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Neutrophil Migration under normal and sepsis conditions

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Abstract

Neutrophil migration is critical for pathogen clearance and host survival during severe sepsis. Interaction of neutrophil adhesion receptors with ligands on endothelial cells results in firm adhesion of the circulating neutrophils, followed by neutrophil activation and directed migration to sites of infection through the basement membrane and interstitial extracellular matrix. Proteolytic enzymes and reactive oxygen species are produced and released by neutrophils in response to a variety of inflammatory stimuli. Although these mediators are important for host defense, they also promote tissue damage. Excessive neutrophil migration during the early stages of sepsis may lead to an exaggerated inflammatory response with associated tissue damage and subsequent organ dysfunction. On the other hand, dysregulation of migration and insufficient migratory response that occurs during the latter stages of severe sepsis contributes to neutrophils' inability to contain and control infection and impaired wound healing. This review discusses the major steps and associated molecules involved in the balance of neutrophil trafficking, the precise regulation of which during sepsis spells life or death for the host.

Keywords

Sepsis; neutrophil; cell migration; endothelial cell; cell adhesion; cytokine; chemokine

Overview of Sepsis

Severe sepsis, a systemic inflammatory response to infections, associated with acute organ failure, is an increasing cause of mortality among children and adults in the US intensive care units (ICU) [1]. It has been estimated that there are approximately 750,000 cases and 215,000 deaths due to severe sepsis annually [2]. This is comparable to the number of people who die annually from acute myocardial infarction. Furthermore, the incidence of

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severe sepsis has steadily increased over the past several decades with larger increases projected over the next twenty years [2].

During the early stages of sepsis, pro-inflammatory chemokines and cytokines prime and guide neutrophils out of circulation to the sites of infection, thus allowing efficient bacterial phagocytosis and apoptotic cell clearance to occur. Proteolytic enzymes stored in azurophilic granules within neutrophils are released in response to a variety of stimuli at the infected sites. To combat the infection, neutrophils also produce and release reactive oxygen species, such as hydrogen peroxide, superoxide, and nitric oxide [3]. While these inflammatory mediators are important for host defense, they promote endothelial damage [3]. Thus, in uncontrolled inflammatory conditions, such as severe sepsis, during which many neutrophils become activated at the endothelial surface and in the underlying extravascular space, excessive inflammation leads to severe microvascular damage and dysfunction [4].

Neutrophil migration in health

Granulopoiesis

Neutrophil homeostasis within the body is maintained via a careful balance between neutrophil production and release from the bone marrow and neutrophil clearance from the periphery. Under normal conditions, 1×10^{11} neutrophils are generated in the human bone marrow daily [5, 6], where they undergo terminal differentiation from the myeloid precursors. Bone marrow hematopoietic cells can be subdivided into three groups: the stem cell pool, the mitotic pool, and the post-mitotic pool. The stem cell pool consists of undifferentiated hematopoietic stem cells, the mitotic pool refers to the multipotent progenitor cells that are undergoing differentiation, and the post-mitotic pool is comprised of fully differentiated cells [7]. As new leukocyte production is required to replenish the dead and dying cells, multipotent progenitor cells differentiate into either a lymphoid or myeloid lineage by producing either the common lymphoid progenitor cells (CLPs) or the common myeloid progenitor cells (CMPs) respectively [8]. In the absence of infection or inflammation, myeloid differentiation commitment pathway serves as the default [9]. CMP's, in turn, can give rise to either the megakaryocyte-erythrocyte progenitor cells (MEPs) or the granulocyte-monocyte progenitor cells (GMPs) [10]. Following the GMPs' commitment to granulocyte lineage development, terminal neutrophil differentiation includes the myeloblast, promyelocyte, myelocyte, meta-myelocyte, band and segmented (mature) neutrophil stages.

