Impaired neutrophil chemotaxis in sepsis associates with GRK expression and inhibition of actin assembly and tyrosine phosphorylation

Short title: GRK and neutrophil chemotaxis failure in sepsis

Sandra Mara A. Arraes, Marta S. Freitas, Simone V. da Silva, Heitor A. de Paula Neto, José Carlos Alves-Filho, Maria Auxiliadora Martins, Aníbal Basile-Filho, Beatriz M. Tavares-Murta, Christina Barja-Fidalgo, and Fernando Q. Cunha

From the Departments of Immunology and Biochemistry, Surgery and Anatomy, and Pharmacology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP; Department of Pharmacology, Institute of Biology, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, RJ; and Department of Biological Sciences, Federal University of Triângulo Mineiro, Uberaba, MG, Brazil.

Supported by grants from the FAPESP, CNPq, CAPES and FAPERJ (Brazil).

Total text word counts: 3727

Abstract word count: 199

Scientific heading: Immunobiology

Reprints: Prof Fernando Q. Cunha, Department of Pharmacology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Avenida Bandeirantes, 3900, Ribeirão Preto, São Paulo, Brazil, 14049-900; e-mail: fdqcunha@fmrp.usp.br, phone: 55 16 6023205 fax: 55 16 6332301.

Sandra M. A. A. Arraes, Present address: Department of Clinical Analysis, State University of Maringá, Maringá, Paraná, Brazil.
Abstract

The deregulation of inflammatory response during sepsis seems to reflect the overproduction of mediators, which suppress leukocyte functions. We investigated the intracellular mechanisms underlying the inability of neutrophils from severe septic patients to migrate towards chemoattractants. Fifty-two septic patients and 15 volunteers were prospectively enrolled. Patients presented increased circulating levels of tumor necrosis factor-α, interferon-γ, interleukin (IL)-8 and IL-10. Patients showed reduced neutrophil chemotaxis to formyl-methionyl-leucyl-phenylalanine (FMLP), leukotriene (LT)B₄ or IL-8. No difference in the transcription or expression of the IL-8 receptor, CXCR1, was detected in neutrophils from controls and patients. However, septic neutrophils failed to increase tyrosine phosphorylation and actin polymerization in response to IL-8 or LTB₄. In contrast, septic neutrophils, similar to controls, showed phagocytic activity that induced actin polymerization and augment in phosphotyrosine content. Treatment of control neutrophils with cytokines and lipopolysaccharide (LPS) to mimic endogenous septic environment inhibited actin polymerization and tyrosine phosphorylation in response to IL-8 or LTB₄. High expression of G-protein-coupled receptor kinase (GRK)2 and GRK5 was detected in septic neutrophils and control cells treated with cytokines plus LPS. Data suggest that endogenous mediators produced during sepsis might continually activate circulating neutrophils, leading to GRK activation, which may induce neutrophil desensitization to chemoattractants.
Introduction

Sepsis is a complex clinical syndrome resulting from a damaging host response to infection. In the United States over 700,000 patients per year develop sepsis with mortality rate reported to vary between 30 and 70%, despite the best available supportive care.

PMN play the first line in the host defense against microorganisms, being recruited to the inflammatory sites by chemoattractants such as LTB$_4$ and chemokines. Once emigrated, these leukocytes are able to phagocytose and to generate large amounts of reactive oxygen and nitrogen species, such as hydrogen peroxide and nitric oxide, which are crucial products for the microbicidal activity of these cells. As neutrophils appear to play a crucial role in the control of the infectious process, one can hypothesize that a deficient migratory ability of neutrophil may aggravate infections. Indeed, impairment of neutrophil migration has been reported in leukemia, diabetes and AIDS, diseases associated with high susceptibility to infection. Furthermore, previous studies from our group showed that failure of neutrophil migration is observed in severe sepsis induced by cecal ligation and puncture and *Staphylococcus aureus* administration. In these lethal models, failure of neutrophil migration to the site of infection was accompanied by increased number of bacteria in the peritoneal fluid and blood. Conversely, in sub-lethal infection in which massive neutrophil migration was observed, bacterial infection was restricted to the peritoneal cavity and the animals exhibited low mortality rate. More recently, we have also reported that blood neutrophils obtained from septic patients failed to respond *in vitro* to the chemotactic stimuli FMLP and LTB$_4$. This unresponsiveness was directly associated to a poor prognosis.
Evidence from literature suggests that the high levels of circulating cytokines/chemokines observed in severe sepsis may mediate the impairment of neutrophil migration, in addition to being involved in the deleterious physiopathological findings of the disease, such as coagulation disorders, cardiovascular collapse and organ failure.\textsuperscript{13} The intravenous administration of TNF-\(\alpha\) or IL-8 inhibited neutrophil migration to mouse peritoneal cavity and anti-TNF-\(\alpha\) antibody partially prevented the inhibition of neutrophil migration in endotoxemia model.\textsuperscript{14} However, the molecular mechanisms involved in the reduced ability of neutrophils to migrate during sepsis were not completely clarified.

