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Signal integration in forward and reverse neutrophil migration: fundamentals and emerging mechanisms

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Abstract

Neutrophils migrate to sites of tissue damage, where they protect the host against pathogens. Often, the cost of these neutrophil defenses is collateral damage to healthy tissues. Thus, the immune system has evolved multiple mechanisms to regulate neutrophil migration. One of these mechanisms is reverse migration – the process whereby neutrophils leave the source of inflammation. *In vivo*, neutrophils arrive and depart the wound simultaneously – indicating that neutrophils dynamically integrate conflicting signals to engage in both forward and reverse migration. This finding is seemingly at odds with the established chemoattractant hierarchy *in vitro*, which places wound-derived signals at the top. Here we will discuss recent work that has uncovered key players involved in retaining and dispersing neutrophils from wounds. These findings offer the opportunity to integrate established and emerging mechanisms into a holistic model for neutrophil migration *in vivo*.

Keywords

chemotaxis; neutrophil; reverse migration

Introduction

Neutrophils are often the first immune cells to arrive at sites of injury and infection. There, they mount a potent defense response that must be tightly regulated to limit collateral damage to the host [1]. Indeed, excessive or inappropriate neutrophilic inflammation can tip the balance from host protection to autoimmunity and tissue damage [1]. Neutrophils and other leukocytes exhibit complex decision making within interstitial tissues in response to various cues to maintain tissue homeostasis and enable tissue repair.

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Declaration of interests

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An example of this complex decision making is the neutrophil response to sterile tissue injury. Until recently, neutrophil recruitment to injury sites was considered unidirectional - with neutrophils undergoing apoptosis and subsequent clearance by macrophages at the wound [2]. However, it is increasingly evident that a subset of neutrophils leave inflamed tissues and re-enter the circulation [3–6]. The reverse migration and reverse transmigration of neutrophils into the vasculature have now been described in zebrafish, mice, and humans, suggesting they play a critical role in local inflammation resolution [3–6]. Indeed, in addition to clearing apoptotic neutrophils, macrophages have also been reported to repel wound-associated neutrophils [7,8] or "cloak" sites of sterile damage, thereby limiting chemoattractant signaling and neutrophil inflammation [9•].

The mechanism underlying the prioritization between tissue damage cues and reverse migration is not fully understood. In part, this is due to the daunting complexity of the physical and chemical landscape neutrophils encounter *in vivo* [10,11]. Furthermore, the signals that regulate reverse migration are not well-defined. Recent work *in vivo* and *in vitro* has just begun to uncover some of the molecular players and signaling pathways that regulate reverse migration. Here, we discuss how neutrophils prioritize chemoattractant signals *in vitro* and more complex *in vivo* contexts and discuss how these mechanisms may provide insight into the complex prioritization needed to reverse migrate and resolve a local response.

Reverse neutrophil migration conundrum: The end is not always the end

Neutrophils are inherently motile cells that migrate randomly (chemokinesis) and directionally (chemotaxis) in response to numerous chemical signals [12–14]. During episodes of inflammation, a torrent of overlapping directional cues guide neutrophils toward the afflicted tissue (Figure 1A–D) [10,11]. To effectively reach their target, they must integrate and prioritize these signals [15]. Indeed, there is a clear hierarchy of recruitment signals. Early studies *in vitro* revealed that attractants emanating from the wound, such as formylated peptides and complement components, take precedence over long-range signals like Interleukin-8 (IL-8, also CXCL8) and Leukotriene B4 (LTB4) (Figure 1C) [15–18]. This observation led to the classification of chemoattractants by their prioritization status - with high-priority signals termed "end-target" attractants and low-priority signals designated as "intermediary" attractants [15].

These terms were coined when the wound was considered neutrophils' final destination, however now we know this is not true. Interestingly, *in vivo*, neutrophils can be seen arriving and departing the wound simultaneously (Figure 1D,J)[3] - suggesting recruitment signals are still present when neutrophils leave. It is unclear how the chemoattractant hierarchy plays out in damaged interstitial tissue highlighting the need for an updated model of prioritization that accounts for the fact that the end is not always the end.

To begin addressing this conundrum, we consider prioritization mechanisms *in vitro* and *in vivo* during the recruitment phase of inflammation and what they might tell us about the integration and prioritization of signals at the wound.

