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Series: Neutrophils in Action

Feature Review

Reverse Migration of Neutrophils: Where, When, How, and Why?

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Neutrophil migration to injured and pathogen-infected tissues is a fundamental component of innate immunity. An array of cellular and molecular events mediate this response to collectively guide neutrophils out of the vasculature and towards the core of the ensuing inflammatory reaction where they exert effector functions. Advances in imaging modalities have revealed that neutrophils can also exhibit motility away from sites of inflammation and injury, although it is unclear under what circumstances this reverse migration is a physiological protective response, and when it has pathophysiological relevance. Here we review different types of neutrophil reverse migration and discuss the current understanding of the associated mechanisms. In this context we propose clarifications to the existing terminology used to describe the many facets of neutrophil reverse migration.

Trends

Neutrophils can exhibit abluminal-to-luminal motility through endothelial cells (rTEM) and reverse interstitial migration (rIM) in models of inflammation. Emerging data suggest that the former can mediate systemic dissemination of a local inflammatory response whereas the latter is a protective physiological event that facilitates inflammation resolution.

In models of inflammation, neutrophil rTEM is enhanced and reduced in EC JAM-C$^{-/-}$ and neutrophil elastase (NE)$^{-/-}$ mice, respectively, demonstrating novel roles of these molecules in regulation of neutrophil trafficking.

Neutrophil rIM is suppressed by genetic activation of the HIF pathway in zebrafish, an intervention that causes a delay in inflammation resolution.

Targeting neutrophil reverse migration represents a novel approach for development of drugs aimed at modulating inflammation.

Introduction

The motility of leukocytes from the bloodstream to interstitial tissues is a fundamental host defence reaction [1]. In the context of neutrophils and innate immunity, this process is largely initiated by pathogen-associated molecular patterns (PAMPs), released from invading microorganisms, or by damage-associated molecular patterns (DAMPs), derived from damaged, dead, or environmentally stressed cells [2,3]. Such danger signals can be detected by sentinel cells including mast cells, macrophages, and dendritic cells, which in turn can release a variety of mediators that promote leukocyte recruitment [1,4]. The mechanisms that regulate leukocyte accumulation into tissues are complex and need to be tightly regulated because defective leukocyte migration leads to immune deficiency disorders while excessive or aberrant leukocyte trafficking can be damaging to the host [3,5,6]. Broadly, this event involves breaching of venular walls and leukocyte crawling within the interstitium to sites of tissue injury or infection (Box 1; the reader is referred to recent comprehensive reviews for mechanistic details [1,7–9]). Once arrived at inflammatory sites, neutrophils can exhibit numerous cellular responses such as release of additional mediators, generation of reactive oxygen species (ROS), phagocytosis, and extrusion of neutrophil extracellular traps (NETs) [3,6], functions that are all ultimately aimed at eliminating the cause of the inflammatory reaction and promoting resolution of inflammation.

The efficient migration of neutrophils to sites of inflammation is governed by the ability of these cells to rapidly detect and respond to attractant molecules [1]. This ensures movement of leukocytes, classically in an amoeboid and polarised manner, towards the foci of the

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Box 1. Neutrophil Migration from the Vascular Lumen to the Interstitial Tissue

The migration of leukocytes out of the vascular lumen and within the interstitial tissue is commonly mediated by coordinated presentation of directional cues to leukocytes on the surface of cells (e.g., ECs and pericytes) and extracellular matrix structures (e.g., heparin sulphate glycosaminoglycans, HS GAGs). Once leukocytes have encountered stimulatory molecules within the lumen of microvessels, a series of adhesive pathways, classically described by the 'leukocyte adhesion cascade', guide them out of the vasculature and into the surrounding tissue [14]. This initiates with leukocyte rolling, mediated by weak and reversible attachment of leukocytes to ECs, followed by further activation of leukocytes leading to leukocyte arrest and crawling on the inner aspect of venular walls, as previously detailed [7,14] (see also Figure 1).

Following luminal interactions, leukocytes need to breach ECs and the second cellular layer of venular walls, the pericyte sheath. Pericytes are mural cells that are typically embedded within the vascular basement membrane (BM). Our understanding of this stage of leukocyte migration is scant, but recent evidence has indicated that neutrophils and monocytes breach venular walls by migrating through gaps between adjacent pericytes and sites within the venular BM exhibiting lower deposition of BM extracellular matrix protein constituents [78,79]. In addition, high-resolution confocal intravital imaging of neutrophil behaviour within mouse cremasteric venular walls identified significant neutrophil sub-EC crawling, as supported by ICAM-1-expressing pericyte processes [29]. Together, it is now apparent that, beyond the vascular lumen, full breaching of the venular wall by leukocytes involves an additional cascade of molecular cues and adhesive mechanisms [1,30]. Of relevance, there appears to be a steep gradient of HS scaffolds between the vascular lumen and the basolateral aspect of endothelial cells [80]. Such a profile could provide a means through which a gradient of chemokines is established across the vessel wall, aiding the passage of leukocytes out of the vasculature in a luminal-to-abluminal manner. Once the leukocytes have fully breached venular walls, they are required to migrate within the interstitial tissue to reach the foci of infection or injury. This phase of leukocyte migration has been the subject of several elegant works involving different modes of advanced confocal intravital microscopy, studies that have begun to shed light on the mechanisms through which efficient leukocyte interstitial motility is achieved [31,32,35,81]. Such investigations have identified multiple patterns of leukocyte migration, and numerous models of cellular and molecular cascades have been proposed [8,9,35,82].

