Neutrophil Migration in Polymicrobial Sepsis: Receptors Accounts for the Impairment of Signaling via Platelet-Activating Factor

Susana E. Moreno, José C. Alves-Filho, Fabrício Rios-Santos, João S. Silva, Sérgio H. Ferreira, Fernando Q. Cunha and Mauro M. Teixeira

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Signaling via Platelet-Activating Factor Receptors Accounts for the Impairment of Neutrophil Migration in Polymicrobial Sepsis

Susana E. Moreno, José C. Alves-Filho, Fabrício Rios-Santos, João S. Silva, Sérgio H. Ferreira, Fernando Q. Cunha, and Mauro M. Teixeira

Sepsis is a systemic inflammatory response that results from the inability of the immune system to limit bacterial spread during an ongoing infection. Recently, we have documented an impaired neutrophil migration toward the infectious focus in severe sepsis. This impairment seems to be mediated by circulating cytokines, chemokines, and NO. Platelet-activating factor (PAF) plays an important role in the orchestration of different inflammatory reactions, including the release of cytokines, chemokines, and free radicals. Using a PAFR antagonist, PCA-4248, and PAFR-deficient mice, we investigated whether signaling via PAFR was relevant for the failure of neutrophils to migrate to the site of infection after lethal sepsis caused by cecum ligation and puncture in mice. In PAFR-deficient mice or mice pretreated with PCA-4248 (5 mg/kg) and subjected to lethal sepsis, neutrophil migration failure was prevented, and bacterial clearance was more efficient. There was also reduced systemic inflammation (low serum cytokine levels), lower nitrate levels in plasma, and higher survival rate. Altogether, the results firmly establish a role for PAFR in mediating the early impairment of neutrophil migration toward the infectious focus. Blockade of PAFR may prevent the establishment of severe sepsis. The Journal of Immunology, 2006, 177: 1264–1271.

Platelet-activating factor (PAF) is a potent phospholipid mediator synthesized by a large number of cells, including platelets, endothelial cells, macrophage, and neutrophils. Its biological activity is mediated through a G protein-linked receptor (PAFR) that is expressed on the surface of a variety of cell types and regulated through a rapid degradation by a subfamily of phospholipases A2, the PAF-acetylhydrolases. In the inflammatory response, PAF has well-characterized actions, mediating the activation of leukocytes to produce cytokines, NO, and other inflammatory mediators and recruitment of these cells to inflammatory site. Because of its widespread production and action, PAF production and action on PAFR have been implicated in the pathophysiology of various inflammatory diseases, including asthma, systemic lupus erythematosus, rheumatoid arthritis, and Crohn’s disease.

Sepsis is an inflammatory systemic response that results from the inability of the immune system to control bacterial spread during an ongoing infection. There are evidences that PAF concentrations are increased and PAF acetylhydrolase activity decreased in plasma of septic patients or experimental endotoxemia, suggesting that PAF might be a mediator of sepsis and septic shock. In fact, it is reported that PAF itself is capable of eliciting many symptoms associated with endotoxic shock. Moreover, the overexpression of the PAFR increases lethality in response to LPS administration in mice, and the administration of PAFR antagonists to animals and humans protect them from the deleterious effects of LPS. Clinical trials using recombinant human PAF acetylhydrolase or PAFR antagonists failed to reduce the mortality of severe septic patients, although a substantial reduction in organ dysfunction was achieved.

Recently, we have shown that severe sepsis induced by cecal ligation and puncture (CLP) or Staphylococcus aureus inoculation is associated with impaired neutrophil recruitment into sites of infection. This impairment of neutrophil migration resulted in augmented number of bacteria in the peritoneal cavity and blood which was associated with high mortality. On the other hand, in sublethal sepsis, the bacterial infection was restricted to the peritoneal cavity, neutrophil migration was not suppressed and no significant mortality was observed. The mechanisms involved in the impairment of neutrophil migration are not completely understood, but it may be due to excessive release of proinflammatory chemokines/ cytokines and a concomitant increase in NO derived from inducible NO synthase.