Attractant detection systems in neutrophils

Scientists first described neutrophils' ability to migrate directionally toward chemical attractants in the late 19th century [19]. It would take almost 100 years before we began to understand the complexity and sophistication of their navigational system. In the 1970s, seminal work distinguished neutrophils' attractant sensing mechanism from that of bacteria [12,20]. Bacteria employ temporal sensing in which chemoattractant concentration is compared over time. When higher concentrations of attractant are detected, the length of the subsequent "run" increases, resulting in biased random movement up the gradient [21]. In contrast, neutrophils can compare receptor occupancy levels across their length with astounding sensitivity [12,22]. These spatial calculations result in dynamic polarization of intracellular signaling and motility components, giving rise to neutrophils' ability to steer toward a gradient source [23–26].

The polarization of intracellular signaling molecules presents a challenge for neutrophils that detect end-target and intermediary attractants on opposing sides. Indeed, *in vitro* studies using human neutrophils revealed that in competing gradients of end-target and intermediary attractants, PTEN, a negative regulator of actin polymerization, becomes uniformly distributed on the plasma membrane. In this case, end-target signals can activate an alternative motility program mediated by the MAPK, p38 – allowing them to migrate toward the end-target source [16,17].

More recently, multiple groups have observed temporal sensing by human and mouse neutrophils *in vitro,* which raises the possibility that differences in sensing strategies between high and low-priority chemoattractant receptors might contribute to some signals' dominance over others [22,27–29]. Furthermore, Chandrasekaran et al. propose an intriguing model in which neutrophils initially engage in spatial sensing but require temporal gradients for persistent migration toward fMLF. The authors suggest that switching between these two sensing mechanisms supports robust recruitment while, at the same time, limiting neutrophil accumulation [22].

Receptor-level regulation of motility in forward and reverse migration

In addition to inherent differences in sensing mechanisms, chemoattractant receptors are also subject to variations in receptor-level regulation. In neutrophils, the vast majority of chemoattractants are detected by G-protein-coupled receptors (GPCRs) [30–32]. Typically, GPCR phosphorylation results in desensitization and/or receptor endocytosis [14,30–32]. While most chemoattractant receptors undergo homologous desensitization, end-target signals also impose heterologous desensitization on intermediary attractant receptors [14,33,34]. As a result, neutrophils become ignorant to intermediary signals in the presence of end-target attractants (Figure 1C) [14,35,36].

Of course, receptor inactivation is not always permanent. Internalized receptors can be degraded or separated from their ligand and recycled [37]. Trafficking dynamics vary between chemoattractant receptors. For example, in migrating neutrophil-like PLB985 cells, internalized c5a receptor 1 is degraded, while FPR1 is internalized from the cell rear

and shuttled back to the plasma membrane [38]. The same study revealed that LTB4R is resistant to internalization and that mutations that affect LTB4R trafficking disrupt neutrophil migration toward fMLF, suggesting that like desensitization, receptor trafficking is subject to cross-talk.

These receptor-level regulation mechanisms can be enacted and reversed relatively quickly compared to responses that require transcription, for example. It is no surprise then that these mechanisms are implicated in inflammation resolution – where neutrophils go forward to the wound and then reverse migrate. CXCR1 and CXCR2 both bind variants of CXCL8 (IL8), have redundant roles in neutrophil recruitment but promote cell clustering and dispersal in wounds, respectively (Figure 1E,G) [39,40••]. Specifically, zebrafish CXR2 mediates neutrophil chemokinesis at the wound and subsequent reverse migration (Figure 1J) [39]. Differences in trafficking dynamics between these receptors ensure these functions happen in sequence. In the wound, activated CXCR1 is rapidly internalized, whereas CXCR2 persists on the plasma membrane [40••]. This persistence enables sustained signaling by CXCR2, which supports the dissolution of neutrophil clusters and chemokinesis (Figure 1E,G) [40••]. Indeed, in zebrafish, loss of CXCR2 results in neutrophil retention at the wound [39,40••]. Interestingly, activated CXCR2 is rapidly internalized outside of the wound microenvironment – making it a particularly compelling example of how receptor-level regulation can enable context-specific responses.