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inflammatory response. As with all motile cells, neutrophils are required to integrate numerous signals to choreograph their movement within complex 3D structures. At sites of inflammation, this is largely regulated by presentation of directional cues in soluble form or, more commonly, in an immobilised fashion, providing a haptotactic gradient. Although the details of how attractant molecules are presented in tissues remain unclear, there is now solid evidence for the existence of functional chemotactic gradients *in vivo* [10]. Additional factors that can modulate movement of neutrophils include shear force (relevant to luminal leukocyte responses), the cellular and molecular composition of the interstitial tissue, and the potential existence of repulsive molecules. The profile and directionality of neutrophil migration out of the vascular lumen and within the interstitial tissue is thus regulated by the resultant processing of multiple signalling factors, both mechanical and biochemical. This commonly leads to a net migratory response of recruited neutrophil populations towards the core of an inflammatory insult, from which it is proposed they are subsequently cleared through apoptosis and uptake by tissue macrophages. Within the past 10 years, investigations of neutrophil behaviour at single cell level have also shown that neutrophils can migrate away from sites of inflammation. We review here the existing evidence for this enigmatic cellular behaviour within inflammation and immunity, describe the different types reported, discuss the proposed mechanisms and, importantly, the potential physiological and pathological roles of this phenomenon. Furthermore, because there is some ambiguity regarding the terms used to describe the various modes of neutrophil reverse migration, we propose some clarity on nomenclature.

Neutrophils Can Show Different Modes of Reverse Migration

Since the first reports of neutrophil reverse migration [11–13], several types of this response have now been identified in different stages and contexts of leukocyte trafficking (Figure 1, Key Figure). These events are loosely referred to as 'neutrophil reverse migration', an expression that requires optimisation and clarity (Box 2 and Figure 1). Of note, this term does not distinguish between neutrophils that exhibit a U-turn and cells that show a true reversal of polarity, or any in-between responses that reflect altered gradient sensing. While acknowledging this limitation, for simplicity