In the present study, using a PAFR antagonist and PAFR-deficient (PAFR−/−) mice, we investigated the role of PAFR signaling for the failure of neutrophil migration into the infectious focus and in the outcome of polymicrobial sepsis induced by CLP. We found that PAFR signaling results in an impaired neutrophil migration toward the infectious focus followed by bacteremia, increase of systemic cytokines, and high mortality. Thus, PAFR signaling is detrimental in severe polymicrobial sepsis.

Materials and Methods

Animals

BALB/C (8– to 10-wk-old) mice obtained from the facility of the School of Medicine of Ribeirão Preto were housed in cages in temperature-controlled...
rooms and received food and water ad libitum. PAFR−/− mice were gen-
erated as previously described and backcrossed or at least 10 generations
into a BALB/c background (30). All experiments were conducted in accor-
dance with the ethical guidelines of the School of Medicine of Ribeirão
Preto, University of São Paulo.

Sepsis model
Sepsis was induced through CLP as previously described with slight mod-
ification (31). Briefly, mice were anesthetized with tribromoethanol (250 mg
kg−1), a 1-cm midline incision was made on the anterior abdomen,
and the cecum was exposed and ligated below the ileocecal junction with-
out causing bowel obstruction. A single puncture was made through the
cecum using a 21- or 16-gauge needle to induce nonlethal (NL-CLP) and
lethal (L-CLP) sepsis, respectively. In another set of experiment, seven
punctures were made through the cecum using a 21-gauge to induce mod-
erate sepsis (M-CLP). The cecum was returned to the abdomen, and the
peritoneal wall and skin incision were closed. All animals received 1 ml of
saline s.c. immediately after the surgery.

Experimental protocol
The mice were pretreated s.c. with vehicle (saline; 0.2 ml) or with the
PAFR antagonist 2-(phenylthio)ethyl-5-methoxy carbonyl-2,4,6-trimethyl-
1,4-dihydropyridine-3-carboxylate (PCA-4248; 5 mg kg−1). Thirty min-
utes later, mice were subjected to NL-CLP, M-CLP, or L-CLP. In another
set of experiments, the animals subjected to L-CLP were treated after CLP
procedure (5 mg/kg, s.c., 4 and 24 h after L-CLP). After sepsis induction,
we determined neutrophil migration into peritoneal cavity, leukocyte rolling,
and postcapillary venous adhesion in neutrophils. In a different experi-
ment, the animals were subjected to L-CLP, or L-CLP by CLP model. As shown in Fig. 1,
all vehicle- and PCA-4248-pretreated animals subjected to NL-CLP survived
throughout the observation period (21 days). Moreover, both vehicle-
and PCA-4248-pretreated animals subjected to M-CLP pre-
sented survival rates of ~50% (Fig. 1A). On the other hand, 100%
of vehicle-treated mice subjected to L-CLP died within 3 days, and the
pretreatment with PCA-4248 significantly protected the ani-
mals against L-CLP-induced lethality. There was 50% of survival at
day 6 in PCA-4248-treated mice, and protection was sustained
throughout the observation period (Fig. 1B). Since the clinical in-
tervention is not routinely performed as preventive, we also per-
formed a set of experiment using a posttreatment protocol. PCA-
4248 was given in two doses with a 24-h interval after CLP,
beginning 4 h after the lethal CLP procedure. Although the post-
treatment was less effective than the pretreatment, it also conferred
significant protection against lethality (25%) until day 9 after sep-
sis induction. After this point, the survival rates of the posttreated
group was not significantly different to the untreated animals (Fig.
1B). Altogether, these findings suggest that PAFR signaling is det-
imental in the early phase of lethal sepsis, whereas this receptor
appeared to play no major role in nonlethal or moderate sepsis.
Thus, the remaining of this investigation focused on identifying the
detrimental role of PAF on lethal sepsis using the pretreatment
protocol.

Effect of PAFR blockade on rolling, adhesion, and migration of
neutrophil and on chemokine production in mice subjected to
polymicrobial sepsis
We have demonstrated that a marked impairment of neutrophil
migration into the infectious focus is observed in lethal sepsis,
Effects of PAFR blockade on rolling, adhesion, and migration of neutrophil in mice subjected to polymicrobial sepsis.