So far, we have considered two examples that point to a loss of directed motility as an essential mechanism for overcoming recruitment signals. First, a reliance on temporal sensing for continued migration toward end-target signals may limit neutrophil accumulation once the concentration of damage signals are no longer rising [22]. Second, by regulating the time and place in which chemoattractant receptors function, neutrophils can become unresponsive to signals that undermine their transition to the resolution phase [40••,41]. In agreement with a random component of the resolution phase of acute injury has been work from several groups using mathematical modeling to analyze neutrophil migration patterns away from the wound (Figure 1J) [42–44]. They found that the experimental observations of neutrophils leaving the wound are better captured by a model in which neutrophils randomly diffuse from the wound as opposed to one in which directive signals are included [42]. Importantly, neutrophil velocity and directionality are comparable in neutrophils migrating to and from a wound, suggesting reverse migration is supported by directional cues outside of the wound [3,6]. It is possible that initially, neutrophils stop responding to recruitment signals resulting in random migration and dispersal before becoming engaged by signals that lead them back to the vasculature. Identifying purported vascular homing signals is an ongoing area of investigation.

Overcoming retention signals

In vivo, where the chemical and physical landscapes are very complex, it is likely that numerous signaling events and processes coordinate to regulate reverse migration and local resolution. In addition to recruitment signals, neutrophils must also overcome retention signals to leave the wound. Of course, both continued neutrophil recruitment and retention

are important until host defenses have gained adequate control of an infection or injury. Thus, when functioning properly, many retention signals likely indicate an active threat.

Recently, several studies have identified molecules that promote neutrophil retention in wounds (Figure 1D,J) [45••–49••]. These findings are of particular clinical interest as persistent neutrophilic inflammation precludes proper wound healing and is associated with a number of inflammatory conditions [1]. Hypoxia-inducible factor 1 is a transcription factor expressed in activated neutrophils and macrophages. HIF-1a is targeted for degradation by oxygen-dependent prolyl hydroxylases (PHDs) [47]. During hypoxia or in response to bacterial infections under normoxic conditions, PHD activity is suppressed, resulting in HIF-1 accumulation[50,51]. Activated HIF-1a stimulates pathways that promote neutrophil survival and defense functions. In tail transected zebrafish, neutrophils expressing constitutively active HIF-1a continuously surveil the wound even during the resolution phase suggesting that negative regulation of retention signals is an important mechanism for resolving neutrophilic inflammation [47].

Disrupting the CXCR4/CXCL12 signaling axis accelerates neutrophilic inflammation resolution by increasing reverse migration [45••]. Interestingly, CXCR4 also plays a role in retaining neutrophils in the bone marrow [52,53]. In this context, neutrophil retention is dependent on the small GTPase, Rac2, which regulates F-actin assembly and cell polarization (among other things) [52]. The finding that CXCR4 governs neutrophil retention in hematopoietic tissue (limiting inflammation) and at wounds (promoting inflammation) is interesting as it suggests its role in neutrophil retention may be more general.

On the other hand, in contrast to Rac2, which promotes F-actin assembly, sema3f was recently identified as a neutrophil retention signal that acts by inducing F-actin disassembly resulting in reduced migration velocity [49••]. Neutrophil-specific depletion of sema3f in zebrafish and Sema3f in mice accelerated neutrophil egress from tail wounds and sites of acutely inflamed lungs, respectively [49••].

Lipid signaling in reverse migration

Leukotriene B4 is a well-characterized chemoattractant that has evolved specialized roles in both forward and reverse neutrophil migration. LTB4 is one of many derivatives of arachidonic acid produced and secreted by neutrophils during inflammation [51]. Neutrophil release of LTB4 helps recruit other neutrophils by amplifying chemoattractant gradients and enhancing migration toward fMLF [51,54]. In mice, LTB4 is required for the formation of neutrophil swarms [55].

At the wound, neutrophils shift from the production of pro-inflammatory LTB4 to Lipoxin A4, an anti-inflammatory derivative of arachidonic acid (Figure 1H) [56,57]. In neutrophils, macrophage-derived Prostaglandin E2 (PGE) inhibits the translocation of 5-LO, a critical enzyme required for LTB4 synthesis, from the cytoplasm to the nucleus [57]. As a result, LXA4 may become the predominant lipid produced from arachidonic acid at wounds, and reverse migration would become the favored outcome, promoting inflammation resolution.

Intriguingly, in mice, LTB4 plays a role in reverse transendothelial migration (rTEM) - the process by which neutrophils cross the blood vessel lumen to re-enter the circulation [4,58]. Endogenously produced LTB4 promotes neutrophil rTEM by stimulating neutrophil-elastase mediated cleavage of vascular Junctional Adhesion Molecule C (JAM-C) (Figure 1L) [4,58]. It will be interesting to learn whether neutrophils revert to LTB4 production or whether LTB4 is produced by other cells near the vasculature.

