EXPERT REVIEWS

Targeting neutrophils in sepsis

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Sepsis continues to have a high mortality rate worldwide. The multi-step effects of this syndrome make it difficult to develop a comprehensive understanding of its pathophysiology and to identify a direct treatment. Neutrophils play a major role in controlling infection. Interestingly, the recruitment of these cells to an infection site is markedly reduced in severe sepsis. The systemic activation of Toll-like receptors and high levels of TNF-α and nitric oxide are involved in the reduction of neutrophil recruitment due to down-regulation of CXCR2 in neutrophils. By contrast, CCR2 is expressed in neutrophils after sepsis induction and contributes to their recruitment to organs far from the infection site, which contributes to organ damage. This review provides an overview of the recent advances in the understanding of the role of neutrophils in sepsis, highlighting their potential as a therapeutic target.

KEYWORDS: chemotaxis • innate immunity • neutrophil • organ damage • sepsis

Sepsis is one of the most deadly medical conditions, and it can be defined as the body's uncontrolled inflammatory response due to an infection. Most often, sepsis is caused by bacterial infection, although viruses, parasites and fungi might also be causes [1]. Sepsis frequently progresses to severe sepsis or septic shock, causing organ dysfunction and/or hypotension that is unresponsive to vasoconstrictor agents, resulting in high mortality rates [2]. Furthermore, immunosuppression to secondary infections induced by sepsis also leads to high mortality rates in mouse and human subjects after their survival to a septic episode [3,4]. Numerous studies have investigated the mechanism underlining the sepsis-induced immunosuppression. This topic has been recently reviewed [5] and will not be the focus of this review.

Efforts to improve sepsis outcomes have resulted in the Surviving Sepsis Campaign undertaken in 2002, which established standards for the diagnosis of sepsis and supportive therapies for septic patients [6]. This effort has generated positive results, demonstrated by a reduction in the sepsis mortality from 39% in 2000 to 27% in 2007. However, 27% remains a high rate of mortality when considering that the same study reported an increased incidence of sepsis over the period from 2000 to 2007 [7].

This increased incidence highlights the need for the development of a new and efficient pharmacological therapy to treat sepsis. Recently, drotrecogin alpha, the only approved medicine for sepsis treatment, was withdrawn from the market because a multicenter clinical trial failed to demonstrate its efficacy [8]. Therefore, there is no currently approved drug therapy for sepsis. The complexity generated by the multiple events observed in sepsis makes investment by biotechnology companies both challenging and risky. The current understanding of sepsis pathophysiology must certainly be re-evaluated, and new approaches must be developed to identify new targets for treating sepsis.

Neutrophils are key cells in sepsis, and more attention should be given to their role. This review revisits recent findings regarding the role of neutrophils in the pathophysiology of sepsis, highlighting the positive and negative aspects of these cells and identifying them as possible targets for new therapies.

Neutrophils: the fighters of the infection

The successful resolution of an infection requires both recognition of the invading pathogen and initiation of the host's immune response to sequester and kill the invader, preventing its multiplication. Neutrophil recruitment is one of the most important features of the innate immune response to control

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pathogen multiplication [9]. However, it has been noted that in pathological conditions such as severe sepsis, the neutrophil recruitment process is disrupted. Moreover, disturbances in neutrophil recruitment are associated with the severity of sepsis. For example, mice subjected to nonsevere cecum ligation and puncture (CLP)-induced sepsis showed effective neutrophil recruitment to the infection site. These mice demonstrated an ability to control the bacterial burden and prevent the spread of bacteria in the blood, resulting in the survival of all of challenged mice. By contrast, mice that were subjected to severe CLP-induced sepsis exhibited a marked failure of neutrophil recruitment to the infection site even a few hours after sepsis induction. As expected, the bacterial burden at the infection site was not controlled, and the spread of pathogens to the blood was observed [10]. Moreover, an intense systemic inflammatory response was observed in these mice, which presented a 100% mortality rate within 2 days of sepsis induction [10,11]. The same profile of neutrophil recruitment failure was also observed in rats subjected to CLP [12]. Interestingly, the failure of the neutrophil migration to the infection site occurred despite high levels of chemoattractants in the local environment [10,13]. Based on that observation, it can be postulated that the failure of neutrophil recruitment result not from an inefficient establishment of the inflammatory response but rather from an impaired interaction between the endothelium and neutrophils and/or a defective chemotactic response to the chemoattractants. Indeed, circulating neutrophils isolated from septic mice exhibit a defective chemotaxis response to CXCL2 [13]. Corroborating the findings in mouse, neutrophils isolated from septic patients also show an impaired chemotactic response to N-formyl-L-methio-nyl-L-leucyl-L-phenylalanine (fMLP) and leukotriene B4. Most importantly, neutrophil paralysis toward chemoattractants is more pronounced in nonsurvivors than in surviving septic patients [14].