PAFR antagonist, PCA-4248, improves resistance against polymicrobial sepsis. A, Survival rates of animals pretreated with vehicle (control) or PCA-4248 (PCA; 5 mg · kg⁻¹) and submitted to NL-CLP and M-CLP were determined daily up to 21 days after surgery. B, Survival rates of animals that receive vehicle (control), pretreated (5 mg · kg⁻¹, 30 min before CLP), or posttreated with PCA-4248 (5 mg · kg⁻¹, s.c., 4 and 24 h after CLP) and submitted to L-CLP were determined daily up to 21 days after surgery. The experiment was repeated three times. Results are expressed as percent survival. The survival rate of the PCA-4248-pretreated animals was significantly different from animals that received only vehicle after L-CLP with p < 0.05, Mantel-Cox log-rank test (n = 12–20). which is associated with a high mortality rate (24, 26). In an attempt to investigate whether PAF is involved in this process, the recruitment of neutrophils into the peritoneal cavity was determined in vehicle- and PCA-4248-pretreated mice subjected to L-CLP. In agreement with our previous data (24, 26), the results in Fig. 2A show that animals subjected to NL-CLP presented a marked neutrophil migration into the peritoneal cavity, increasing gradually from 6 to 12 h after surgery. However, mice subjected to L-CLP displayed an impaired neutrophil migration. Indeed, in the latter group, the migration of neutrophils was 5-fold smaller than that observed in mice subjected to NL-CLP (Fig. 2A), despite the fact that the number of holes and the number of bacteria that fall into the peritoneal cavity of L-CLP mice are higher than those of NL-CLP mice. Moreover, using intravital microscopy to visualize leukocyte-endothelial cell interactions, we found that the impairment of neutrophil migration was correlated with a significant reduction in the number of rolling and adherent leukocytes to postcapillary venules of the mesentery when compared with NL-CLP mice (Fig. 2B). When mice subjected to L-CLP were pretreated with PCA-4248, the reduction of rolling and adhesion of leukocytes to postcapillary venules (Fig. 2B) and the impairment of migration of neutrophils into peritoneal cavity (Fig. 2A) were reduced.

Because neutrophil migration is a complex process that involves several chemotactic factors that include CXC chemokines, we also evaluated the production of the CXC chemokine KC after blockade of PAFR. We observed that production of KC at the site of infection was not affected by blockade of PAFR as compared with untreated L-CLP group (NL-CLP, 3.34 ± 0.43; L-CLP, 4.70 ± 0.35; and L-CLP + PCA-4248, 4.14 ± 0.27 ng/ml; n = 5). Thus, the beneficial effect of PAFR inhibition did not correlate with an enhancement of CXC chemokines at the site of infection. Taken together, these results suggest that PAFR signaling underlies the failure of neutrophils to migrate into the infection focus during lethal sepsis.

PAFR blockade improves bacterial clearance in mice subjected to polymicrobial sepsis

The next series of experiments was designed to investigate the effects of PAFR blockade on the bacterial load in the infectious focus (peritoneal exudate) and bacteremia (blood) 6 h after CLP. As shown in Fig. 3, A and B, vehicle-treated mice subjected to NL-CLP did not present detectable bacterial counts in exudate or blood. In contrast, vehicle-treated mice subjected to L-CLP failed to control infection, as demonstrated by the increased number of bacteria in the peritoneal cavity and blood. Pretreatment with PCA-4248 induced a marked reduction of bacterial counts in the peritoneal cavity and blood in mice subjected to L-CLP (Fig. 3),
clearly demonstrating that the PAFR blockade results in the improvement of bacterial clearance. It is important to reinforce that the number of bacteria that transmigrate to peritoneal cavities of L-CLP mice is higher than that of NL-CLP mice. Thus, although the number of neutrophil that migrated to peritoneal cavity of L-CLP mice treated with PAFR antagonist was similar to that observed in NL-CLP mice (Fig. 2A), the ratio of neutrophil:bacteria present in peritoneal cavity is different. It appears that the blockade of PAFR re-established the impaired neutrophil migration only partially, possibly explaining why the control of infection was not totally efficient in PAFR antagonist-treated mice.