Key Figure

Leukocytes Exhibit Different Modes of Reverse Motility *In Vivo*

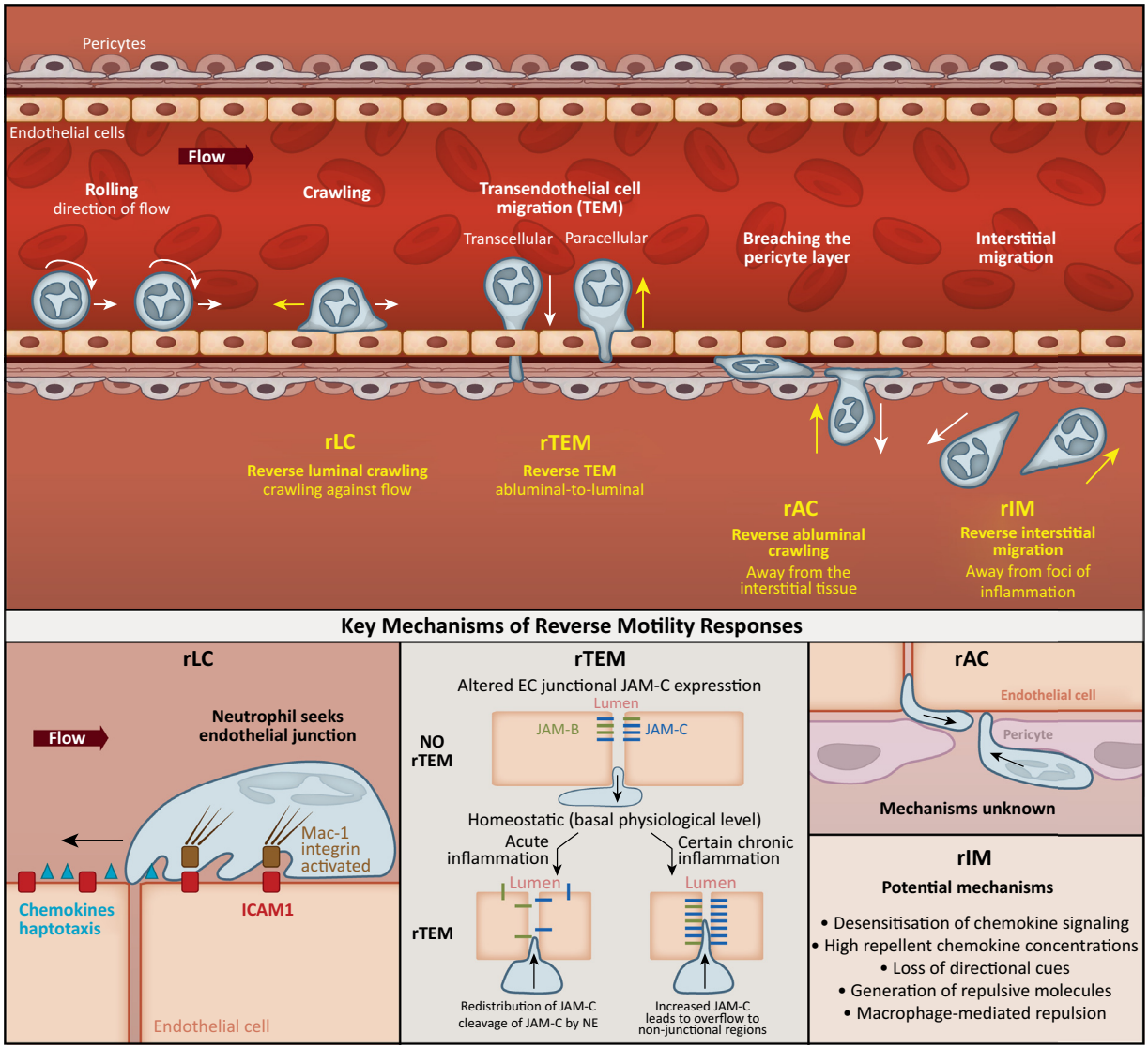

Figure 1. (Upper panel) In response to an inflammatory insult, blood leukocytes initiate a series of adhesive interactions with venular walls, as described by the leukocyte adhesion cascade (see also Box 1). This sequence of events involves leukocyte rolling and crawling on the luminal aspect of endothelial cells (ECs), transendothelial cell migration (TEM; paracellular or transcellular), and breaching of the pericyte layer (and associated venular basement membrane). This cascade facilitates the net migration of leukocytes from the vascular lumen to the interstitial tissue and eventually tissue migration towards the core of the inflammatory insult (indicated by white arrows). Although leukocyte rolling occurs in the direction of flow, other responses within this cascade can occur in a reverse mode (indicated by yellow arrows). We propose here the following terms to describe different types of reverse motility responses (see also Box 2): 'reverse luminal crawling' (rLC) for crawling against vascular flow, 'reverse TEM' for leukocyte motility through ECs in an abluminal-to-luminal direction, 'reverse abluminal crawling' (rAC) for movement of leukocytes within the pericyte layer in the opposite direction to that of the interstitial tissue, and 'reverse interstitial migration' (rIM) for dispersion and/or migration of cells away from the foci of an inflammatory insult within the interstitial tissue. (Lower panels) The panels highlight key molecular interactions involved in the reverse migration responses detailed above. rLC: chemokine-induced activation of neutrophil Mac-1 supports Mac-1/ICAM-1-mediated neutrophil crawling which can occur against flow. rTEM: JAM-C is maintained at EC junctions through interactions with its ligand JAM-B. Disruptions of this interaction or physiological levels of EC JAM-C (i.e., reduced or enhanced) can lead to neutrophil rTEM. rAC and rIM: at present almost nothing is known about the mechanisms that mediate rAC; however, it may be caused by similar mechanisms to those associated with rIM (listed).

Box 2. Proposed Terminology To Describe Leukocyte Reverse Migration Responses

At present the literature is made unnecessarily opaque by multiple terms used to describe the same phenomenon or, even more confusingly, by similar terms used to describe very different types of leukocyte reverse migration responses. This issue has contributed to misunderstandings in the field, particularly for those not working directly on these reactions in their own laboratories. To address this important point, we propose the following terms to provide a more accurate and consistent terminology for describing the wide range of leukocyte reverse migration events that are emerging (see also Figure 1).

Reverse migration: term to describe the general phenomenon of leukocytes moving in the opposite direction to that expected. In some circumstances, this will be in the opposite direction taken by the net leukocyte population, and in others it will describe migration away from a stimulus which has previously been described as being chemotactic for leukocytes.

Reverse abluminal crawling (rAC): abluminal (sub-EC) crawling is commonly associated with neutrophils exhibiting numerous oscillatory movements in conjunction with the formation of multiple protrusions into the venular basement membrane and the pericyte layer. This enables neutrophils to seek essential directional cues to fully breach venular walls. The retrograde motility of neutrophils in the venular wall away from exit points could be defined as neutrophil reverse abluminal crawling (rAC), in other words reverse migration away from the direction of the interstitial tissue.

Reverse interstitial migration (rIM): dispersion and/or migration of cells away from the foci of an inflammatory insult within interstitial tissues.

Reverse luminal crawling (rLC): crawling of leukocytes in the opposite direction to that of blood flow *in vivo* or, under *in vitro* conditions, against flow.

Reverse transendothelial cell migration (rTEM): transendothelial cell migration, in other words migration through the EC layer (junctional or non-junctional), in an abluminal-to-luminal direction.

we propose to use the term 'reverse migration' to describe the general concept of retrograde neutrophil motility. However, because such events have been observed at different stages of neutrophil trafficking, for lucidity we propose to adapt this terminology to defined steps of leukocyte trafficking as necessary. These are detailed in Box 2 and illustrated in Figure 1.

Luminal Motility

The migration of leukocytes out of the vasculature is described by the leukocyte adhesion cascade (Figure 1 and Box 1). As illustrated in this scheme, key early luminal leukocyte motility responses are leukocyte rolling and crawling. The former is principally a selectin-dependent reaction (cells exhibit velocity of ~50 μm/s) that is evident and enhanced under conditions of hydrodynamic shear force, and hence exclusively occurs in the direction of blood flow [14]. Neutrophil luminal crawling (~1–3 μm/min and hence ~100-fold slower than rolling) is a post-firm adhesion event that is mediated by a Mac-1/ICAM-1 interaction [15]. This response can occur both in the direction of blood flow (forward luminal crawling), against blood flow (reverse luminal crawling; rLC), and, more commonly, perpendicularly to the direction of the bloodstream [15]. Of note, luminal crawling (both forward and reverse) was first reported in the context of natural killer T (NKT) cells in liver sinusoids [16] and has also been noted with patrolling monocytes [17,18].

Breaching Venular Walls

For leukocytes to exit the vascular lumen they are required to breach the endothelial cell (EC) barrier that lines the inner aspect of venular walls. This appears to involve use of ventral protrusions extended through the endothelium during crawling [19,20], providing a means through which leukocytes can detect directional cues beyond the venular lumen [1]. Neutrophil migration through the EC barrier can occur via both transcellular and paracellular modes [1]. Although significant use of the transcellular route has been reported across the blood–brain and blood–retina barriers during inflammatory pathologies [21], paracellular diapedesis appears to be the most prevalent mode (~80–90%) of breaching ECs both *in vitro* and *in vivo* [1,14,22,23].

The latter response is supported by an elaborate series of interactions between leukocytes and EC junctional adhesion molecules including PECAM-1, JAM-A, JAM-C, ICAM-2, CD47, ALCAM-1, ESAM-1, MCAM, and CD99 (for reviews on this topic, see [1,24,25]). There is strong evidence to indicate distinct and/or sequential roles for these molecules in different stages of leukocyte movement through venular walls [14].