Triggering the innate immune response

The recognition of an invading pathogen by the host is crucial to triggering the innate immune response. Toll-like receptors (TLR) are a family of receptors responsible for the recognition of a wide number of specific pathogen- and damage-associated molecular patterns [15,16]. The activation of TLRs by their ligands has been associated with the production of chemokines, cytokines and other mediators to establish the inflammatory response aimed at the elimination of the pathogenic agents and restoration of the homeostasis [15]. For example, mice with a functional mutation in the gene encoding the TLR4 receptor that recognizes lipopolysacharide (LPS) are highly susceptible to a low dose of *Salmonella typhimurium*, a Gram-negative bacteria, that does not induce mortality in wild-type mice, suggesting that the absence of TLR4 signaling causes impaired recognition of the pathogen [17,18]. Moreover, it has been shown that incubation of bovine neutrophils with low concentrations of LPS enhances neutrophil migration *in vitro*, supporting the role of TLR4 in cellular recruitment during an inflammatory condition [19]. However, incubation of these cells with high concentrations of LPS impairs neutrophil recruitment *in vitro* [19]. Similarly, it has been shown that rats systemically challenged with LPS have impaired thyoglicollate-elicited neutrophil recruitment into the peritoneal cavity [20]. Collectively, these data indicate that intense activation of the TLR4 pathway is deleterious. Corroborating this hypothesis, a large body of evidence supports the deleterious role of TLR4 in the pathophysiology of sepsis. Indeed, TLR4 mutant mice are more resistant to LPS challenge and to CLP-induced severe sepsis [18,21,22]. Similar to TLR4 mutant mice, TLR2 and TLR9 knockout mice also show increased resistance to CLP-induced severe sepsis [13,23]. Moreover, TLR2 and TLR9 knockout and TLR4 mutant mice show increased neutrophil migration to the infection site when subjected to CLP-induced sepsis. It is important to note that in these cases, the establishment of the inflammatory response occurs in the absence of one of the mentioned TLRs due to the activation of other TLRs by bacterial components released during the polymicrobial infection induced by CLP surgery. Nevertheless, these observations support the hypothesis that the systemic activation of several TLRs is deleterious in sepsis due to their involvement in the failure of neutrophil recruitment to the infection site.

Failure of neutrophil recruitment

TLRs are triggers of neutrophil migration failure

CXCR1 and CXCR2 are the main chemokine receptors expressed on the neutrophil surface and mediate the neutrophils' response to CXC chemokines. CXCR1 selectively interacts with CXCL1 and CXCL6, while CXCR2 promiscuously interacts with CXCL1-3 and CXCL5-8 [24,25]. Neutrophils functionally express similar levels of CXCR1 and CXCR2 in physiological conditions, and similar to other G protein-coupled receptors, their expression is precisely regulated. Indeed, prolonged or repeated exposure to ligands induces desensitization of both receptors, which are promptly internalized under ligand binding *in vitro* [26]. G protein-coupled receptor kinases (GRKs) have been shown to promote desensitization and subsequent internalization of G protein-coupled receptors. GRKs phosphorylate the intracellular domains of the activated receptors, leading to the recruitment of arrestins, which decouple the G protein from the receptor and trigger its internalization [27,28]. It has been shown that CXCR1 internalization is a rapid process, given that its surface expression on neutrophils is restored within minutes, therefore maintaining nearly unaltered expression levels. By contrast, the restoration of CXCR2 on the neutrophil surface is slower and leads to a notable reduction in expression levels over the time [25]. This observation is supported by evidences in human neutrophils isolated from septic patients, which show reduced expression of CXCR2 but unaltered expression of CXCR1 [25,29,30]. Moreover, CXCR2 expression is also reduced in circulating neutrophils of septic mice and this alteration in the profile of chemokine receptor expression in neutrophils results in a reduced chemotactic response toward CXCR2 ligands [13,31]. In addition to CXCR2 ligands, TLR agonists have been shown to

be involved in the induction of the internalization of chemokine receptors. Neutrophils stimulated with LPS, LTA and CpG-ODN also show CXCR2 internalization and a consequent reduction in chemotaxis toward CXCL2 *in vitro*. Furthermore, CXCR2 expression was also found to be reduced in wild-type mice that were subjected CLP but not in mice lacking TLR2, 4 or 9 signaling [13,18,23]. In addition, treatment with CXCR2 antagonists such as repertaxin or SB225 reduced neutrophil recruitment to the infection site and worsened the mortality rate after CLP surgery, reinforcing the importance of CXCR2 in neutrophil recruitment during sepsis [31,32]. Therefore, the internalization of CXCR2 induced by TLR ligands has been associated with increased expression of GRK2 in activated neutrophils [13,29,32]. In addition, it has been shown that the internalization of CXCR2 requires proteic synthesis because cyclohexamide blocks LTA-induced reduction of chemotaxis *in vitro* [13]. Together, the above observations strongly suggest that the inhibition of TLR2, 4 or 9 activation would be a valuable tool in the treatment of polymicrobial sepsis.