The systemic inflammatory response is attenuated after PAF blockade in mice during polymicrobial septic peritonitis

High levels of systemic inflammatory cytokines might contribute to organ injury and shock during sepsis. To elucidate the potential role of PAFR signaling in controlling the cytokine storm that accompanies a lethal septic response, we next investigated serum levels of cytokines 6 h after CLP. The results in Fig. 4 show that serum concentrations of TNF-α, IL-6, and IL-10 were significantly increased in vehicle-treated mice subjected to L-CLP as compared with the NL-CLP group. Pretreatment with PCA-4248 markedly reduced the systemic cytokine levels in mice subjected to lethal sepsis (Fig. 4), indicating that the systemic inflammatory response is attenuated in these animals.

PAFR<sup>−/−</sup> mice are also protected from the lethal effects of polymicrobial sepsis

To confirm the detrimental role of PAFR signaling in lethal sepsis, we also conducted experiments using PAFR<sup>−/−</sup> mice. The results in Fig. 5 show that PAFR-deficient mice presented a significant enhancement of the survival rate after L-CLP as compared with wild-type (WT) mice (Fig. 5A). In animals subjected to NL-CLP, there was no detectable lethality in PAFR<sup>−/−</sup> and WT mice (Fig. 5A). In PAFR<sup>−/−</sup> mice subjected to L-CLP, there was a reduction of the impairment of migration of neutrophils to the infectious focus, and the bacterial counts in blood were significantly reduced as compared with WT mice (Fig. 5, B and C). Although the bacterial counts in Figs. 5C and 3B differ in intensity, probably reflecting interassay variation, it is clear that either pharmacological or genetic inhibition of PAFR promoted similar reduction of infection indices. Peritoneal exudate levels of KC were similar in WT and PAFR<sup>−/−</sup> mice were similar in L-CLP (L-CLP WT, 4.70 ± 0.35; and L-CLP PAF<sup>−/−</sup>, 4.26 ± 0.23 ng/ml; n = 5). These results are consistent with data obtained with the PAF antagonist.

We have previously demonstrated that the systemic production of NO induced by circulating inflammatory cytokines contributes to impairment of neutrophil migration into the infectious focus in lethal sepsis (24, 26, 28). Thus, we assessed serum levels of cytokines and nitrate in WT and PAFR-deficient mice submitted to L-CLP. The results in Fig. 5D and Table I show that serum concentrations of TNF-α, IL-6, IL-10, and nitrate were significantly increased in WT mice subjected to L-CLP as compared with NL-CLP group. In contrast, the circulating levels of TNF, IL-6, and nitrate reduced significantly in PAFR<sup>−/−</sup> mice subjected to L-CLP.

**Discussion**

Studies from our and other laboratories demonstrate that neutrophil migration to the infectious focus is extremely important for the local control of bacterial growth, preventing bacterial dissemination, and, consequently, evolution of sepsis. It was observed
that neutrophil migration to the infectious focus is impaired in severe sepsis induced by CLP or S. aureus infection, and this phenomenon is associated with an increase of bacterial count at the site of infection, bacteremia, and high mortality (24–26). It seems that an early and inappropriate systemic inflammatory response, characterized by elevated levels of plasma cytokines, chemokines, and NO mediates the impairment in the migration of neutrophils (25, 26, 28). There are evidences indicating that PAF, an inflammatory phospholipid mediator, induces several pathophysiological dysfunctions, which are observed in both animal models and in human sepsis (6, 35). In this study, we investigated whether PAFR signaling is involved on the failure of neutrophil migration to the infectious focus observed in lethal sepsis.