Leukocyte transendothelial migration (TEM) is considered to be largely a one-way trafficking process. This stems from the general concepts that leukocytes can sense chemotactic gradients across the thin depth of ECs and also the rapid sequential opening and closing of EC junctions. For a substantial percentage of tissue-infiltrated leukocytes this scenario holds true. However, there is now evidence for the ability of leukocytes to exhibit abluminal-to-luminal migration through the endothelium, in other words they exhibit reverse TEM (rTEM). This concept was first reported on by Gwendalyn Randolph and Martha Furie in the context of human monocyte TEM through cultured ECs [26], and has since been observed for numerous other leukocyte subtypes including neutrophils (Table 1). Specifically, neutrophils have now been shown to exhibit rTEM through cultured ECs *in vitro* [12,22] and also in some murine models of inflammation *in vivo* [22,27]. The latter studies were performed on inflamed cremaster muscle tissues of neutrophil reporter mice (i.e., *LysM-GFP-ki* mice [28]) in which the cremasteric venules were immunofluorescently labelled for PECAM-1 to delineate EC junctions. The application of high-resolution 3D real-time confocal microscopy to this model provided the first conclusive evidence for the occurrence of neutrophil rTEM *in vivo* [22,27]. This response was most notable in tissues subjected to ischaemia–reperfusion injury (~10–15% of total TEM events), following LTB_4-driven reactions (~20–25% of total TEM events) and also under conditions of pharmacological blockade or genetic deletion of EC JAM-C (>30% of all TEM events) [22,27]. The latter highlights the importance of this EC junctional molecule in maintaining normal luminal-to-abluminal neutrophil TEM, and also provides an important mechanistic insight to regulation of neutrophil rTEM (see below for more details). In these scenarios, neutrophils were observed to exhibit multiple forms of rTEM: (i) partial or full breaching of EC junctions in a luminal-to-abluminal direction, followed by reverse migration (abluminal-to-luminal) through the same junction back into the vascular lumen, and (ii) breaching of EC junctions in a luminal-to-abluminal direction,

Table 1. Leukocyte Reverse Transendothelial Cell Migration (rTEM) Has Been Reported for Multiple Leukocyte Subtypes.

Study	Leukocyte	Model	Refs
Randolph and Furie (1996); Randolph *et al.* (1998)	Human monocytes	Monocytes co-cultured with HUVECs in the presence of various stimulants (*in vitro*)	[26,83]
D'Amico *et al.* (1998); Bianchi *et al.* (2000)	Human dendritic cells	Migration of DCs through polycarbonate filters coated with matrix proteins on the upper side and HUVECs on the lower side (*in vitro*)	[84,85]
Llodra *et al.* (2004)	Mouse monocyte-derived cells	Emigration of monocyte-derived cells from atherosclerotic plaques after transfer from atherogenic background to normal mice, as assessed by indirect methods (*in vivo*)	[86]
Buckley *et al.* (2006)	Human neutrophils	Neutrophils co-cultured with TNF-stimulated HUVECs (*in vitro*)	[12]
Bradfield *et al.* (2007)	Human monocytes	Monocytes co-cultured with TNF-stimulated HUVECs under flow (*in vitro*)	[50]
Lee *et al.* (2009)	Human T cells	T cells co-cultured with HUVECs with CXC12 in the sub-EC compartment (*in vitro*)	[87]
Woodfin *et al.* (2011); Colom *et al.* (2015)	Mouse neutrophils	Inflamed mouse cremaster muscle as analyzed by confocal intravital microscopy (*in vivo*)	[22,27]

followed by sub-EC motility and rTEM through a different EC junction to that in which the TEM response initiated. Neutrophil rTEM was predominantly paracellular in nature, although very rarely transcellular rTEM was observed, indicating that, as seen with normal luminal-to-abluminal TEM, rTEM can also occur via both EC junctions and the cell body of the endothelium [22]. Importantly, regardless of the type of rTEM, on re-entering the vascular lumen the neutrophils exhibited rolling or crawling along the luminal aspect of the vessel wall and eventually migrated away from the local site of inflammation. The implications of this blood re-entry phenomenon are discussed below.

Once through the endothelium, neutrophils encounter the second cellular component of venular walls, pericytes. As with neutrophil rTEM, the application of high-resolution 3D real-time confocal intravital microscopy has shed much light on the dynamics of this aspect of neutrophil trafficking, as briefly detailed in Box 1. Specifically, through the use of genetically modified mice exhibiting RFPcherry-pericytes (under the control of the αSMA promoter) and GFP-neutrophils (*LysM-GFP-ki* mice), real-time imaging has revealed that post TEM, neutrophils exhibit significant crawling along pericyte processes [29]. During this stage, neutrophils extend multiple protrusions through the pericyte layer and its associated venular basement membrane, suggesting an attempt at seeking further directional cues necessary for full breaching of venular walls [29,30]. This response can often result in oscillatory movements of protruding neutrophils into the venular basement membrane, an event that could be defined as a mode of neutrophil reverse migration, in other words neutrophils crawling away from the interstitial tissue. We propose the term neutrophil 'reverse abluminal crawling' (rAC; Box 2) for this cellular behaviour.