The granulopoiesis process is closely regulated by cytokines and transcription factors [reviewed in [11, 12]]. Granulocyte-colony stimulating factor (G-CSF) has been shown as the principal cytokine governing granulopoiesis, with its effects extending from progenitor cell commitment [13] and precursor proliferation to reduced transit time thru the granulocytic compartment [14], and mature and immature neutrophil release from the bone marrow [15]. Mice lacking G-CSF or the G-CSF receptor and humans with a dominant negative receptor mutation are severely neutropenic, largely due to impaired granulopoiesis [16-19]. Other cytokines, such as IL-6, GM-CSF, IL-3 and stem cells factor (ligand for c-kit), also stimulate granulopoiesis in vivo, but with a much smaller effect [20-22]. Indeed, IL-6, GM-CSF, and IL-3 single knock-out mice showed normal granulopoiesis [23-25],

while basal granulopoiesis in the double knock-out of G-CSF and GM-CSF was impaired [26]. Importantly, LPS and inflammatory cytokines, such as TNF- α , IL-1 β , IL-6, and IL-17 stimulate G-CSF production during infection [27].

Neutrophil egress from the bone marrow

Under normal conditions, only a small fraction (1-2% in mice) of mature neutrophils (not neutrophil precursors) is released from the bone marrow [28]. During severe infection or systemic inflammation, however, increasingly immature neutrophil populations become released from the bone marrow stores to replenish the circulating granulocyte pool, as more neutrophils are recruited and marginate to the peripheral tissues. Neutrophil migration in the bone marrow requires them to leave the parenchyma and cross into the vascular sinusoids and the draining central sinus of the bone marrow. The discontinuities in the basement membrane layer and the bone marrow endothelial cells (BMECs) of myeloid sinusoids render these sinusoidal capillaries less restrictive to blood cell transmigration. Interestingly, neutrophils have been reported to migrate through, rather than between endothelial cells lining the sinusoids in regions where endothelial luminal and abluminal cell membranes are fused (called diaphragmatic fenestrae) [29]. Chemokine gradients and adhesion molecules expressed on neutrophils and BMECs, such as integrins and selectins, are central players in regulating neutrophil release from the bone marrow.

Two chemokine receptor axes – CXCR4 interacting with its ligand SDF-1 α (CXCL12) and CXCR2 interacting with either KC/GRO- α (CXCL1) or MIP-2/GRO- β (CXCL2) – regulate neutrophil retention or release from the bone marrow. Studies in *Cxcr2*^{-/-} and *Cxcr4* myeloid-specific conditional knock-out mice suggest that these two chemokine receptors provide opposing signals that regulate neutrophil traffic in and out the bone marrow [30-32]. Under normal conditions, the balance of the chemokines favors neutrophil retention within the bone marrow, with only a small fraction released into the circulation. As such, SDF-1 α is constitutively highly expressed by the bone marrow osteoblasts, reticular and endothelial cells. CXCR4 expression on bone marrow neutrophil surface is low, but shows high intracellular levels [33], characteristic of constitutive G-protein coupled receptor (GPCR) desensitization and internalization.

Studies in patients with WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) confirmed the crucial role for CXCR4 signaling in neutrophil release from the bone marrow. The majority of these patients has truncation mutations in *Cxcr4* and is neutropenic despite increased numbers of neutrophils in the bone marrow [34, 35]. The role of SDF-1/CXCR4 interaction in neutrophil egress from the bone marrow is further supported by the observations that treatment with CXCR4 antagonist or blocking antibodies results in an increased neutrophil release from both human and mouse bone marrow [36-38]. Co-administration of G-CSF with a CXCR4 antagonist results in synergistic neutrophil release. G-CSF treatment decreases stromal cell SDF-1 production [28], which correlates with increased neutrophil mobilization. Transgenic mice harboring various G-CSF receptor mutations showed a strong correlation between the reduction in SDF-1 protein levels in the bone marrow and neutrophil egress [39].

In addition to its role in retention of bone marrow neutrophil stores, CXCR4 has been implicated in homing of senescent neutrophils back to the bone marrow for clearance. Aged neutrophils express higher levels of CXCR4 on their surface, and this increased expression corresponds to enhanced migration toward SDF-1 α [33, 40]. Blocking antibodies to CXCR4 hinder neutrophil trek back to the bone marrow, and the number of CXCR4-deficient neutrophils homing to the bone marrow is reduced [31]. However, since neutrophils can be cleared in the spleen and liver in addition to the bone marrow, overall clearance of CXCR4-deficient neutrophils is similar to that of wild-type mice [31].