Based on the wider evidence of the deleterious role of TLR4 activation in sepsis, a synthetic antagonist of LPS-dependent TLR4 activation, eritoran, was tested in septic patients. Contrary to expectations, treatment with eritoran did not improve survival in a Phase III Trial [33]. However, this observation does not exclude the role that LPS-induced TLR4 activation plays in aggravating sepsis because the design of the trial might not have been optimal [34]. Therefore, the effect of eritoran on sepsis, as well as of other TLRs antagonist, should be evaluated.

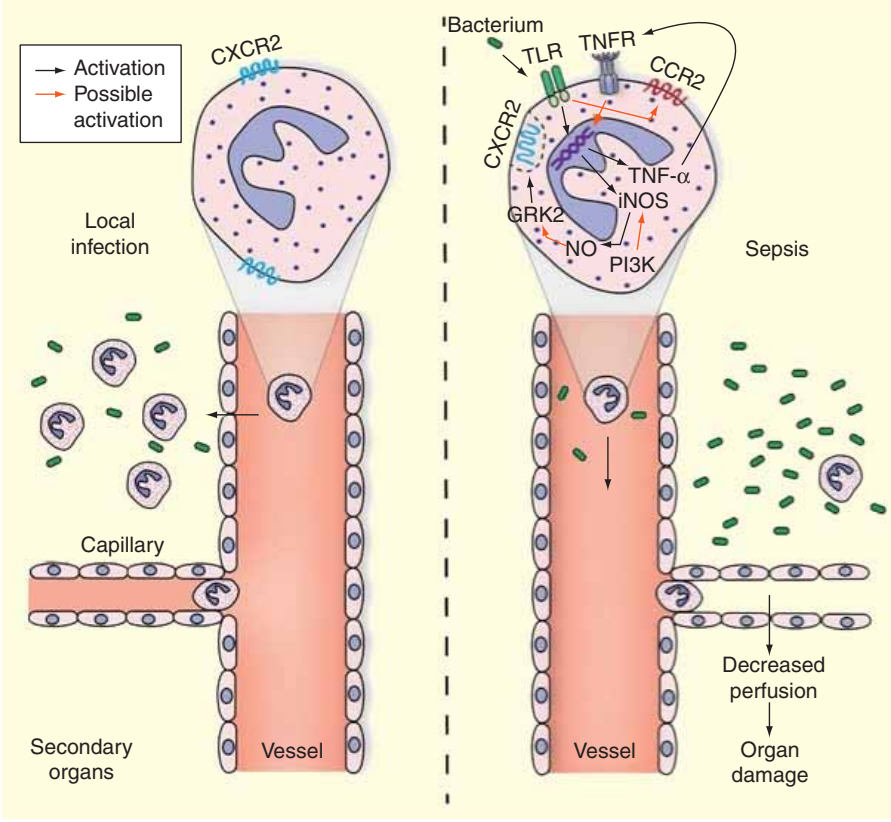

Figure 1. Schematic comparison of the events involving neutrophils during local infection and severe sepsis. While circulating neutrophils expressing CXCR2 migrate to the infection site during a local infection, in a severe septic episode neutrophils show reduced migration due to decreased expression of the chemokine receptor. It is hypothesized that bacteria can activate TLRs on circulating neutrophils leading to the production of TNF-α, which might upregulate the expression of iNOS via TNFR. Phosphoinositide 3-kinase might activate iNOS and via sGC upregulates GRK2 expression, which leads to CXCR2 desensitization. In parallel, TLRs are involved in the expression of CCR2 on the surface of neutrophils, probably contributing to their recruitment to secondary organs as well as organ damage (mechanism not show in the figure). In addition, the organ damage can also be aggravated by the sequestering of activated neutrophils in the capillaries, inducing decreased perfusion and hypoxia in the secondary organs.
GRK2: G protein-coupled receptor kinase; iNOS: Inducible nitric oxide synthase; PI3K: Phosphoinositide 3-kinase; sGC: Soluble guanylate cyclase; TLR: Toll-like receptors; TNFR: TNF receptor.