Interstitial Tissue Migration

Once effector leukocytes have fully breached blood vessel walls and have entered the interstitial space, they are guided to their target sites by gradient sensing [10] (Box 1). This response is additionally regulated by a vast array of cell-intrinsic and cell-extrinsic factors that collectively ensure the correct positioning, shape and forward propulsion of immune cells in an amoeboid and integrin-independent manner [8–10]. High-resolution imaging methods, such as intravital confocal and multi-photon microscopy, have yet again been instrumental in aiding our understanding of the cellular and molecular mechanisms that underlie these events. Most notably, such studies have identified several key phenomena that regulate leukocyte motility in the extravascular space [8,31,32]. These include the ability of neutrophils to provide relay signals as a means to amplify the recruitment process [31] and the existence of mediator hierarchies to facilitate efficient movement of neutrophils from the vascular lumen to sites of tissue injury [32]. The latter was originally indicated in elegant *in vitro* studies that highlighted the power of combinatorial determinants in regulating leukocyte positioning and motility in complex microenvironments [33,34]. Once in the interstitial tissue, neutrophils can move into cellular clusters, exhibiting 'neutrophil swarming' [35]. Some of these swarms are 'temporary', and neutrophils may move away from the inflammatory focus over time, enabling them to participate in other swarms; ultimately swarm resolution may occur with neutrophils dispersing from the inflammatory site [35]. The profile and dynamics of such responses are very much governed by the nature (e.g., pathogen or sterile injury) and magnitude of the inflammatory trigger [31,32,35–43]. In the above-cited murine models of inflammation [35] the focus has been very much on swarm assembly and dynamics, with less emphasis being placed on the mechanisms of swarm resolution or the signals preventing swarm resolution. These phenomena of neutrophil migration away from the foci of inflammation have been extensively studied in the zebrafish [44]. The first such studies were conducted by Anna Huttenlocher and colleagues using a zebrafish embryo tailfin injury model [13]. Zebrafish inflammation models are perhaps the simplest vertebrate systems for the study of inflammation dynamics and have since been widely used for the study of neutrophil interstitial migration. In the works of Huttenlocher and colleagues, and subsequent studies performed in several other laboratories, a wound to the tailfin leads to neutrophil

recruitment with numbers peaking around 6 h, followed by dispersion and migration of the neutrophils away from the wound beginning as soon as 3 h after injury [13,45–48]. To distinguish this phenomenon from other modes of neutrophil reverse motility, we propose the term neutrophil 'reverse interstitial migration' (rIM). Although it has been suggested that following rIM neutrophils can re-enter the circulation [13], such an event has not been observed in murine models of sterile or infectious inflammation [22,31], or in all tailfin injury models of the zebrafish [46]. Characterisation of the profile of these reverse migrated neutrophils *in vivo* suggests that no major changes in phenotype occur [49]. For tissue infiltrated neutrophils to re-enter the blood vascular compartment, neutrophils will be required to link together rIM, followed by reverse migration through the pericyte layer and the venular basement membrane (i.e., exhibit rAC) and finally undergo reverse migration through the endothelium (i.e., rTEM). The possible occurrence of this coordinated sequence of events (i.e., rIM + rAC + rTEM) will require further investigations through advances in transgenic and imaging technologies.

Mechanisms of Leukocyte Reverse Migration

As detailed above, numerous types of neutrophil reverse migration responses have been reported, but it remains unclear whether common mechanisms are involved in supporting these phenomena (Figure 1). With respect to luminal neutrophil–vessel wall interactions, at present it is unknown whether the frequency of forward and reverse luminal crawling (rLC) is a result of random migration patterns or is governed by luminal haptotactic gradients. However, in general, shear flow induces higher affinity of integrin-mediated leukocyte attachment and this can explain the ability, and indeed desire, of the cells to crawl against shear forces along the luminal side of blood vessels.

More is known about neutrophil reverse motility at the level of EC junctions, in other words during the process of breaching the endothelium. Tracking of neutrophils within inflamed murine tissues by confocal intravital microscopy demonstrated that neutrophils could exhibit significant reverse transendothelial cell migration (rTEM) under inflammatory conditions where there was reduced expression of EC JAM-C [22,27]. These findings are in line with earlier works showing that loss of EC JAM-C functionality promotes monocyte rTEM *in vitro* [50]. Collectively, there is now conclusive evidence in both human and murine systems for the ability of EC JAM-C to maintain ECs in a polarised manner such that a one-way gate is established for leukocytes moving from the vascular lumen to the abluminal tissue compartment. For JAM-C to achieve this, it must be expressed at EC junctions at basal physiological levels [50]. Specifically, diminished expression of EC JAM-C, as achieved by its redistribution from EC junctions in some acute inflammatory conditions (e.g., post ischaemia–reperfusion injury) [22,51] or following its enzymatic cleavage by neutrophil elastase [27], will disrupt this gate and promote neutrophil rTEM. Similar results are obtained under conditions of pharmacological blockade and/or genetic deletion of EC JAM-C, providing direct evidence for its involvement as a regulator of polarised leukocyte motility [22,50].

The precise mechanism through which EC JAM-C can mediate luminal-to-abluminal neutrophil migration remains unclear, but several possibilities can be speculated upon. For example, as JAM-C is a high-affinity ligand for its family member JAM-B, and JAM-C–JAM-B interaction maintains JAM-C at EC junctions, the ratio of expression of these molecules controls their localisation within EC tight junctions [52]. This could impact on EC barrier function and hence directional cell migration through EC junctions. In contrast to the reduced expression of JAM-C noted in acute inflammatory conditions, chronic inflammatory scenarios such as atherosclerosis appear to be associated with increased expression of EC JAM-C (P.F. Bradfield and B.A.I., unpublished). Paradoxically such circumstances also seem to cause redistribution of JAM-C from junctional regions to the luminal aspect of the endothelium, possibly owing to saturation of JAM-B binding sites. This response could also promote neutrophil rTEM by (i) compromising EC junctional integrity, and (ii) providing a haptotactic gradient of JAM-C, via interactions with its

leukocyte ligand Mac-1, for neutrophils away from the junctions to the luminal aspect of the vessel. As JAM-C plays a key role in the assembly of the PAR3/PAR6 cell polarity complex [53–55], loss of junctional JAM-C could additionally result in disrupted EC polarity that could influence directional motility of neutrophils through EC junctions, for example through disrupted generation and/or expression of leukocyte directional cues. Disrupted expression of EC junctional molecules could also regulate the dynamics of opening and closing of the cytoskeleton-dependent endothelial barriers that could impact on directional cell migration.