Independent of their chemical nature, most chemoattractants exerts their action via binding to specific G protein-coupled receptors (GPCR) controlling complex cascades of signaling events. Among these, activation of the tyrosine kinase pathway is a key event to mediate actin filament assembly, a fundamental step for neutrophil responses, including cell locomotion and phagocytosis.\textsuperscript{15} However, desensitization of GPCR family receptor is an important determinant of the intensity and duration of agonist stimulation. GRKs, specific kinases interacting with GPCR-protein, induce receptor phosphorylation and thereby signal GPCR desensitization in the continuing presence of chemoattractants\textsuperscript{16}. Therefore, an increased expression of GRKs could augment chemotactic receptor desensitization and in turn reduces neutrophil migratory response.\textsuperscript{17}

In the present study, we hypothesized that the impairment of neutrophil migration observed in sepsis could be resultant from signal receptor desensitization mediated by continuous and excessive chemotactic receptor activation. To address this question, we investigated the intracellular mechanisms underlying the inability of neutrophils
from severe septic patients to migrate towards IL-8 and LTB$_4$. We have demonstrated that the systemic septic environment can overstimulate circulating neutrophils, therefore inducing GRK2 and GRK5 expression and receptor desensitization. GRKs, probably leading to GPCRs phosphorylation, might impair chemoattractant-induced tyrosine kinase activity and the subsequent rearrangement of actin network, thus compromising the ability of neutrophils from septic patients to migrate.
Methods

Patients

The prospectively enrolled patients attended for sepsis in the Intensive Care Unit of the Department of Surgery, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brazil, from June 2001 to June 2003. All patients presented clinical and/or laboratory variables that fulfilled the criteria for sepsis. Healthy male and female volunteers served as controls. The study was approved by the Human Subjects Institutional Committee of the Faculty of Medicine of Ribeirão Preto, University of São Paulo (HCRP 4989/99) and written informed consent was obtained from patients (or their caretakers) and volunteers. Patients were excluded if they were >75 or <15 years age; mean arterial pressure <50 mmHg; bradicardia (heart rate <50 beats per min) or tachycardia (heart rate >125 beats per min); intervention with high doses of vasopressor agents (dopamine >7,5 µg/ kg/ min, dobutamine >10 µg/ kg/ min), oliguria (urine output <50 mL/ h), irreversible circulatory shock and when informed consent could not be obtained. All patients enrolled were evaluated according to the APACHE II score.

Neutrophil isolation

Blood neutrophils from septic patients or healthy volunteers were isolated by Percoll gradient and suspended in RPMI medium (97% of viable cells, as assessed by trypan blue exclusion). In some experiments, neutrophils isolated from healthy subjects were pre-treated (2 h, 37ºC) with IL-1β (2 ng/ mL) plus IFN-γ (10 ng/ mL) and LPS (10 µg/ mL), and further incubated (1 h, 37ºC) with IL-8 (10⁻⁹ M) or LTB4 (10⁻⁸ M).
Neutrophil migration assay
Chemotaxis was assayed in 48-well microchemotaxis chamber (Neuro Probe Inc.)
using 5-µm PVP-free polycarbonate filter. Neutrophils (10^6 cells/ mL in RPMI-0.01%
BSA) were allowed to migrate towards FMLP (10^-7 M), LTB₄ (10^-8 M) or IL-8 (10^-9 M) or
medium alone (random migration) (37°C, 5% CO₂). After 1 h, filters were removed,
fixed, stained and neutrophils which migrated through the membrane were counted
under a light microscope on at least five randomly selected fields. Each sample was
assayed in triplicate. Results are expressed as number of neutrophils per field.