TNF-α & nitric oxide involvement in CXCR2 desensitization

It is well known that TLR activation induces TNF-α production [35]. Among other effects, TNF-α signaling is involved in the impairment of neutrophil recruitment both *in vivo* and *in vitro*. Neutrophils isolated from mouse and rabbit treated with TNF-α show reduced chemotaxis toward CXCL2 and fMLP, respectively [36,37]. Intriguingly, Khandaker *et al.* were not able to detect TNF-α in the first hour of neutrophil incubation with LPS and discarded the participation of TNF-α in the LPS-induced reduction of neutrophil chemotaxis [38]. On the other hand, LPS- and LTA-induced reduction in chemotaxis toward CXCL2 was prevented in neutrophils isolated from TNF receptor knockout mice. Moreover, TNF receptor 1/R2-deficient mice demonstrate increased chemotaxis toward CXCL2 and higher expression of CXCR2 on the neutrophil surface compared with wild-type mice under polymicrobial sepsis [36].

The downstream pathway of LPS and TNF-α, which leads to enhanced GRK2 expression, still needs to be further investigated. However, it can be suggested that LPS- and TNF-α-dependent GRK2 induction is mediated via nitric oxide (NO) (Figure 1). It is widely acknowledged that LPS and TNF-α can upregulate inducible NO synthase (iNOS) [39]. Moreover, genetic and pharmacological inhibition of iNOS prevented the failure of neutrophil migration in mice that underwent CLP surgery [11,31]. This event was associated with reduced

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GRK2 expression in neutrophils and consequently increased CXCR2 expression on the surface of neutrophils [31,40]. In addition, the prophylactic effect of iNOS inhibitor treatment was abolished in mice pretreated with the CXCR2 blocker repertaxin [31]. Moreover, treatment with the iNOS inhibitor aminoguanidine improved survival rates after sepsis induction, even though the selective iNOS inhibitor 1400W did not increase survival [11,40]. These discrepant responses may result from differences in the dose administered or from differential effects on NO production, which could be beneficial or deleterious depending on the context (see dual effect of NO below). The effect of NO has been shown to be mediated by soluble guanylate cyclase (sGC) [41]. It has also been documented that the expression of GRK2 can be modulated by sGC because the selective inhibitor of sGC inhibits LPS-induced GRK2 expression and prevents the failure of neutrophil recruitment to infection sites after CLP surgery. Similarly, the treatment of septic mice with a selective inhibitor of sGC (ODQ) resulted in an enhanced survival rate [40]. Although sGC is a potential therapeutical target, the available pharmacological tools used to inhibit it fail to specifically bind to sGC, interfering with several heme-dependent processes and inducing severe toxic effects [42]. Interestingly, although mice treated with selective sGC inhibitor showed increased survival rates, their systemic levels of cytokines were comparable to those of untreated mice. Similar results were obtained under pharmacological and genetic inhibition of phosphoinositide-3 kinase (PI3K)γ. While the cytokine levels were not altered, the lack of PI3Kγ was also associated with reduced expression of GRK2 and increased expression of CXCR2 on the neutrophil surface, resulting in higher survival rates [43]. Therefore, this set of results supports the hypothesis that PI3Kγ is potentially upstream of sGC. Although it has been extensively demonstrated that PI3Kγ induces NO via endothelial NO synthase, evidence shows that it could be involved in the dimerization of iNOS, an essential process for the activity of this enzyme [44]. However, further investigations of the signaling pathway that leads to GRK2 expression during polymicrobial sepsis are crucial to the discovery of effective targets. Given that previous clinical trials reported no effect for anti-TNF treatment, a reasonable potential approach would be to identify and validate downstream effectors such as PI3K and sGC and modulate them to reverse the failure of neutrophil migration induced during sepsis.

Pharmacological tools for preventing GRK2 expression

The induction of GRK2 expression was also demonstrated to be modulated by IL-33 treatment, where exogenous IL-33 has been shown to increase the survival of septic mice due to increased neutrophil recruitment to the infection site. Neutrophils and mice treated with IL-33 *in vitro* or *in vivo,* respectively, showed reduced GRK2 expression and increased CXCR2 expression compared with control groups [32]. In addition, this effect induced by IL-33 treatment was also observed in mice subjected to *Candida albicans*-induced peritoneal infection [45]. It can be hypothesized that IL-33 binds to its ST2 receptor and in turn recruits and reduces MyD88 availability for TLR activation pathways. In agreement with this hypothesis, treatment with PPAR activators prevented increases in MyD88 expression during sepsis, and the treatment was associated with increased neutrophil recruitment to the peritoneal cavity and subsequently increased survival [46].