Our data clearly demonstrate that the absence of PAFR signaling, as assessed by using a PAFR antagonist and PAFR-deficient mice, provided significant protection from CLP-induced mortality in lethal sepsis. The survival advantage provided by blockade of PAFR signaling was associated with an enhancement of neutrophil migration into the infectious site, i.e., there was a reduction of failure of the migration of neutrophils in PAFR antagonist-treated or PAFR−/− mice (Figs. 2A and 5B). Moreover, the use of intravital microscopy revealed that the blockade of PAFR avoided the reduction of leukocyte-endothelial cell interactions (rolling and adhesion) observed in mice subjected to lethal CLP (Fig. 2B), supporting the concept that the impairment of an adequate neutrophil-endothelial interaction in lethal sepsis involves PAFR signaling. Reinforcing this hypothesis, it was observed that the peritoneal levels of KC (a neutrophil chemotactic chemokine) in septic mice were not affected by blockade of PAFR or in PAFR−/− mice. The survival rate of animals subjected to moderate septic insult (M-CLP) was not affected by treatment with the PAFR antagonist (Fig. 1A). In this context, in a recent study, we demonstrated that animals submitted to M-CLP did not present failure of neutrophil migration to the site of infection (36). Altogether, these findings suggest that the main harmful effect of PAF in lethal sepsis is the mediation of the failure of the migration of neutrophils.

Although the PAFR blockade increased the rolling and adhesion of the neutrophils on endothelial cells, as discussed above, there is also the possibility that a reduction of apoptosis could account for at least part of the increased number of neutrophils accumulated in the infection focus of L-CLP mice treated with PAFR antagonist. Indeed, there is evidence to suggest that apoptosis is increased during sepsis (37). As the available evidence in the literature suggests that PAF delays neutrophil apoptosis (38, 39), it seems

<table>
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<tr>
<th>Experimental Groups</th>
<th>Serum Nitrate (µM)</th>
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<tr>
<td>NL-CLP WT</td>
<td>17.0 ± 2.3</td>
</tr>
<tr>
<td>L-CLP WT</td>
<td>23.9 ± 2.0*</td>
</tr>
<tr>
<td>L-CLP PAFR−/−</td>
<td>12.5 ± 1.8</td>
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* Data are expressed as mean ± SEM in µM, (n = 6). The experiment was repeated twice.

* L-CLP WT vs. L-CLP PAFR−/−, p < 0.05.
unlikely that blockade of PAFR would lead to an increased accumulation of neutrophils after L-CLP.

PAFR antagonists may improve resistance against the systemic inflammatory response and lethal shock induced by administration of a high dose of LPS (16–20). The CLP sepsis model applied in our study differs from the endotoxic shock model because it also has an infectious process. As a consequence, beneficial effects for the outcome of sepsis caused by CLP may not only require attenuation of the systemic inflammatory response but also depend on activation or re-establishment of an efficient local host defense. We observed that the animals pretreated with PAFR antagonist had a marked reduction of bacterial counts in peritoneal cavity and blood, which was confirmed in PAFR−/− mice (Figs. 3 and 5C). Altogether, the results show that the enhanced neutrophil migration observed in PAFR antagonist-treated or PAFR−/− mice was accompanied by an improvement of bacterial clearance and reduction of the systemic spread of bacteria. Improved bacterial clearance and reduction of bacteremia may account for the increased survival rate after PAFR blockade. In this context, we have previously demonstrated that there was a positive association between bacteremia and mortality (25, 26).

The systemic inflammatory response is considered a central pathogenic event in severe sepsis. High levels of systemic inflammatory cytokines are implicated on the development of multiple organ failure and shock (40, 41). Among the cytokines, several reports have demonstrated that IL-6 levels are a major prognostic indicator of sepsis severity (42–44). Moreover, as mentioned previously, elevated levels of plasma cytokines, chemokines, and NO play a crucial role in mediating the impairment of the migration of neutrophils into the site of infection (24, 26, 28). Our results show that, during lethal CLP, there was a dysregulated elevation of systemic TNF-α and IL-6 levels and that PAFR blockade significantly reduced the levels of these cytokines (Figs. 4 and 5D). Furthermore, nitrite production, an index of inducible NO synthase activity, in response to lethal CLP was diminished significantly in PAFR−/− mice as compared with WT mice, suggesting that PAFR signaling may enhance NO production during severe sepsis (Table I). The latter results are consistent with studies demonstrating a close temporal relationship between the appearance of PAF and NO synthesis during sepsis (45–49) and results showing that PAF contributes to the induction of TNF-α after LPS injection in mice (50) and LPS activation of macrophages in vitro (51). Altogether, these data provide evidence that PAFR signaling is an important determinant of the systemic inflammatory response evolution during lethal sepsis.