Phagocytosis assay
The ability of septic or healthy neutrophils to phagocytose (1 h, 37°C, 5% CO₂) human
plasma-opsonized zymosan (10 particles/ cell) was assayed, in the presence or not
of cytochalasin B (CyB, 15 µg/ mL) or genistein (80 µM), and expressed as the
phagocytic index (% of phagocytic cells x number of interiorized particles). The
contents of F-actin and phosphotyrosine of phagocytizing cells were analyzed, by
fluorescence microscopy.

Cytokine assay
Plasma circulating levels of TNF-α, IFN-γ, IL-8 and IL-10 were determined by double-
ligand ELISA assay. The results are expressed as pg of cytokine per mL of serum.

RNA extraction and RT-PCR analysis
Total RNA extracted from neutrophils by Trizol (Gibco-BRL) was reversed transcribed
using Superscript II reverse transcriptase (Gibco-BRL). The primers for CXCR1
amplification were sense (5’) 5’ CAG ATC CAC AGA TGT GGG AT-3’, and antisense,
(3’) 5’ AGC AGC CAA GAC AAA CAA ACT-3’ amplifying 468 bp. For β-Actin: sense, (5’) 5’-GGC GAC GAG GCC CAG A-3’, and antisense, (3’) 5’-CGA TTT CCC GCT CGG C-3’ amplifying 463 bp. The reverse transcription product was amplified, the PCR products were separated in 1.5% agarose gel and identified by ethidium bromide.36,37 Gel Pro-Analyzer 3.1 software was used for densitometric analysis. Results are shown as units of β-Actin mRNA.

**CXCR1 expression**

Quantification of CXCR1 expression surface antigens was performed in flow cytometry using PE-conjugated antibodies anti-CXCR1 (RD-Systems) or anti- γ1 and γ2 (BD-Pharmingen) using a FACSCalibur flow cytometer and the CellQuest software (Becton Dickinson).24

**Fluorescence microscopy for F-actin, phosphotyrosine or GRK**

The contents of tyrosine phosphorylated residues, F-actin, GRK2 and GRK5 were determined in neutrophils stimulated with IL-8, LTB4 or zymosan, by fluorescence microscopy. After treatment, neutrophils were cytopspined and F-actin was staining with TRITC-labelled phalloidin (Sigma).34 Immunocytochemistry for phosphotyrosine or GRKs was developed using biotin conjugated-primary antibodies (anti-pTyr, anti-GRK2 or anti-GRK5, 1:50; Santa Cruz) and streptavidin-conjugated FITC (1:50, Caltag).38 Microscopic analysis of fluorescent images was done using an epifluorescence microscope (Olympus BX40-F4, Tokyo, Japan) equipped with appropriate filters, for FITC or TRITC, and using 100x oil-immersion objectives. Image capturing was performed with a cooled-charged-coupled device camera (Photometrics, Tucson, AR). All images were captured using identical camera settings as: time of exposure,
brightness, contrast and sharpness, and an appropriated white balance set according to the fluorescence filter and acquired and analyzed by Image-Pro Plus 4.0 (Media Cybernetics). The mean fluorescence density was determined from a linear measurement of individual cells’ fluorescence. All cells of at least five randomly chosen fields of each slide, performed in duplicate, were analyzed from at least ten individual experiments. Results are shown as the mean ± SD of the mean fluorescence densities of each field subtracted from the mean density of the area measured as background for each individual slide. Figures show representative gray images taken by Adobe Photoshop software.

**Immunoblot for GRK**

GRK2 and GRK5 expression in septic neutrophils or in cells treated with cytokines plus LPS and further stimulated with IL-8 or LTB₄ were determined by immunoblots with polyclonal anti-GRK2 or anti-GRK5 ab (1:500, Santa Cruz). Immunoreactive proteins were visualized on the PVDF (Hybond-P, Amersham Biosciences) by 3,3’-diaminobenzidine, and band densitometry was quantified by Scion Image Software.