The expression of GRK2 was also reduced and CXCR2 expression was increased in mice treated with hydrogen sulfide (H_2S) donors. H_2S is a biologically active gas that is generated from cysteine in many cell types via two enzymes: cystathionine-β-synthase and cystathionine γ-lyase. The treatment with H_2S donors increased neutrophil migration elicited by LPS and CLP-induced sepsis [47,48]. The effect of H_2S donor treatment was abolished when mice were treated beforehand with an ATP-dependent K1 channel blocker, suggesting that the protective effects are mediated via this channel [47]. The protective effect of H_2S in sepsis has recently been reinforced. However, the authors justified the protective effects of H_2S based on the inhibition of C/EBP homologous protein 10 in macrophages, which showed increased expression in sepsis and was associated with a high inflammatory response [49].

In addition to CXCR2, GRK2 has been shown to modulate the expression of other chemotactic receptors, such as formyl peptide receptor 1. Neutrophils isolated from septic patients showed reduced chemotaxis to fMLP and increased expression of GRK2 in comparison to healthy controls [14,29]. It has been shown that MAPK can play differential roles in the neutrophil chemotaxis, depending on formyl peptide receptor 1. While p38 MAPK blocks GRK2, Erk2 potentiates its activity, inducing and inhibiting the migration of neutrophils, respectively [50]. Thus, Erk2 could also be considered a potential target for neutrophil recruitment modulation during sepsis.

By contrast, a recent report suggests that the desensitization of CXCR2 is mediated by GRK6 instead of GRK2, which would account for the regulation of CXCR1 expression. Although the study showed that the inhibition of GRK6 expression led to prevention of CXCR2 desensitization [51], our data clearly support the role of GRK2 in the modulation of CXCR2 expression in neutrophils during sepsis. Moreover, the pharmacological inhibition of GRK2 prevents the LTA-induced reduction of CXCR2 expression in neutrophils [13]. These differences could be explained by the cell type utilized and by the inductor agent of the neutrophil migration reduction. In our data, neutrophils were stimulated under inflammatory septic conditions, while the other studies used mainly *in vitro* evaluations of induction by chemokine ligands. Further investigation should be undertaken to address the activity of GRKs on CXCR expression during sepsis.

The negative aspect of neutrophils: aggravators of organ damage

Organ failure is one of the most severe symptoms of sepsis and can often lead to death [52]. Systemic activation of

circulating neutrophils impairs their recruitment to the infection site. However, when these neutrophils remain in circulation, they can cause significant organ damage [53]. Confirming the observation in mice, autopsies of septic patients have revealed accumulation of neutrophils primarily in the lungs and kidneys [52]. Neutrophils from septic patients show notable rigidity associated with high levels of TNF-α and fMLP, as well as accumulation of F-actin below the cell membrane instead of in the cytoskeletal rearrangement. Neutrophils are then sequestered in the capillary beds, occluding the lumen, preventing blood flow and ultimately inducing tissue ischemia. Moreover, neutrophils can worsen tissue damage by releasing lytic factors and pro-inflammatory cytokines [52,53]. It has been shown that depletion of neutrophils 12 h after CLP induction in mice reduces the levels of liver and kidney biochemical markers of dysfunction, suggesting reduced organ damage [54].

It has also recently been shown that neutrophils are potentially recruited to secondary organs, contributing to organ damage during sepsis. Under normal conditions, neutrophils do not express CC receptor on the surface. However, the profile of chemokine receptor expression has been shown to be altered during inflammatory conditions such as sepsis [55,56]. It has been demonstrated that under LPS or LTA stimulation, neutrophils migrate up the CCL2 chemokine gradient. CCR2 expression was observed in neutrophils isolated from mice and patients with sepsis. Moreover, CCR2 expression was higher in nonsurvivors than in surviving patients. CCR2 knockout mice showed reduced neutrophil accumulation in the lungs, kidneys and heart, which was associated with reduced damage indicators in the kidneys and heart. Consequently, CCR2 knockout mice showed higher survival rates than wild-type mice. Furthermore, a CCR2 antagonist improved these parameters enhancing survival rates after sepsis induction [57]. In addition, CCR2 knockout resulted in reduced levels of HMGB-1 [58]. HMGB-1 has been extensively demonstrated to be involved in organ damage, and recent evidence implicates HMGB-1 as a chemoattractant for neutrophils [59]. Therefore, CCR2 and HMGB-1 could also be potential targets for preventing organ damage during sepsis.