Several studies have shown that systemic IL-10 is expressed in elevated concentrations during sepsis (24, 52, 53). Although some studies have shown that IL-10 produced concomitantly with TNF-α and IL-1 may counteract the proinflammatory effects of these cytokines (54, 55), anti-inflammatory predominance in sepsis seems to be associated with increased severity of infection and as a consequence increased end-organ damage and increased mortality (56, 57). In fact, van der Poll et al. (52) reported that plasma concentrations of IL-10 remained invariably high only in nonsurviving patients, while it significantly decreased in survivors. Thus, the imbalance between pro- and anti-inflammatory mediators is related to the severity and mortality of sepsis (57, 58). Our results show that the production of IL-10 increased in the serum of control L-CLP mice and reduced by blockade of PAFR (Figs. 4B and 5D). Interestingly, the reduction of IL-10 occurred concomitantly with the decrease of TNF-α and IL-6, reinforcing that the blockade of PAFR protect animals from established severe sepsis.

The evidence described above argues that the severity of sepsis correlates with levels of cytokines, nitrate, and neutrophil migration to the site of infection. However, the mortality was higher in L-CLP mice treated with PAFR antagonist (~40%) than that observed in NL-CLP (0%) (see Fig. 1). In the two groups, there were similar levels of cytokines, nitrate, and neutrophil migration (see Figs. 2 and 4). Interestingly, a similar percentage of the animals of the L-CLP group treated with the PAFR antagonist also had higher CFU in the exudates and blood than those observed in the NL-CLP group (see Fig. 3), which might explain the increased mortality in the former experimental group. A possible explanation to the greater lethality in PAFR antagonist-treated L-CLP mice is that the number of bacteria that transmigrates to peritoneal cavities of L-CLP is higher than that in NL-CLP, a reflection of the larger bore of the needle used to induce damage in the L-CLP group. Thus, although the number of neutrophils that migrated to peritoneal cavity of L-CLP mice treated with the PAFR antagonist was similar to that observed in NL-CLP mice (Fig. 2A), the ratio of neutrophil: bacteria present in peritoneal cavity was different, i.e., a similar number of neutrophils was involved in the control of different number of bacteria. This may be a possible explanation as to why the control of infection in PAFR antagonist-treated mice submitted to L-CLP was not totally efficient, why the bacteria reached the circulation, and survival was reduced. Taken together, the results demonstrate that the blockade of PAFR re-established only partially the neutrophil migration impairment, suggesting that other mediators, such as cytokines/chemokines (59) and leukotrienes (60), are also mediating the failure of neutrophil migration and other harmful events of L-CLP.

Our results demonstrating that the blockade of PAFR signaling reduced the impairment of neutrophil migration toward the infectious focus and reduced lethality may suggest that the inhibition of PAF signaling might be an adequate strategy for the treatment of sepsis. This tenet is further reinforced by the studies showing that serum levels of PAF acetylhydrolase, which inactivates PAF, are decreased in severe sepsis (61, 62) and that PAFR antagonists improve resistance against the lethal effects of experimental endotoxicemia (16–20). However, clinical trials with recombinant human PAF acetylhydrolase or PAFR antagonists have not been shown to reduce the mortality of patients with severe sepsis (21–23). Similarly, we observed that the protective effect of PAFR antagonist reduced when it was used as posttreatment protocol (Fig. 1B). Our studies may provide an interesting possibility to explain this apparent contradiction between good effects of PAFR antagonists experimentally but not in clinical trials. In the trials, the treatment of patients was started when the shock syndrome was advanced, and, hence, when the impairment of neutrophil migration was already established. In this context, we have demonstrated that neutrophils obtained from patients with severe sepsis present reduced chemotactic activity (34).

In summary, the present study identifies, for the first time to our knowledge, a fundamental role for PAFR in mediating the failure of neutrophils to migrate to infection focus during severe sepsis. The results suggest that PAFR antagonists used in the early stage of shock are potential drugs for immunotherapy of sepsis since they might reduce the impairment of neutrophil migration and, as consequence, avoid the spread of bacteria and the systemic inflammatory response syndrome.

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Disclosures

The authors have no financial conflict of interest.
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