Integrins

Neutrophils under normal conditions express relatively high levels of β_2 integrins (CD18), such as Mac-1 ($\alpha_M\beta_2$) and LFA-1 ($\alpha_L\beta_2$), which are further upregulated in response to inflammatory stimuli. Treatment of mice with blocking antibodies to β_2 integrins augmented neutrophil release from the bone marrow in response to CXCL2 [41], but not in response to LPS, C5a or TNF- α . While β_2 -integrin deficient mice had elevated neutrophil counts [42], this effect was due to a negative feedback loop, whereas the failure of β_2 -integrin deficient neutrophils to emigrate into tissues induced IL-17 and G-CSF, which which stimulate granulopoiesis and neutrophil release. Consequently, this data suggests that β_2 -integrins have only a limited role in neutrophil egress from the bone marrow, which is in contrast to their central role in vascular neutrophil extravasation into the peripheral tissues.

β_1 integrin levels have been shown to become upregulated in the bone marrow of mice in two different models of sepsis (endotoxemia, and cecal ligation & puncture surgery) [43]. Vascular cell adhesion molecule 1 (VCAM-1), a major ligand for $\alpha_4\beta_1$ integrin (VLA-4), is expressed on bone marrow stromal cells, including bone marrow sinusoidal endothelium [44]. Under normal conditions, conditional deletion of $\alpha_4\beta_1$ integrin on hematopoietic stem cells appeared to have no effect on neutrophil trafficking [45]. On the other hand, α_4 -integrin antagonists and blocking antibodies resulted in reduced neutrophil mobilization in response to CXCL2.

Extravasation

Neutrophil intravascular adhesion is mediated largely by the two β_2 integrins, LFA-1 and Mac-1. Considerable redundancy exists between these integrins. For example, several proteins, such as talin and the kindlins, are proximally involved in inside-out activation by binding directly to the cytoplasmic tail of the β_2 subunit, a common β subunit for LFA-1 and Mac-1. In addition, in vitro LFA-1 and Mac-1 bind the same ligand, ICAM-1 [46-48]. An alternative explanation is that each β_2 integrins plays a distinct and sequential role in the recruitment cascade. Indeed, recent intravital imaging studies have shown that LFA-1 and Mac-1 act through distinct mechanism in leukocyte adhesion and crawling [49, 50]. Neutrophil adhesion to endothelium was shown to be mediated by LFA-1 and crawling by Mac-1 [49]. In monocytes and lymphocytes, however, crawling was shown to be dependent on LFA-1 [50, 51]. These data clearly suggest that different regulatory mechanisms that control LFA-1 and Mac-1 activation in leukocytes may predominate at different times, in different cell types, or in different inflammatory models.

The normal vascular endothelium consists of an endothelial cell (EC) layer, connected by the cell-cell junctions, with the glycocalyx on its luminal side and with the basement membrane (BM) below. The glycocalyx consists of proteoglycans and glycolipids, along with other functional proteins on its surface. The BM is composed of collagen IV, laminins, nidogens and perlecan [52]. Embedded in the BM is a non-continuous layer of cells known as pericytes [53]. Neutrophils must traverse all these components during their emigration from the vasculature.

Neutrophil transendothelial migration (TEM) occurs within minutes on stimulated endothelium. A number of adhesion molecules involved in TEM have been identified (Table 1). However, the mechanisms of TEM are still not fully understood. Studies have shown that different combinations of adhesion molecules mediate TEM, depending on the chemotactic stimulus used [54, 55]. Treatment with inflammatory cytokines, TNF α or IL-1 β , also promoted neutrophil TEM in different ways [56]. IL-1 β -stimulated TEM was mediated by intercellular adhesion molecule-2 (ICAM-2), junctional adhesion molecule-A (JAM-A) and platelet endothelial cell adhesion molecule-1 (PECAM-1) on endothelium. In a TNF α -stimulated model, on the other hand, blocking these molecules with monoclonal antibodies did not affect TEM. Further experiments showed that TNF α stimulates neutrophil TEM at least in part via Mac-1 (on neutrophils) and JAM-C (on ECs).