Metalloproteinases (MMPs) have been suggested to play a role in the pathophysiology of sepsis by modulating neutrophil migration to the infection site and secondary organs. However, the data described so far are controversial. Broad-range inhibition of MMPs reduce chemokine levels and neutrophil sequestration in lung after CLP-induced sepsis [60]. Comparable results were obtained in MMP-9-deficient mice under CLP-induced sepsis [60]. On the other hand, sepsis induced by *Escherichia coli* has been shown to be aggravated in MMP-9-deficient mice. Neutrophil migration to the infection site was reduced most probably due to the absence of degradation of extracellular matrix components by MMP-9. Consequently, MMP-9-deficient mice showed higher bacterial load than the wild-type ones and had increased accumulation of neutrophils in liver and lung [61].

Neutrophils have also been shown to be involved in organ damage during sepsis due to the formation of neutrophil extracellular traps (NETs). It has been extensively demonstrated that stimulated neutrophils are able to release extracellular nucleic acids coated with histone and granular proteins that capture and eliminate extracellular bacteria. The formation of NETs can be associated with cellular death (NETosis) or with maintenance of chemotaxis and phagocytosis of live bacteria (Vital NETosis) [62]. It has been suggested that NETs play a protective role during sepsis. Neutrophil migration to the liver has been observed and associated with the release of NETs, entrapping bacteria and reducing organ damage. Treatment with DNAse, which degrades NETs, resulted in increased neutrophil accumulation in the liver and lungs, exacerbating tissue damage. In addition, treatment with DNAse was associated with higher bacterial load at the infection site in the first hours of infection, but it did not improve the survival after CLP-induced sepsis [63]. By contrast, Gram-negative sepsis, induced by *E. coli*, resulted in hepatic damage that was reduced after DNAse treatment, even though the bacterial load in blood was increased after NET degradation. Importantly, NET has been involved in thrombogenesis and it could contribute to the disseminated intravascular coagulation, aggravating the organ damage [64]. Moreover, the deleterious role of NETs in organ damage during LPS challenge has been described [65,66]. Therefore, the role of NETs in the control of bacteria and organ damage during sepsis should be further investigated.

Just like bacterial sepsis, which is the main focus of this review, systemic inflammatory response triggered in sterile condition such as ischemia-reperfusion, trauma and hemorrhagic shock might also induce organ damage. In fact, sterile and infectious sepses often activate comparable inflammatory pathways and show indistinguishable clinical features [67]. It was observed that neutrophils isolated from patients under these clinical conditions show an activation profile similar to the one found in neutrophils during bacterial sepsis. This includes the sequestration in the vasculature and reduced chemotactic response *in vitro*, among others [68]. In contrast to infectious sepsis, the reduction on the neutrophil recruitment to the inflammatory site due to impaired chemotactic activity of the neutrophils during sterile sepsis is not deleterious because the infectious component is lacking in the latter.

Neutrophils in the comorbidities of sepsis

Several pathological conditions have been associated with the impairment of neutrophil function or reduced number of this cell type in circulation. Patients undergoing chemotherapy treatment and immunosuppressed HIV patients are highly susceptible to infections, and their neutrophils show impaired chemotaxis [69]. Diabetes has similar effects and has been considered a risky factor for the development of sepsis [70]. Indeed, alloxan-induced diabetes is associated with higher mortality rates after polymicrobial sepsis in murine animals. Moreover, non-obese diabetic mice are approximately 25% more susceptible to mild polymicrobial sepsis than prediabetic mice.

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Diabetic mice have significantly worse parameters associated with sepsis, such as bacterial load, inflammatory mediator production and organ damage, which are associated with reduced neutrophil recruitment to the infection site. Neutrophils from diabetic mice also show reduced chemotaxis due to increased GRK2 expression and consequent reduction in CXCR2 expression on the surface [71]. It has also been shown that neutrophils from diabetic patients have impaired microbicidal and phagocidal activity and superoxide production [72]. Interestingly, insulin treatment in healthy subjects induced higher chemotaxis, phagocytic and microbicidal indexes on neutrophils [73]. Additionally, the treatment with insulin prevented high mortality rates in diabetic mice subjected to CLP-induced sepsis [71]. Reduced mortality after sepsis was also observed in diabetic patients treated with insulin [74]. Overall, it is plausible that this effect is a result of the insulin independently acting on neutrophils rather than the control of glycemia as studies have failed to show increased survival in patients with strict control of glucose levels during a sepsis event [75]. Furthermore, treatment with insulin prevented increases in α1 acid glycoprotein (AGP) mRNA during sepsis, which has been shown to impair neutrophil migration (see below) [71].