Other factors and scenarios that could cause neutrophil rTEM include diminished attractant molecule generation following inflammation resolution. This could lead to insufficient guidance cues being presented to migrating leukocytes to facilitate their continued motility through venular walls, in other words through the pericyte layer and the venular BM. Such a scenario can potentially result in reverse movement of leukocytes from the sub-EC space back into the vascular lumen. Conversely, enhanced mediator generation, such as that encountered during prolonged and/or excessive pathological inflammation, may lead to loss of responsiveness of leukocytes through desensitisation of receptors and/or their associated signalling pathways. Under such conditions leukocytes may also show a defective ability to correctly integrate competing directional cues for correct luminal-to-abluminal movement through venular walls. Of note, numerous chemokines, including CXCL12 (stromal cell-derived factor-1, SDF-1), CCL26 (eotaxin-3), and CXCL8 (IL-8) have been reported to be chemorepellent for leukocytes, responses that were shown to be mediated by chemokine receptors and dependent on the concentration of the molecule in question [11,56,57]. In neutrophils, as found with chemoattraction, chemorepulsion is reportedly pertussis toxin-sensitive and dependent on phosphoinositide-3-kinase, RhoGTPases, and associated proteins [11]. Furthermore, disruption of other signalling molecules, such as levels of cAMP and the activity of PKC isoforms, could revert a chemorepellent to a chemoattractant response [11]. Other intriguing mechanisms that could mediate neutrophil rTEM include the potential establishment of reverse chemokine gradients by intravascular crawling monocytes patrolling the vascular luminal surface, and by the possible generation of chemorepulsive factors such as axon-guidance repellant molecules that are gaining much interest as regulators of immune cell migration [58–60]. The potential role of such pathways in neutrophil reverse migration *in vivo* remains to be explored.

Some of the possibilities discussed above also apply to neutrophil rIM. Specifically, the concept of neutrophil migration against a chemokine gradient (fugetaxis) was first introduced by Poznansky and colleagues using a microfluidic migration assay [11]. This phenomenon has been studied more extensively in recent years using zebrafish models of inflammation where two competing hypotheses remain to be resolved. Neutrophils can respond to directional cues (either chemotactic or fugetactic signals) that dictate their migration away from inflammatory sites [61]. Alternatively, neutrophils may lose sensitivity to chemotactic gradients over time (for example by receptor desensitisation) and revert to a default programme of 'patrolling' the tissues [45,46,62]. Such mechanisms could account for the formation of transient neutrophil swarms that are followed by cellular dispersion and/or cellular recruitment to other swarms [35]. The potential existence and nature of chemorepulsive signals driving neutrophils away from inflammatory sites remains unknown [61]. Intriguingly, a recent study has suggested that macrophages at sites of inflammation can cause neutrophil rIM through a contact-mediated mechanism [63].

Neutrophil rIM can be delayed by genetic and pharmacological approaches targeting hypoxia inducible factor (HIF) [45]. Signalling through HIF pathways is an important regulator of neutrophil function, prolonging neutrophil functional lifespan and enhancing inflammation in mammalian systems [64]. This suggests that the response might be regulated by transcriptional changes in

receptor levels, leading to changes in sensitivity to tissue gradients, akin to those responsible for neutrophil recruitment. Specifically activating the HIF transcriptional response in zebrafish neutrophils by overexpressing a dominant active form of HIF alone is enough to cause neutrophils to stay at inflammatory sites [45]. Hence, receptor downregulation at sites of high ligand levels, with subsequent transcriptional regulation of a chemokine receptor, would be a strong candidate for the altered sensitivity of neutrophils to their local environment over the course of inflammation. These processes have parallels with 'survival signals' regulating neutrophil survival during inflammation resolution [65]. Signals that regulate reverse motility and neutrophil apoptosis in tandem might best be considered as 'retention signals'. Certainly, hypoxia has been shown to delay neutrophil apoptosis and retention signalling in parallel [45], although whether this is true for other survival signals remains to be determined. In this paradigm, the nature of the gradients to which neutrophils respond, and the signalling pathways that modulate sensitivity to them, are key questions for future studies.

Collectively, considering the diverse range of reverse migration events noted to date, it is not surprising that different mechanisms have been associated with distinct responses as detailed above. However, because all reverse motility responses ultimately relate to altered directional migration of cells, the existence of common mechanisms appears plausible. In this regard, potential contributing factors include (i) the impact of antiadhesive or repellent local mechanical or chemical signals, (ii) disrupted generation and/or presentation of attractant cues, (iii) existence of competing gradients of attractants and repellents, and correct integration of these by migrating leukocytes, and (iv) desensitisation of leukocyte attractant cell surface receptors and/or their associated signalling pathways following high receptor occupancy. Such mechanisms have been extensively studied in the context of bacteria, single cell organisms such as *Dictyostelium discoideum* (*D. discoideum*), and axonal growth cones [66–68], and attaining direct *in vivo* evidence for their involvement in regulating neutrophil migration will form the basis of future studies.

Physiological and Pathological Relevance of Leukocyte Reverse Migration

The functional role of neutrophil reverse motility within inflammatory scenarios requires further exploration. At present there exists evidence to suggest that this phenomenon can be both a physiologically protective response, facilitating an efficient and resolving innate immune reaction, and also a pathological tissue-damaging event, depending on its nature and context (Table 2).