Two pathways for leukocyte TEM have been reported – paracellular [reviewed in [57]] and transcellular [reviewed in [58]]. Leukocytes transmigrating within the peripheral vasculature predominantly use the paracellular route by preferentially migrating thru the tri-cellular junctions (intersection of the borders of three adjacent ECs). Due to the discontinuity of junctional proteins, such as cadherin, occludin and ZO-1 [59], as well as inflammatory stimuli-induced enhanced expression of ICAM-1 [60] in these regions, the tri-cellular junctions serve as portals for leukocyte TEM. Leukocyte transmigration can be further regulated by targeted translocation of PECAM-1 to endothelial junctions from cell-surface connected vesicular compartments [61]. Additionally, findings by Shaw et al. demonstrated that VE-cadherin can undergo rearrangement, loosening the adhesive interactions between adjacent ECs and promoting TEM [62]. Signaling crosstalk via common cytoplasmic partners between junctional molecules may further promote leukocyte TEM. For example, endothelial cell-selective adhesion molecule (ESAM) activates Rho and leads to destabilization of VE-cadherin interactions and increased endothelial permeability during neutrophil extravasation [63].

Transcellular diapedesis is the route of emigration for only a minority of transmigrating cells (~ 5-20% of transmigrated cells through cytokine-activated HUVECs) [64]. The evidence for transcellular leukocyte migration at various sites, including bone marrow, lymphoid organs and peripheral and central nervous system vasculature, has been previously reviewed [58], and adhesion molecules involved in transcellular TEM are becoming characterized. Similar to paracellular TEM, neutrophil transcellular migration is favored by enhanced endothelial ICAM-1 expression in a manner largely dependent on complimentary LFA-1 enrichment on the closely apposed segments of transmigrating leukocytes. Williams et al. showed that the C-terminal cleavage product of annexin A1, secreted by neutrophils, promoted ICAM-1 clustering around adherent neutrophils in order to anchor them to the

endothelium and promote transmigration via transcellular route. Blocking with a monoclonal antibody against annexin A1 reduced transcellular TEM [65]. Ligation of apical ICAM-1 on endothelial cells triggers cytoplasmic signaling events that lead to its translocation to caveolae- and F-actin-rich regions and to its eventual transport with caveolin-1 to the basal plasma membrane [66]. Such events could promote 'pore' formation through which leukocytes can migrate. Both transcellular and paracellular transmigration involve changes in endothelial membrane shape to produce cup-like protrusions, surrounding the adherent leukocyte [67, 68]. In vivo, transcellular neutrophil migration is associated with areas of endothelial cell thinning [69], thus shortening the transmigration path. Neutrophil integrin-ligand interactions have also been implicated in determining the preference for paracellular vs. transcellular mode of transmigration. Mac-1-deficient mice had delayed TEM in response to MIP-2 and an increased propensity for transcellular transmigration (~ 65% compared to 15% in wild-type neutrophils) [49].

After finishing transendothelial migration and prior to proceeding across the basement membrane, leukocytes must detach from the basolateral side of the endothelial layer. Therefore, leukocyte uropod detachment is the final step in completion of leukocyte extravasation. Using intravital microscopy, we recently showed that extravasating leukocytes (neutrophils, monocytes and T-cells) become extremely elongated and exhibit slow uropod detachment from the endothelium after TEM [70]. Additionally, these transmigrating leukocytes deposited CD18⁺ microparticles on the subendothelium before retracting the stretched uropod. Experiments using knock-out mice and blocking antibodies revealed that this uropod elongation is the result of two opposing forces: 1) the retention force of the LFA-1-mediated leukocyte adhesion to the basolateral membrane of vascular endothelium and 2) the propelling force of integrin $\alpha_3\beta_1$ (VLA-3) -mediated migration thru the vascular basement membrane during inflammation. Using in vitro stimulation, Evans et al. previously suggested that shedding of an active heterodimeric fragment of LFA-1 from human leukocytes may play a role in leukocyte detachment following TEM in cantharidin blister fluid [71]. Interestingly, LFA-1 was shed only from neutrophils and monocytes, but not lymphocytes. Matrix metalloproteinase-9 (MMP-9), which is enriched in activated neutrophils and monocytes, was demonstrated to cleave between the Ala⁷⁰⁵ and Ile⁷⁰⁶ residues of the β_2 subunit and, thus, promote its shedding from the cell surface [72]. In addition to integrin shedding, contractile force generation by non-muscle myosin type IIA (MyH9) was demonstrated to be critical for uropod retraction of migrating T lymphocytes [73]. It is likely that a combination of contractile and protease-dependent forces drives leukocyte-endothelium detachment in vivo following TEM. Much remains to be learned about this late phase of leukocyte transendothelial migration.

