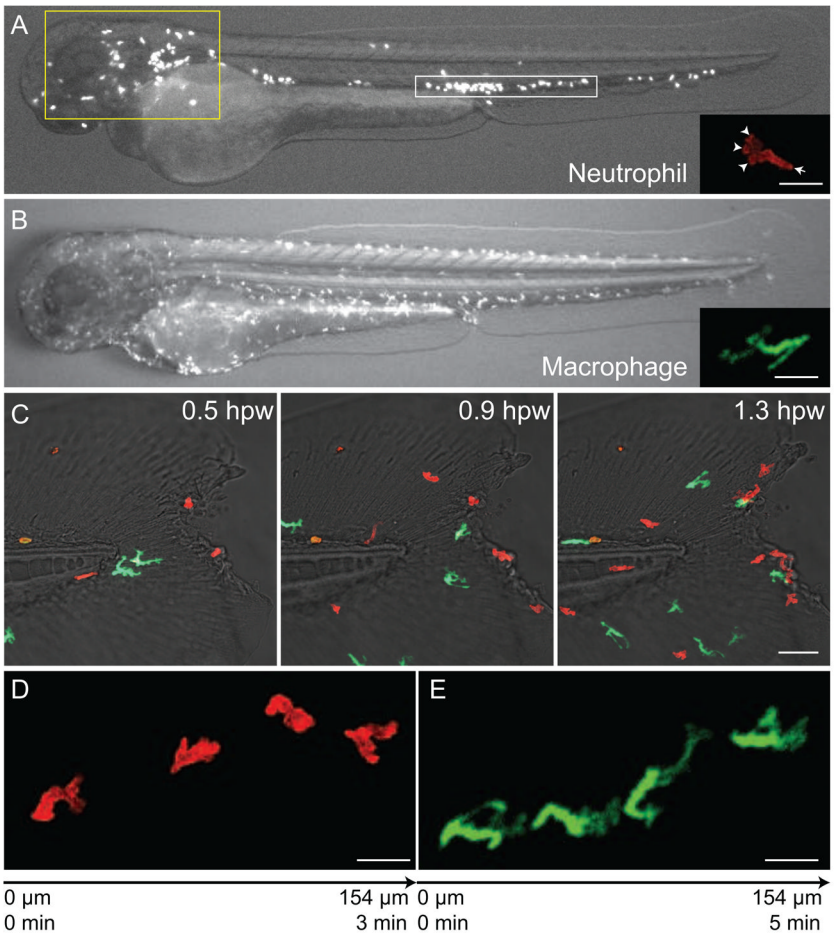

Figure 1. Neutrophil and macrophage distribution, morphology and response to wounding in zebrafish embryo at 3 days post fertilization

(A) Neutrophils distribute mainly in the mesenchymal tissue in the head region (yellow box) and in the caudal hematopoietic tissue (white box). (Inset in A) A typical migrating neutrophil has multiple pseudopodia (arrow heads) and a uropod (arrow). Scale bar = 20 μm. (B) Macrophages distribute in the whole embryo with no clear tissue localization. (Inset in B) A typical migrating macrophage shows multiple spindly protrusions. Scale bar = 20 μm. (C) Time series image of neutrophils (red; lifeact-Ruby) and macrophages (green; dendra) migrate to tail fin wound. Neutrophils have arrived at the wound at 0.5 hours post wounding (hpw) and macrophages arrived at later time points. Scale bar = 50 μm. Time series image of a neutrophil (D) or a macrophage (E) migrating from left to right towards the wound. Scale bar = 20 μm.

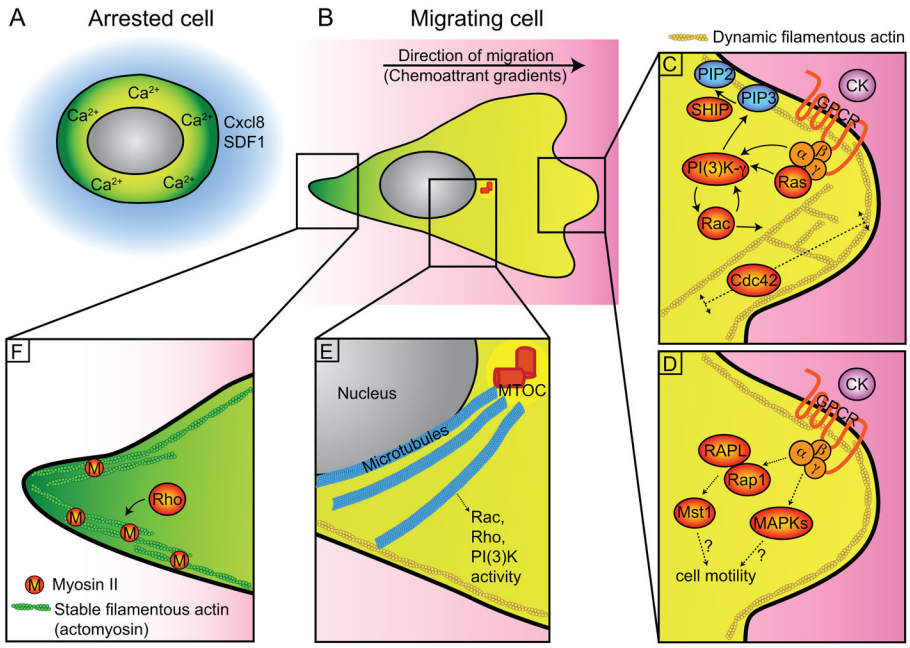

Figure 2. Cell Signaling and morphology in leukocyte interstitial migration *in vivo*