Luminal crawling (forward, reverse, or perpendicular to direction of blood flow) was first reported in the context of NKT cells and monocytes, and is now considered to be a physiological response that provides intravascular immune surveillance [17,18]. Whether neutrophil luminal crawling also provides such a patrolling role is at present unknown, but it is feasible to speculate that luminal crawling neutrophils are a subset of high-migratory cells that rapidly respond to chemotactic cues and may provide the first line of cells to be recruited to sites of inflammation. These 'lead' neutrophils could subsequently promote the migration of 'follower' neutrophils, possibly via a relay mechanism [69], establishing the well-known phenomenon of neutrophil 'hotspots' within the vascular lumen [27]. Irrespective of whether it is exhibited by a subset of cells or a generalised phenomenon, neutrophil luminal crawling appears to be vital for continued migration of the cells through venular walls [15], providing a mechanism through which the cells find their preferred sites for penetrating the endothelium.

Neutrophil rIM is also a potential physiological response. More than 10 years ago Poznansky and colleagues proposed that neutrophil migration away from sites of inflammation may contribute to the downregulation of an inflammatory response [11]. Findings in zebrafish embryos, where neutrophils have been observed to migrate away from the site of injury, support the hypothesis that leukocyte rIM may provide a means to resolve an inflammatory reaction [70]. For example,

Table 2. Leukocyte Reverse Migration Can Be Potentially Physiological or Pathological.

Response	Effect	Refs
Physiological		
Reverse luminal crawling (rLC)	rLC of NK cells and monocytes contributes to immunosurveillance in multiple murine models	[16–18]
	rLC of neutrophils contributes to the finding of TEM sites in stimulated murine cremasteric venules	[15]
Reverse interstitial migration (rIM)	Genetic or pharmacological activation of HIF signalling pathway suppresses neutrophil rIM, promoting antibacterial responses in zebrafish larvae	[45,70,71]
	The anti-inflammatory agent Tanshinone IIA, promotes inflammation resolution by accelerating neutrophil rIM in zebrafish larvae	[72]
Pathological		
Reverse transendothelial cell migration (rTEM)	Neutrophil rTEM caused by murine hind-limb or cremaster muscle ischaemia–reperfusion injury is associated with development of remote organ (lung) inflammation	[22]
	Neutrophil rTEM through mouse cremasteric venules induced by activation of LTB_4–neutrophil elastase axis causes remote organ injury	[27]
rIM and rTEM	rIM and rTEM of neutrophils in murine skin is implicated in dissemination of vaccinia Ankara virus	[74]

neutrophil rIM in zebrafish larvae is suppressed by proinflammatory stimuli. Genetic activation of the HIF pathway by expression of constitutively stabilised (and therefore activated) HIF1α isoforms in zebrafish delays inflammation resolution by suppressing neutrophil apoptosis and neutrophil reverse migration in parallel [45]. Numerically, suppression of reverse migration seems to be the more crucial mechanism, supporting a key physiological role of reverse migration in inflammation resolution. Inhibition of rIM through manipulation of the HIF pathway was also accompanied by an increase in scar deposition at the site of tissue injury [71]. This important result shows that persistent inflammation, caused by suppression of neutrophil rIM, can have consequences in terms of defective wound-healing.

Further evidence to support a physiological role for neutrophil rIM *in vivo* comes from studies on Tanshinone IIA, a compound derived from the roots of a medicinal herb, *Salvia miltiorrhiza*, that is used in Chinese medicine. In zebrafish inflammation resolution screens of compound libraries, Tanshinone IIA was identified by its ability to accelerate inflammation resolution [72]. Subsequent studies showed that Tanshinone IIA has an effect on increasing neutrophil apoptosis in human peripheral blood neutrophils and in zebrafish tailfin injury models. In zebrafish, the pro-resolution effects of Tanshinone IIA are mediated, at least in part, by promoting rIM of neutrophils away from the wound. The ability to positively and negatively modulate tissue reverse migration suggests an important physiological role for this response in determining the fate of the tissue neutrophil in zebrafish models. A parallel phenomenon has been described for human neutrophils within microfluidic devices where neutrophils can be seen to migrate towards and then away from chemotactic gradients, the latter being enhanced in the presence of pro-resolving molecules such as lipoxin A4 [73]. Confirmation of this concept in mammalian *in vivo* systems will be important in determining the potential value of manipulating neutrophil rIM for therapeutic benefit (Box 3).

In contrast to the above descriptions of potential physiological roles for neutrophil rIM, vascular re-entry of reverse-migrating neutrophils could have a detrimental pathological effect on the

Box 3. Therapeutic Potential of Targeting Neutrophil Reverse Motility

It is anticipated that, as our understanding of the physiological and pathological relevance of neutrophil reverse migration increases (Table 2), so will opportunities to target this phenomenon for therapeutic gain. At present the evidence for a physiological role for neutrophil rIM suggests that promoting this response could be of benefit as a means of inducing resolution of inflammation. In support of this possibility, neutrophil reverse migration in zebrafish can be induced by Tashinone IIA (the extract of the root of the plant danshen) [72], already in use in Chinese medicine to treat inflammatory disorders. How much of the therapeutic effect of this drug in humans is accounted for by its effect on neutrophil rIM remains to be determined. This finding potentially opens the door to a new class of pro-resolution therapeutics that drive inflammation resolution by accelerating rIM of unwanted activated neutrophils away from inflammatory sites. The molecular pathways governing this process and the therapeutic targets they contain are currently unknown but suggest exciting areas for future study.