While leukocyte migration through the endothelial layer is rapid (<2-5 min), transmigration through the endothelial basement membrane occurs more slowly (>5-15 min) [74]. The thin tightly crosslinked network of the vascular basement membrane consists of laminin isoforms, collagen IV, nidogen, perlecan and some glycoproteins, and it acts in part as both a structural support for the vessel and a filtration barrier. Using immunofluorescent confocal microscopy and various inflammatory stimuli to induce ex vivo inflammation of the exteriorized mouse cremaster muscle, the Nourshargh group demonstrated in a series of studies [53, 75, 76], the existence of the basement membrane regions with reduced

expression of laminin and collagen IV. These regions, termed the 'low expression regions' (LERs) were shown to align with the EC junctions and pericyte gaps. Different leukocytes exhibited different modes of migration thru the LERs. Monocytes changed their morphology to a greater extent in order to squeeze through the LER. Neutrophils, on the other hand, could enlarge the LER by degrading the matrix, as demonstrated by the presence of laminin on the surface of 20% of transmigrated neutrophils. Treatment with neutrophil elastase (NE) inhibitor in this study led to significant reduction of CCL-2-induced neutrophil transmigration and LER remodeling, but no effect on monocyte infiltration. Interestingly, an MMP-2/MMP-9 inhibitor had no effect on CCL-2-induced neutrophil or monocyte transmigration, but suppressed neutrophil migration in TNF α -stimulated tissues, suggesting that the role of specific proteases in neutrophil transmigration in vivo is governed by inflammatory stimulus [77-81]. Neutrophil β_1 - and β_3 -integrins are known receptors for basement membrane proteins. Neutrophil-EC interactions via adhesion molecules such as PECAM-1 [78] or others during EC layer transmigration may play a role in upregulation of the neutrophil β_1 integrins [82-85] required for the basement membrane transmigration and migration within the extracellular matrix.

Neutrophil migration is sepsis

Neutrophil release from bone marrow

In light of the increasing understanding of the tug-of-war model between CXCR2 and CXCR4 in neutrophil egress from the bone marrow [32], Delano et al. tested the respective contributions of these two receptors to neutrophil mobilization in a CLP model of sepsis [30, 86]. The study showed that CXCL12 mRNA decreases, while CXCL1 expression increases in the bone marrow within 12h of CLP induction. This is in contrast to the homeostatic conditions, where CXCL12 is highly expressed by the bone marrow stromal cells, allowing for neutrophil retention [87]. Further experiments using monoclonal blocking antibodies suggested a prominent role for CXCR4, but not CXCR2 signaling during infectious stress. TLR 4, MyD88 or TRIF signaling were not required to stimulate neutrophil egress from the bone marrow after CLP.

Changes in neutrophil rigidity

Pro-inflammatory mediators and bacterial products released during sepsis result in increased leukocyte rigidity [88]. As a consequence of the altered deformability, neutrophils become sequestered in the capillary beds, such as those in the lungs and liver. Reduced leukocyte rolling during sepsis may further contribute to neutrophil sequestration and vascular occlusion, thereby promoting tissue ischemia and multiple organ dysfunction [4, 89]. These data suggest that changes in neutrophil rigidity correlate to sepsis severity. The increased rigidity is associated with F-actin accumulation, and can be induced in vitro by stimulation with TNF α [90]. The inhibition of neutrophil chemotaxis, and increased actin polymerization have been reported to be mediated by the activation of peroxisome proliferator-activated receptor gamma (PPAR γ), a ligand activated nuclear transcription factor, which becomes upregulated during sepsis [91].