**Statistical analysis**

Data were analyzed by Prism 3 software. Kolmogorov-Smirnov test was used to verify data distribution. Results are expressed as mean ± SD, except for cytokine levels, expressed as medians and 25th and 75th percentiles. Unpaired t-test and Mann-Whitney were used for comparing means or medians between two groups, respectively. ANOVA followed by Bonferroni was used for multiple comparisons for unpaired data. P < 0.05 was taken as statistically significant.
Results

Clinical data

Fifty-two patients with sepsis from different sources were enrolled in this study. The main diagnoses included pneumonia in 17 cases (35.4%), intra-abdominal infection in 6 (12.5%) and trauma in 4 cases (8.3%). The remaining 21 cases (43.7%) consisted of diverse causes and miscellaneous (Table 1). Characteristics of the patients concerning to age, gender, Acute Physiology and Chronic Health Evaluation (APACHE) II score and bacteriological data are summarized in table 1. Controls consisted of 15 healthy volunteers, 7 (46.7%) male and 8 (53.3%) female, with mean age of 30.9 ± 7.4 years (range, 23-47 years).

Cytokine concentrations

Cytokine levels were determined in serum samples of septic patients and controls. The median time between the onset of sepsis and blood collection was 3 days (range, 1-5 days). Table 2 shows that median serum levels of TNF-α, IFN-γ, IL-8 and IL-10 were significantly increased in septic patients compared to controls.

Neutrophil chemotaxis

The ability of neutrophils obtained from 20 septic patients (mean age ± SD, 56.3 ± 17.2, range 26-83) and 15 normal donors to migrate in vitro towards the chemoattractants was assayed. Figure 1A shows that even random migration was found to be reduced in patients. Moreover, migration of neutrophils towards all chemoattractants tested was significantly inhibited (greater than 50%) in septic
patients, when compared to controls. Interestingly, although controls and patients differed in age distribution, when septic patients (n=6, mean age ± SD, 39.2 ± 7.6, range 23-47) were analyzed within the same range of controls, impaired chemotactic response of septic neutrophils was either observed vs control cells (* P<0.05, unpaired t test) respectively, for RPMI (3.5 ± 3.1 vs 7.2 ± 2.9), fMLP (13.8 ± 9.4 vs 30.5 ± 12.1), LTB₄ (9.5 ± 8.8 vs 21.2 ± 5.2) and IL-8 (7.2 ± 6.0 vs 14.3 ± 5.3).

**IL-8 receptor expression**

To evaluate whether inhibition of chemotactic response of septic neutrophils was related to alterations in chemoattractant receptor expression, CXCR1 mRNA and protein levels were determined by RT-PCR and flow cytometry, respectively. As seen in Figure 1 B there was no significant difference in CXCR1 mRNA expression in neutrophils from controls or septic patients. In agreement, no difference between the median fluorescence intensity for CXCR1 was detected between groups (Fig. 1 C), indicating that cell membrane CXCR1 expression was similar in neutrophils from controls and septic patients.

**Actin polymerization and phosphotyrosine content**

During migration, concurrent changes in actin cytoskeleton enable cells to move toward the stimulus. In response to external stimuli, there is a dynamic remodeling of the cytoskeleton, which is involved with changes in cell shape and motility and signal transduction.¹⁸,¹⁹ We investigated the alterations in actin cytoskeleton dynamic and the pattern of tyrosine kinase activation in neutrophils from septic patients and controls after stimulation with IL-8 or LTB₄. Figure 2 shows that under basal conditions (non-stimulation) no significant difference in F-actin content was observed in neutrophils
from both controls (Fig. 2 Aa) and septic patients (Fig. 2 Ad). However, while treatment of control cells with IL-8 (Fig. 2 Ab) or LTB₄ (Fig. 2 Ac) increased actin polymerization leading to an increase in cell fluorescence, no alteration in actin assembly was observed in neutrophils from septic patients treated with IL-8 (Figure 2 Ae) or LTB₄ (Figure 2 Af). A similar pattern of results was observed by immunocytochemistry for phosphotyrosine in both groups. There was no difference in the fluorescence intensity for phosphotyrosine in cells from controls (Figure 2 Ba) or septic individuals (Figure 2 Bd) incubated with medium alone. However, while the addition of IL-8 or LTB₄ to cultures significantly increased the phosphotyrosine content in control cells (Figure 2 Bb and 2 Bc, respectively), neutrophils from septic patients were not able to respond to IL-8 (Figure 2 Be) or to LTB₄ (Figure 2 Bf). Differences in the intensity of fluorescence among all treatments are demonstrated in Figure 2 C and 2 D, that clearly show the inability of septic neutrophils in signaling with an increase in actin polymerization (Figure 2 C) and tyrosine phosphorylation (Figure 2 D) after chemoattractant stimulation.