Moreover, neutrophil migration and bacterial control during sepsis in diabetic mice was demonstrated to be higher in mice with genetic or pharmacological inhibition of mast cell function [76]. The pharmacological depletion of the mast cell population also prevented neutrophil migration failure in nondiabetic mice [77]. Additionally, diabetic mice showed improved outcomes resulting from CLP-induced sepsis after the blockage of the histamine 2 (H2) receptor. GRK2 was reduced and CXCR2 expression was increased in mice treated with an H2 receptor blocker and under mast cell depletion, contributing to the enhanced survival after CLP-induced sepsis [76].

Furthermore, neutrophils have recently been associated with increased susceptibility of elderly patients to sepsis. It has been estimated that 60% of septic patients are older than 65 years. Similarly, older mice (20–24 months) are more susceptible to CLP-induced sepsis than younger mice. Despite the higher number of neutrophils present in the bone marrow of older mice compared with young mice, older mice display a reduced number of phagocytic neutrophils, in addition to reduced chemotaxis toward CXCL1, leading to reduced bacterial control compared with young mice [78]. These data suggest that neutrophils might be potential targets for treating elderly septic patients and therapies that enhance neutrophil function should be investigated.

Dual effect of NO

As previously noted in this review, NO is involved in the internalization of CXCR2. NO also regulates the expression of adhesion molecules, reducing leukocyte rolling and adhesion to the endothelium and further contributing to neutrophil migration failure in sepsis. NO donors reduce the expression of E-selectin, P-selectin, VCAM-1 and ICAM-1 [41,79,80]. Additionally, iNOS knockout mice show enhanced interaction of neutrophils with the endothelium during endotoxemia and CLP-induced sepsis, resulting in increased neutrophil recruitment to the peritoneal cavity of septic mice [11].

NO-derived mediators have also been shown to be harmful to neutrophil migration. In contact to the superoxide anion, NO induces peroxynitrite production. It has been demonstrated that the administration of a peroxynitrite scavenger prevents reductions in the neutrophil migration to the infection site, resulting in improved survival rates following treatment [81].

Based on these results, it is plausible that NOS inhibition and/or NO blockage are a potential approach to treating sepsis. These compounds could also be suggested for the treatment of hypotension in sepsis, in which NO seems to be a key player [82]. Although NOS inhibition and/or NO blockage are interesting and valuable approaches, the use of these compounds in sepsis must be carefully evaluated. While it would be expected that iNOS knockout mice display enhanced neutrophil migration and reduced mortality rates during sepsis, a higher mortality rate was observed in these mice due to a marked reduction in bacterial burden control. NO is crucial to the microbicidal activity of neutrophils, and the total absence of this mediator could be dangerous in infectious conditions [11].

Reinforcing the importance of NO in the microbicidal ability of neutrophils, IL-17 knockout mice show increased susceptibility to CLP-induced sepsis, which was associated to diminished production of NO by neutrophils, leading to reduced microbicidal activity. Moreover, the IL-17-deficient mice showed reduced neutrophil migration to the infection site, which was associated to the chemoattractant properties of IL-17 [83].

Similarly to the dual effect exerted by NO, the enzyme heme oxygenase (HO)-1 also appears to play a dual role in sepsis. HO-1 catalyzes the degradation of heme in biliverdin into free iron and carbon monoxide. Biliverdin is converted to bilirubin, which has anti-inflammatory properties. Carbon monoxide plays distinct roles and shares some of the effects induced by NO [84]. HO metabolites induced impaired neutrophil migration elicited by carrageenan. Furthermore, the inhibition of HO-1 increased neutrophil migration induced by carrageenan [85]. Even the pretreatment of mice with a specific inhibitor of HO-1, zinc deuteroporphyrin 2, 4-bis glycol, prevented the failure of neutrophil migration in both *Klebsiela pneumoniae*- and CLP-induced sepsis. The treatment was also associated with improved outcomes during a septic episode [86,87]. However, this effect can vary under different therapeutic conditions. Post-treatment with the same inhibitor during CLP-induced sepsis resulted in worsening of sepsis due to the high levels of heme in circulation, which were associated with tissue damage and failure of neutrophil migration [86]. Corroborating these data, HO-1 knockout mice also showed increased susceptibility to CLP-induced sepsis, which was also associated with the high levels of circulating heme [88].