Changes in neutrophil-endothelial interactions

Sepsis-induced changes in expression profiles of adhesion molecules on both neutrophils and ECs further promote neutrophil firm adhesion and sequestration in the vasculature. Sharp increases in ICAM-1 occurred on endothelial cells of different organs in LPS-injected and CLP surgery-treated mice, as well as LPS-stimulated human endothelium in vitro [92, 93]. Modest VCAM-1 upregulation (mRNA and protein expression) was observed on the endothelium of LPS-treated mice [92, 93]. Mice with CLP-induced sepsis, on the other hand, showed a slight increase in endothelial VCAM-1 mRNA during the initial phase, followed by a sharp reduction at 12h and later post-surgery [94, 95]. Protein levels of VCAM-1 on endothelium were reduced at 6h after CLP surgery and returned to normal homeostatic levels thereafter [94]. Interestingly, LPS pre-treatment for 48 h in endotoxin tolerance experiments on human endothelial cells inhibited VCAM-1 expression (mRNA and protein), but did not alter ICAM-1 levels compared to those after a single LPS stimulation [96].

Soluble VCAM-1 (sVCAM-1) and soluble ICAM-1 (sICAM-1) appear in the serum of CLP- and LPS- treated mice [94, 97]. Such increases in circulating adhesion molecules have also been found in humans and correlate with multiple organ dysfunction and death in neonatal and adult sepsis [98]. These findings are substantiated by the observations that genetic deletion of ICAM-1 reduces the severity of sepsis and sepsis-induced organ dysfunction [99, 100].

Schmidt et al. [101] recently highlighted the importance of endothelial glycocalyx in the development of sepsis-associated acute lung injury (ALI). Using intravital microscopy in LPS-treated mice, they found rapid glycocalyx degradation, specifically the loss of heparin sulfate, due to TNF α -dependent activation of endothelial heparanase. Heparanase inhibition reduced endotoxemia-associated glycocalyx loss, neutrophil adhesion and inflammation-induced damage to the lung endothelium. Human data further substantiated these findings, whereas lung biopsies from patients with nonpulmonary sepsis showed greatly increased heparanase immunofluorescence around the capillaries in conjunction with diffuse alveolar damage. The authors proposed that glycocalyx degradation exposed the previously hidden endothelial adhesion molecules, allowing for their recognition and binding to by neutrophils.

L-selectin shedding and rapid leukocyte upregulation of surface β_2 integrins (via granule exocytosis) in response to pro-inflammatory mediators further enables firm adhesion of neutrophils to the vascular endothelium. Intravenous injection of LPS was reported to inhibit neutrophil migration to a variety of chemotactic airway stimuli, and this inhibition was selective for stimuli that require β_2 integrins [102]. Interestingly, β_2 integrin upregulation is not observed on intravascular neutrophils, but becomes apparent on interstitial neutrophils when a β_2 integrin-dependent stimulus, such as *E.coli* endotoxin, is used for recruitment [103]. By contrast, β_2 integrin expression is upregulated in both intravascular and emigrated neutrophils in response to β_2 integrin-independent stimuli, such as *S. pneumoniae*, Group B *Streptococcus*, *S. aureus* and C5a. IFN- γ induced by *S. pneumoniae* does not upregulate ICAM-1 on the endothelium and correlates with the β_2 integrin-independent migration. Therefore, cytokines initially induced by a particular stimulus may dictate which pathway is used.

Guo et al. found that during CLP-induced sepsis in mice, both β_1 and β_2 integrins on blood neutrophils became upregulated [104]. Blocking of C5a after CLP reduced the increase in β_2 , but not β_1 integrin levels. In another study, blood neutrophils from septic, but not healthy, volunteers expressed $\alpha_4\beta_1$ integrin, which resulted in increased adhesiveness to immobilized VCAM-1 [105]. Coupled with upregulated VCAM-1 expression during the initial stages of sepsis, these observations suggest that neutrophils may also use β_1 integrin-dependent crawling on vascular endothelial cells during sepsis.