Emerging mechanisms

While neutrophils' attractant detection systems are sophisticated, there are boundaries within which they can successfully operate. For example, spatial sensing requires an adequately steep gradient (~2–5% difference in ligand concentration across the cell length) and temporal sensing fails when attractant concentrations are no longer rising [12,22,29]. Given the long distances they travel and the reliability with which neutrophils reach their targets *in vivo*, there must be mechanisms in place to ensure that chemoattractant fields are maintained within these bounds. In some cases, neutrophils themselves play an active role in shaping gradient fields. Neutrophils amplify chemoattractant gradients by producing and secreting LTB4, which also enhances neutrophil migration toward fMLF [38,51,54,59].

More recently, "self-generated gradients," whereby cells create or sharpen attractant gradients by sequestering receptor-bound ligands or via attractant degradation, are emerging as an important mechanism for maintaining optimal attractant concentration [60–65••,66•] [60–62•]. Indeed, chemotaxis along self-generated gradients has been observed both *in vitro* and *in vivo* in various contexts, including nutrient acquisition, embryogenesis, and cancer cell metastasis [60,61,67]. Although this mechanism seems more applicable to collective cell migration, individual yeast cells enhance their ability to find mating partners by degrading and thereby sharpening pheromone gradients suggesting such a phenomenon is possible at the single-cell level [68]. In the context of neutrophil migration, the role of self-generated gradients remains to be determined. It is particularly intriguing to consider the role of self-generated gradients in cancer-cell dissemination from tumors as a similar mechanism may help explain neutrophil dispersal from wounds [61]. In any case, future studies on neutrophil migration should account for the role of neutrophils in gradient formation and/or degradation in design and interpretation.

Conclusion

There is a famous adage attributed to Aristotle: "The more you learn, the less you know." Over a century of study on leukocyte migration has produced a wealth of knowledge on the fundamental principles of cellular navigation. Reductionist, *in vitro* approaches have allowed us to define the basic algorithms that govern signal integration in neutrophils. Through more complex studies, we have come to appreciate that neutrophil intrinsic mechanisms (e.g., dynamic regulation of attractant receptors and signaling pathways) work in concert with extrinsic signals (damage and retention signals) to ensure neutrophils carry out their functions at the right place and right time. At the same time, we recognize that *in vivo*, the signaling landscapes are dynamic, imprecise, and subject to randomness that can be difficult to predict or measure. This imprecision can go awry and in some cases, neutrophil reverse

migration can contribute to human diseases, like lupus [69•]. We may feel like we know less, but we appreciate the complexity that inspires us to learn more.

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Highlights

- Neutrophil reverse migration has emerged as a critical component of inflammation resolution
- In the wound microenvironment, neutrophils must integrate and prioritize among pro-inflammatory recruitment and retention signals and anti-inflammatory dispersal signals
- Recently, key molecular players and signaling pathways regulating reverse migration have provided insights into the mechanisms underlying these complex signaling decisions
- In combination with what we know about prioritization during the recruitment phase of inflammation, a more holistic model of signal integration in migrating cells is emerging