Neutrophils & acute phase proteins

Despite the effect of cytokines and bacterial components, it has also been shown that proteins elicited during the acute phase of inflammation also play roles in neutrophil migration failure during sepsis. Administration of fractions isolated from the serum of septic patients in mice showed impairment in neutrophil migration elicited by carrageenan. Mass spectrometry identified these fractions as two acute phase proteins: AGP and hemopexin [89,90]. The administration of purified AGP to rats reduced rolling and adhesion of leukocytes to the endothelium in a NO-mediated mechanism, reducing neutrophil migration to the infection site. As a consequence, survival rates were reduced after AGP administration during sepsis in rats [89].

Similarly, hemopexin also reduced neutrophil migration to the infection site in CLP-induced sepsis. Moreover, hemopexin reduced both neutrophil chemotaxis toward C5a and CXCL2 and the expression of CXCR2 in neutrophils. The result was an increased bacterial burden in mice treated with hemopexin and reduced survival rates after sepsis induction. Additionally, hemopexin knockout mice showed improved outcomes following CLP-induced sepsis [90].

Based on these observations and evidence that plasma transfusions in newborns with sepsis restore neutrophil function, it is plausible that the blockage of acute phase proteins could be a potential therapeutic target in the treatment of sepsis [91].

Expert commentary & five-year view

Sepsis is a challenging condition that continues to have high mortality rates worldwide due to the lack of an effective pharmacological treatment. The high complexity of this syndrome contributes to an incomplete understanding of its pathophysiology. Therapies targeting the systemic overresponse of pro-inflammatory mediators, such as IL-1 and TNF-α, have been rejected due to a lack of protective effects. Moreover, drotrecogin alpha, a drug commonly used for the treatment of sepsis, was removed from the market because it did not improve the survival of treated patients. The latest failure in sepsis therapy implementation was eritoran, a synthetic antagonist of LPS-dependent TLR4 activation. A series of failures in drug therapies for sepsis has raised concerns regarding the translation of results obtained through basic research to the clinical environment. Furthermore, the necessity of reproducing clinical preconditions in sepsis has also recently been discussed. Indeed, our understanding of the physiopathology of sepsis needs to be further developed, and basic research could definitely contribute to that understanding. CLP remains as the gold standard model for studying sepsis, as the kinetics of infection in mice reproduces the one observed in sepsis patients; however, experimental strategies must be carefully chosen. In the scenario of this review, it should be noted that the number of circulating neutrophils is higher in humans than in mice and the chemokine repertoire diverges between the two species. Although the existing differences might induce distinct responses, they do not exclude mouse as a valuable model for investigation of human sepsis. Indeed, as mentioned above, the mechanism of paralysis of the neutrophil migration described in the murine model of sepsis was confirmed in humans. Nevertheless, similar to other diseases, results obtained in mice should be prudently analyzed and extrapolation should be confirmed in human if possible. It is also important to consider the stratification of patients regarding their preclinical conditions. Approaches such as the induction of conditions considered comorbidities should be adopted in future studies to provide new insights regarding the physiopathology of sepsis. In addition, monomicrobial sepsis should also be carefully evaluated. Currently, the recommended treatment for patients with sepsis is administration of antibiotics and support of organ functions during the septic episode. Although the broad spectrum antibiotics can control pathogen growth and mimic the role played by neutrophils, they cannot treat the damage induced by these cells. Indeed, activated neutrophils that do not migrate to the infection site remain in the circulatory system and potentially migrate to organs far from the primary infection, which can contribute to organ damage during sepsis. Therefore, early treatment of septic patients that targets the restoration of neutrophil migration is a potentially effective tool for controlling infection, as it may prevent organ failure and increase patient survival. In this review, several targets are suggested, and the development and evaluation of drugs directed at these targets should be considered by future studies.

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Key issues

- Sepsis still challenges researchers and clinicians worldwide due to the absence of effective direct treatments.
- Neutrophils are key players in the innate immune response, and their recruitment to infection sites is crucial to controlling bacterial/fungal growth.
- The chemotactic response of circulating neutrophils is impaired in septic patients and mice. Moreover, neutrophil migration to the infection site is markedly reduced in septic mice.
- Circulating neutrophils isolated from septic patients and mice exhibit diminished expression of CXCR2 via a process dependent on G protein-coupled receptor kinase 2 upregulation. Systemic activation of toll-like receptors, TNF-α and high levels of nitric oxide are involved.
- Although they are essential to controlling infection, neutrophils can be harmful and induce secondary organ damage during infection. Neutrophil recruitment to organs far from the infection site is facilitated by the expression of CCR2 under septic conditions.
- Impairment of neutrophil function has been reported in diabetic and immunosuppressed patients. Interestingly, these conditions have been implicated as sepsis comorbidities.
- Acute phase proteins released under sepsis conditions contribute to the impairment of neutrophil migration to the infection site.
- Neutrophils may be potential targets for treating sepsis.

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