Changes in G-protein coupled receptor expression and signaling

Large quantities of chemokines become released due to sepsis-induced inflammation. Such continuous exposure to high ligand concentrations within a short period of time desensitizes the chemokine receptors, all of which are G-protein coupled receptors (GPCRs), in a GPCR kinase (GRK)-dependent manner. GPCR signaling, in a negative feedback loop, induces GRK2 recruitment to the plasma membrane. GRK2 phosphorylation of a GPCR on its C-terminus results in the receptor downregulation and recycling by the β -arrestin and clathrin mediated mechanism. Sepsis patients were shown to have reduced surface CXCR2 expression [106]. Moreover, neutrophils from sepsis patient displayed decreased chemotaxis in response to fMLP, IL-8 or LTB₄ in vitro compared to cells from healthy controls. This correlated with the high expression of GRK2 and GRK5, and inhibition of tyrosine phosphorylation and actin polymerization in sepsis patient cells as opposed to healthy volunteers [107]. Concurrent in vitro stimulation with LPS + IL1 β + IFN of naïve neutrophils induced similar responses. TLR2 and TLR9 activation have been shown to upregulate GRK2 expression, and thus downregulate CXCR2 and neutrophil chemotactic responses in CLP-induced sepsis in mice [108, 109]. Conversely, IL-33 cytokine blocked TLR-4 mediated CXCR2 internalization by inhibition of GRK2 [108].

In contrast to CXCR2, other chemokine receptors can express during sepsis. For example, CCR2, normally not expressed by neutrophils, is upregulated in CLP-, but not LPS-treated mice [110]. This upregulation is TLR activation-dependent and results in increased neutrophil migration and accumulation in remote organs rather than chemotaxis to infectious locus [111]. Increased expression of CXCR4 on extravasated lung neutrophils and increased CXCL12 levels within the injured lung demonstrate a novel application of the CXCR4/CXCL12 signaling axis in promoting neutrophil accumulation in the late stages of LPS-induced lung injury [112].

Signaling molecules downstream of chemokine receptors can also modulate chemotaxis. Receptor for activated C kinase 1 (RACK1) serves as a negative regulator for leukocyte chemotaxis through competitive binding to G $\beta\gamma$ and blockade of its binding interface for the downstream effectors, phosphatidylinositol 3-kinase γ (PI3K γ) and phospholipase C β (PLC β), involved in directional migration [113].

Many chemokines can bind to more than one signal-transducing chemokine receptor, as well as decoy receptors with different affinities. Chemokine concentrations at any particular time will dictate whether the higher affinity and/or lower affinity receptors will be occupied. Herrmann et al. illustrate this by using different concentrations of fMLP to stimulate opposite cell responses [114]. At 100 nM, fMLP binds to its high affinity receptor and

induces chemotaxis without inducing oxidative burst. At 10 μM , fMLP can bind to its low affinity receptor, producing oxidative burst and blocking chemotaxis.

Non-cytokine mediators

Gaseous mediators, such as nitric oxide (NO) and hydrogen sulfide (H_2S) have become established as important regulators of directed leukocyte migration and the pathogenesis of sepsis. NO is synthesized by three known isoforms of NO synthase: endothelial (eNOS), inducible (iNOS) and neuronal (nNOS). The induction of inducible NOS isoform has been implicated in the elevated nitric oxide production during sepsis. Due to sepsis-associated inflammatory response, iNOS is upregulated in leukocytes [115-117], as well as endothelial and certain parenchymal cells [118, 119]. NO was reported to promote leukocyte effector functions, such as phagocytosis and cytokine secretion. In contrast, it inhibits neutrophil migration in CLP-induced murine sepsis [120] and in NO pre-treated human neutrophils from healthy donors [121]. This defect appears to be, at least in part, dependent on the reduced expression of β_2 integrins [120], and further compounded by the NO-mediated reduction of CXCR2 surface expression on circulating neutrophils [122]. Nitric oxide reaction with reactive oxygen species (ROS) during inflammation produces peroxynitrite, which further decreases neutrophil adhesion and migration by inhibiting actin polymerization via actin S-nitrosylation [7, 123].