**Effect of phagocytic activity of septic neutrophils on actin polymerization and phosphotyrosine content**

Phagocytosis is absolutely dependent on actin cytoskeleton integrity and can modulate tyrosine kinase activation in leukocytes. The ability to phagocytose opsonized zymosan and the modulation of this process by tyrosine kinase was evaluated in septic neutrophils. As shown in Figure 3A, neutrophils from septic patients (gray bars) presented greater phagocytic activity (P<0.05) compared to control neutrophils (open bars). Incubation with either CyB (15 µg/mL), a disruptor of microfilament organization, or genistein (80 µM), an inhibitor of tyrosine kinases, reduced
phagocytosis of opsonized targets in controls and septic cells (Fig. 3A). Cytochemistry analysis of cells under phagocytic activity for F-actin (Fig. 3B) or phosphotyrosine (Fig. 3C) showed that neutrophils from both groups, control (Fig. 3Ba, 3Ca) and septic (Fig. 3Bb, 3Cb) were able to respond to opsonized zymosan stimulation, increasing acting polymerization (B) and phosphotyrosine content (C).

**Effect of cytokine-LPS treatment on LTB₄- or IL-8-induced actin assembly and tyrosine phosphorylation**

Bacterial products and cytokine serum levels are elevated in sepsis, and, for this, we have investigated by fluorescence microscopy whether pretreatment of neutrophils from healthy volunteers with cytokines and LPS would interfere with actin assembling (Figure 4 A) and tyrosine phosphorylation (Figure 4 B) in response to chemoattractants. Basal level of F-actin (Figure 4 Aa) and phosphotyrosine (Figure 4 Ba) in blood neutrophils from controls increased significantly after stimulation with IL-8 (Figure 4 Ab and 4 Bb) or with LTB₄ (Figure 4 Ac and 4 Bc). In contrast, pretreatment of neutrophils with cytokines (IL-1β+IFN-γ) plus LPS (Figure 4 Ad and 4 Bd) impaired actin polymerization (Figure 4 A) and tyrosine phosphorylation (Figure 4 B) induced by IL-8 (Figure 4 Ae and 4 Be) or LTB₄ (Figure 4 Af and 4 Bf). The quantification of cell fluorescence, as described in Methods, is shown in Figure 4 C and 4 D. Treatment with cytokines and LPS was not able to induce, per se, significant changes in actin cytoskeleton, or in phosphotyrosine content, when compared to non-treated control cells, but prevented the increase in actin polymerization (Figure 4 C) and tyrosine phosphorylation (Figure 4 D). Accordingly, pre-treatment with LPS and cytokines also inhibited the chemotactic response of neutrophils to IL-8 (Non-treated cells: 29.3 ± 3.5; LPS+Ctk: 13.4 ± 2.3; n= 5; p< 0.05).
GRK2 and GRK5 expression

GRKs have been described as key modulators of GPCR desensitization. The IL-8 receptors, CXCRs, and the LTB₄ receptor, BLT1, can be phosphorylated by GRKs resulting in receptor desensitization.¹⁶,¹⁷ Alterations in GRK2 and GRK5 expression on neutrophils from septic patients were analyzed by immunocytochemistry or immunoblotting (Fig. 5A). Compared to control cells (Fig. 5 Aa and 5 Ac), a significant increase in fluorescence was clearly seen in septic neutrophils, indicating high expression of GRK2 (Figure 5 Ab) and GRK5 (Fig. 5 Ad). Confirming these results, an increase in GRK2 and GRK5 expression was also observed on the immunoblottings of septic neutrophils (Fig. 5B). Moreover, in vitro treatment of healthy neutrophils with LPS+IFN-γ+IL-1β plus IL-8 significantly induced increase in either GRK2 or GRK5 (Fig. 5C) expression. Stimulation with LTB₄ also increased GRK2 and GRK5 expression in neutrophils (Fig. 5C), although with a lower magnitude than IL-8.
Discussion