Detailed *in vitro* findings are reviewed elsewhere [72]. Here, we highlight the *in vivo* data. (A) A round and non-polarized morphology of leukocytes during arrest. (B) A polarized, amoeboid morphology during directed or random motility. (C) Signaling events and cytoskeletal structure at the leading edge. Positive feedback between PI(3)K and Rac support the formation of the leading edge while Cdc42 regulates the actin flow and stability of the protrusion. SHIP hydrolyzes PI(3)K product PIP3 into PIP2 and limits leukocyte motility. (D) GPCR can also activate the small GTPase Rap1; Rap1-RAPL-Mst1 signaling likely contributes to both integrin-dependent and independent leukocyte migration. The regulation of MAPKs on overall leukocyte motility is less clear. (E) Microtubules regulate Rac, Rho and PI(3)K activities, and thus leukocyte motility. (F) Signaling events and cytoskeletal structure at the uropod. Stable filamentous actin present in uropod mediates retraction. CK, chemokines; GPCR, G-protein coupled receptor; MAPKs, Mitogen-activated protein kinases including p38, ERK and JNK; PIP2, PI(3,4)P2; PIP3, PI(3,4,5)P3; ROCK, Rho effector Rho-associated protein kinase; SDF1, stromal cell-derived factor 1 (or CXCL12); SHIP, SH2 domain-containing inositol 5′-phosphatase.

Table 1

Signaling molecules (soluble or tissue-bound) mediate leukocyte directed migration *in vivo*

Tissue/Type of injury	Signaling Molecule	Mode of function	Distance from injury/Destination	Leukocyte	Receptor/Direct downstream target	Outcome	Ref.
Epithelium injury	H_2O_2	Signal peaks at ~20 min after wounding	Extends to ~100 to 200 μm from wound	Neutrophils	Lyn	Initial recruitment to tissue damage	[51] [43]
	(TNF)	--	--	Neutrophils, Macrophages	FAN	Directed interstitial migration	[55]
	(Purine nucleotides)	--	--	Neutrophils	cADPR production	Amplification phase of recruitment	[61]
Oncogene-transformed cells	H_2O_2	Stochastic and transient	In transformed cells and their immediately neighboring cells	Neutrophils, Macrophages	--	Leukocyte recruitment	[52]
Normal lymphatic endothelium	CCL21	Tissue-bound gradient	≤ 90 μm from lymphatic vessel	DCs	CCR7	Directed migration from dermal interstitium into afferent lymphatic vessels	[59]
Normal lymph nodes	CCL21	Tissuebound gradient	Increasing gradient from follicle region to the B/T boundary	B cells	CCR7	Migration from follicle zone to the B/T boundary	[60]
Sterile skin inflammation	ICAM-1	Cell surface protein	On $NG2^+$ pericytes	Monocytes, Neutrophils, Macrophages	--	Increases chemotaxis	[62]
	MIF	Secreted by $NG2^+$ pericytes	On $NG2^+$ pericytes	Monocytes, Neutrophils, Macrophages	--	Increases chemotaxis	[62]
Sterile hepatic inflammation	MIP-2	Intravascular gradient	Decreasing gradient from ~150 μm to 650 μm from the injury border	Neutrophils	CXCR2	Directed intravascular migration	[63]
	Formyl peptides	Released from necrotic cells	Within ~150 μm around the injury	Neutrophils	FPR1	Serves as end-target chemoattractant	[63]
Local bacterial infection	(TNF)	--	--	Neutrophils/Macrophages	FAN	Directed interstitial migration	[55]
	Cxcl8	Tissue-bound gradient	≤ 50 μm from the producing cells	Neutrophils	--	Mediates directional bias and restricts cell motility near the source	[58]
	Cxcl8	--	Systemic	Neutrophils	Cxcr2	Neutrophil mobilization from hematopoietic tissue and recruitment to infection sites	[71]

cADPR, cyclic ADP ribose

DCs, dendritic cells

FAN, Factor associated with neutral sphingomyelinase activity

FPR1, formyl-peptide receptor 1

H_2O_2, hydrogen peroxide

ICAM-1, intercellular adhesion molecule 1

MIF, macrophage migration-inhibitory factor

MIP-2, macrophage inflammatory protein 2, the closest IL-8 homologue in the mouse

TNF-R1, p55 TNF receptor