H_2S is also produced endogenously by a variety of cells via either cystathionine- β -synthase (CBS) or cystathionine- γ -lyase (CSE). It has been implicated as a vasodilator and a neurotransmitter, and more recently as a potent pro-inflammatory mediator in LPS- and CLP-induced sepsis [9, 124]. Prophylactic treatment of CLP-induced septic mice with H_2S donors improved neutrophil rolling/adhesion and directed migration [125, 126]. Moreover, the down-regulation of CXCR2 and L-selectin and the upregulation of GRK2 and CD11b on neutrophils during sepsis was abolished with prophylactic H_2S . H_2S treatment also prevented reduction of ICAM-1 expression on the endothelium of the mesenteric vessels during sepsis, and its effect on cell migration was not observed with ICAM-1-deficient mice [126]. The use of CSE inhibitor in these studies reduced neutrophil emigration to infection sites and greatly increased morbidity and mortality of mice with the non-severe sepsis. By contrast, during acute intranasal LPS-stimulation of the lungs, H_2S inhalation protected against lung injury by attenuating pro-inflammatory responses [10]. Interestingly, the beneficial effects of H_2S were blocked by glibenclamide (a ATP-dependent K^+ channel blocker), while diazoxide (a K^+ ATP channel opener) increased neutrophil migration in vivo [125, 126]. Further research is needed to clarify the mechanisms behind this connection.

Microparticle-mediated effects on leukocyte chemotactic responses

Microparticles (MPs) are small plasma membrane-derived fragments generated after cellular activation or apoptosis from a variety of cells, including leukocytes and endothelium [reviewed in [127]]. MPs can exert either pro-inflammatory or anti-inflammatory effects, altering endothelial barrier function and directed leukocyte migration, depending on the molecules expressed. For example, some neutrophil-derived MPs can enhance leukocyte chemotaxis via L-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) on their surface [128]. Nitric oxide inhibited the release of the MPs. However, the ability of NOS inhibitor,

L-NAME, to enhance migration was dependent on the number of MPs released, rather than increased surface expression of L-selectin or PSGL-1. Alternatively, another type of neutrophil-derived MPs inhibited neutrophil recruitment to the site of inflammation through the anti-inflammatory effects of cytosolic annexin-1 [11]. A large proportion of annexin-1 resides in neutrophil granules and is rapidly mobilized to the plasma membrane upon cell activation.

Conclusion

In severe inflammatory conditions, such as sepsis, many neutrophils become strongly activated within the blood vessels and the underlying tissue. Their protease activity can cause excessive tissue damage and microvascular dysfunction. Antibodies directed against adhesion molecules important for leukocyte extravasation, such as β_2 integrins (LFA-1 and Mac-1) and ICAM-1 (their endothelial counterpart ligand), have shown benefits in some settings, but have been associated with devastating infections. Thus, antagonists designed to completely block neutrophil migration are not the best therapies against sepsis. As alternatives, either partial antagonists or reagents more selective to specific steps in neutrophil migratory cascade may prove more efficacious. The detailed mechanisms and signal transduction molecules controlling the sequential and complementary roles of LFA-1 and Mac-1, for example, in neutrophil migration are only beginning to emerge and will provide rich ground for future exploration and discovery.

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Table 1

Leukocyte integrins and their ligands

Integrin	Expression	Ligand
LFA-1 ($\alpha_L\beta_2$)	Lymphocytes, dendritic cells, NK cells, neutrophils	ICAM-1 ICAM-2 ICAM-3 ICAM-4 ICAM-5 JAM-A
Mac-1 ($\alpha_M\beta_2$)	Neutrophils, monocytes, macrophages, dendritic cells, lymphocytes, NK cells	ICAM-1, -2 ICAM-4 JAM-C C3bi LPS/LPG/APG Bacterial and fungal polysaccharides Fibronectin, laminin, collagen, fibrinogen DC-SIGN
VLA-4 ($\alpha_4\beta_1$)	Lymphocytes, eosinophils, monocytes, NK cells, neutrophils, non-immune cells	VCAM-1, fibronectin, JAM-B