In contrast, because neutrophil rTEM has been strongly implicated in dissemination of systemic inflammation [22,27], most notably following ischaemia–reperfusion injury, blockers of neutrophil rTEM could provide a novel means to suppress distant organ damage after local injury. For example, because reduced expression and/or functionality of EC JAM-C is instrumental at promoting neutrophil rTEM *in vivo*, blocking this could prevent neutrophil rTEM and distant organ damage. In this context, activation of the LTB_4–NE axis was identified as an effective inducer of loss of EC JAM-C, neutrophil rTEM, and distant organ damage [27]. By extension, these results suggest that blocking JAM-C cleavage by inhibition of NE could be a useful preventative strategy in inflammatory conditions associated with local ischaemia–reperfusion injury. Importantly, NE inhibitors are currently in clinical use in Japan in patients with acute respiratory distress syndrome (ARDS) associated with systemic inflammatory response syndrome (SIRS), as well as for reducing surgery-induced pulmonary inflammation [88,89]. Together, blockade of neutrophil rTEM appears to be a novel mechanism of action of NE inhibitors and may account for their efficacy in preventing acute lung injury and secondary ARDS.

host. Although at present there is no conclusive evidence for the ability of neutrophils to reverse migrate back into the vascular lumen from the tissue, there is ample and strong *in vivo* evidence to show that neutrophils that have breached the endothelium can exhibit reverse motility from the sub-EC space back into the circulation [22,27]. Importantly, to date all *in vivo* experimental models where significant levels of neutrophil rTEM have been detected have also been associated with a significant level of remote organ damage. This includes murine models that mimic human pathology such as ischaemia–reperfusion injury, or following pharmacological or genetic interventions (e.g., genetic deletion of EC JAM-C) that enhance neutrophil rTEM [22,27]. Similarly, interventions that suppress neutrophil rTEM, such as pharmacological or genetic inactivation of NE, protect the host from distant organ damage [27]. The precise mechanism through which this occurs is at present unclear, but it is hypothesised that rTEM neutrophils represent a subset of neutrophils that have encountered venular wall components, such as ECs, venular BM, and pericytes, and as such are primed and/or activated for enhanced effector functions. The re-entry of these cells from the sub-EC compartment back into the circulation from a primary site of inflammation could contribute to disseminating a localised inflammatory response to secondary organs (e.g., lungs). Such a response provides a novel paradigm for how a local inflammatory reaction can become systemic, as noted in numerous clinical conditions including trauma and post major surgeries. Of importance, *in vitro* studies have shown that rTEM neutrophils have an altered phenotype that resemble tissue migrated cells, exhibiting increased expression of particular adhesion molecules (e.g., ICAM-1) and effector functions (e.g., increased ROS generation) [12,22]. Furthermore, inflammatory reactions that cause neutrophil rTEM are associated with the presence of ICAM-1-expressing and ROS-generating neutrophils in the pulmonary vasculature, a response that correlated with the extent of lung inflammation [22]. Significantly elevated levels of ICAM-1^{high} neutrophils have also been noted in patients with chronic inflammatory conditions (e.g., atherosclerosis and rheumatoid arthritis) [12], suggesting that neutrophil rTEM may be associated with pathogenesis of both acute and chronic conditions. Greater understanding of neutrophil rTEM could open novel avenues for protection of patients from distant organ damage after severe local injuries (Box 3). In addition to dissemination of inflammation, reverse-migrating neutrophils have also been linked to dissemination of pathogens. Specifically, the findings of a study describing immune responses against vaccinia virus in mice indicated that neutrophils that have homed to the site of infection in skin can transport the pathogen back into blood by rIM followed by rTEM, and from there into bone

marrow [74]. Although direct imaging evidence for this hypothesis remains to be attained, the concept of neutrophils being used as Trojan horses for the dissemination of pathogens is intriguing [75–77].

Concluding Remarks

Advances in intravital imaging have been invaluable in enhancing our understanding of immune cell functions and interactions *in vivo*. This now includes the acquisition of undisputed evidence for the ability of neutrophils to exhibit reverse migration behaviours in numerous inflammatory models and contexts. Studies conducted in the past 10 years have identified the complexity and diversity of these responses, highlighting the need for better understanding of the characteristics, prevalence, and implications of neutrophil reverse migration events (see Outstanding Questions). Of importance, much of our understanding of neutrophil reverse motility in tissues stems from studies performed in zebrafish embryos where tissue migration can be seen clearly, but confirmation in mammalian systems is still required. Despite the limitations of current models, and the fact that the field of leukocyte reverse motility is at a relatively embryonic stage, the existing data suggest a range of beneficial roles for leukocyte reverse migration in regulating the onset of an efficient innate immune reaction and inflammatory response. At the same time, there is growing evidence to suggest a potential damaging role of neutrophil reverse transendothelial cell migration, contributing to pathologies such as lung inflammation after trauma or dissemination of pathogens. The potential physiological and pathological roles of neutrophil reverse migration emphasise the importance of gaining more in-depth insight into these phenomena. This has the potential to identify novel means of modulating inflammation, an issue urgently required for the treatment of inflammatory diseases.

Outstanding Questions

Are the different forms of neutrophil reverse migration mechanistically distinct or part of the same process?

Are the different forms of neutrophil reverse migration functionally distinct and, if so, how?

What factors deter neutrophils from migrating towards foci of infection and injury?

Can tissue infiltrated neutrophils migrate back into the blood circulation?

Is control of leukocyte reverse migration through venules different under homeostatic conditions (e.g., entering of haematopoietic cells from bone marrow or thymus) to conditions of inflammation (chronic vs acute, or sterile vs pathogen-induced)?

Can we turn a pathological reverse transmigration event into a beneficial response?

What EC molecules other than JAM-C (e.g., other junctional molecules, chemokines, and other surface structures) regulate neutrophil reverse motility through the EC barrier?

Is the shape and polarity of the endothelium important, and how does the vascular cytoskeleton control neutrophil motility through ECs?

Do pericytes contribute to and/or regulate neutrophil rTEM?

What are the neutrophil and vascular cell signalling pathways that mediate neutrophil reverse motility phenomena?

What is the functional role of reverse-migrated leukocytes in blood and remote tissues?

Do reverse-migrated cells bring antigen to remote lymphoid organs (other than the tissue-specific raining lymph nodes)?

Can reverse-migrating neutrophils disseminate pathogens?

Is neutrophil reverse motility a target for therapeutic interventions?

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