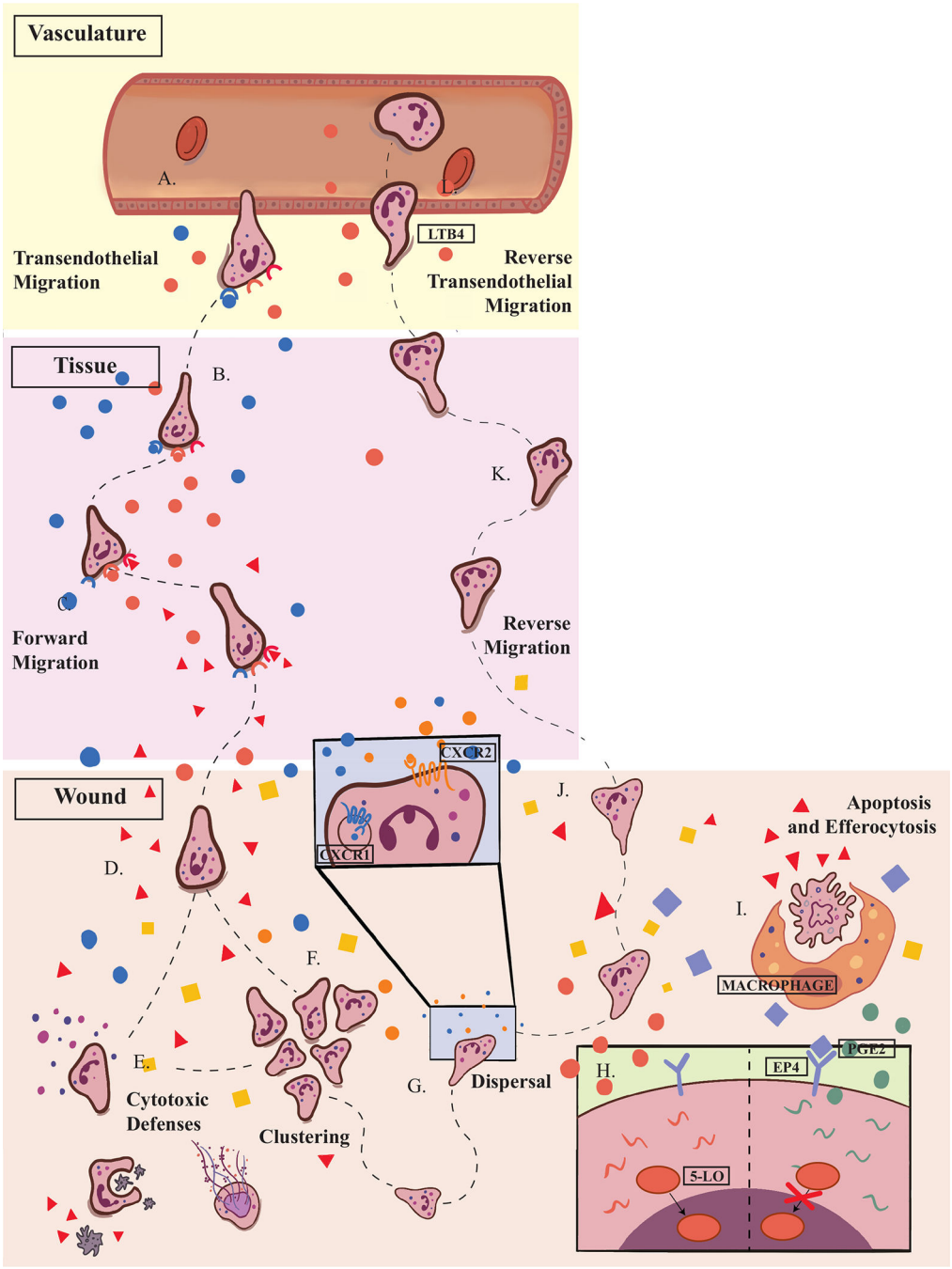

Figure 1. Neutrophils encounter complex signaling environments during forward and reverse migration *in vivo*

A. When tissue damage is detected, neutrophils migrate out of the vasculature in a process called transendothelial migration **B.** In the tissues, neutrophils engage in multi-step navigation toward sources of intermediary chemoattractants. **C.** Once neutrophils detect wound-derived, end-target signals, they ignore intermediary attractants. **D.** In the wound, neutrophils integrate multiple signals, including recruitment and retention signals, pro-inflammatory and anti-inflammatory molecules. **E.** Neutrophil clustering is mediated by

the CXCL1/CXCL8 signaling axis **F.** At the wound, neutrophils mount a cytotoxic response that includes degranulation, phagocytosis, and NET formation. **G.** In the wound, CXCR1 is rapidly internalized, leaving CXCR2 as the primary CXCL8 receptor. CXCR2 promotes cluster dissolution. **H.** Macrophages secrete PGE2, which activates neutrophil EP4 receptors preventing 5-LO translocation into the nucleus. As a result, neutrophils switch from the production of LTB4 to the production of LXA4. **I.** Some neutrophils undergo apoptosis and efferocytosis by macrophages. **J.** After overcoming recruitment and retention signals, some neutrophils are randomly dispersed from the wound microenvironment. **K.** Once they leave the wound, neutrophils reverse migrate back toward the vasculature. **L.** LTB4 mediates rTEM allowing neutrophils re-enter circulation.

CXCL8 ●
LTB4 ●
End-Target Attractant ▼
Retention Signal ■
PGE2 ◆