Arising through the body’s response to infection, sepsis is a life threatening and commonly lethal disorder, accompanied by an inability to regulate the inflammatory responses. The impairment of neutrophil migration towards the inflammatory focus limits the appropriate clearance of microorganisms in the peripheral tissue, and has been ascribed as a harmful feature during severe sepsis.\textsuperscript{10,11} In animal model, the reduction of neutrophil migration to infection sites accompanied by lethality have been directly associated with increased circulating levels of cytokines, chemokines and NO.\textsuperscript{10,11} Furthermore, the unresponsiveness of blood neutrophils from septic patients to chemotactic mediators \textit{in vitro}\textsuperscript{20,21} has been directly correlated to a poor prognosis.\textsuperscript{12}

Confirming the severity of sepsis scored as APACHE II, septic patients enrolled in this study presented a significant increase in circulating pro- and anti-inflammatory cytokines, TNF-\(\alpha\), IFN-\(\gamma\), IL-8 (CXCL8) and IL-10, accompanied by high mortality rate. Supporting previous data,\textsuperscript{12} we are showing that limited chemotactic ability of septic neutrophils occurs for structurally different chemoattractants, including bacterial product such as FMLP, chemokines, as IL-8 (CXCL8) and also lipid mediators, as LTB\(_4\). The failure of septic patient neutrophils to mount a chemotactic response could not be ascribed to a deficiency in the transcription or expression of chemotactic receptors, since CXCR1, an IL-8 (CXCL8) receptor in humans,\textsuperscript{22} was similarly expressed in neutrophils obtained from controls and septic patients. The chemokine receptors CXCR1 and CXCR2 are central for many PMN functions.\textsuperscript{22} Although some reports have shown that \textit{in vitro} treatment with cytokines and/or bacterial products could down-regulate CXCR1 and CXCR2 expression on human neutrophils,\textsuperscript{23,24}
studies with septic patients, confirming our data, showed that expression of CXCR1, the high affinity receptor for IL-8, were not altered in sepsis.\textsuperscript{25,26}

Most neutrophil chemoattractants recognize membrane GPCR on the leukocytes and, as a result of receptor activation by the chemoattractant, a complex sequence of signaling events are triggered, inducing profound alterations in cytoskeleton dynamics and activating signaling pathway, allowing neutrophils to develop motile, adhesive, phagocytic and anti-microbial responses.\textsuperscript{27}

In the classical view of signaling initiated by activation of GPCR by chemoattractants, the G\textsubscript{\beta\gamma} complex activates phospholipase C\textsubscript{\beta} isoforms that, ultimately, results in calcium mobilization and activation of PKC, that mediates the activation of NADPH oxidase complex, regulating the respiratory burst, phagocytosis and bacterial killing in neutrophils.\textsuperscript{28} Additionally, downstream to G-proteins, other intracellular signals are triggered including PI3K and MAPK pathways, Src family of tyrosine kinases, Rho family of small guanosine triphosphate-binding proteins, and phosphatases that impacts on many aspects of neutrophil functioning, particularly chemotaxis and survival.\textsuperscript{29} Activation of these pathways by chemoattractants leads to protein phosphorylation, especially on tyrosine residues of several adapter proteins, which amplifies the signal transduction and priming cells to respond to adhesive interactions via integrins.\textsuperscript{15} Signaling for motility finds its climax with the polymerization of F-actin, which results in lamella formation and overall rearrangement of the cellular cytoskeleton and cell crawling.\textsuperscript{27}

Our data show that rather than a down-regulation of receptor expression on septic neutrophils, a failure of receptor signaling in response to chemoattractants assigned for impairing the neutrophil migration. We have demonstrated that neutrophils from septic patients present a significant reduction in the basal
phosphotyrosine protein content and failed to display increase in tyrosine kinase activity and alterations in actin cytoskeleton dynamics in response to IL-8 (CXCL8) or LTB₄. Moreover, the inability of septic neutrophils to increase actin polymerization and tyrosine kinase activity seems to be restricted to the incapacity of chemoattractants, such as IL-8 or LTB₄, to trigger the signal transduction through their respective receptors. Neutrophils from septic patients showed a high and cyt-B-sensitive phagocytic activity against opsonized particulated stimuli that induced increase and rapid redistribution of F-actin and augment in phosphotyrosine content, indicating that these cells present functional cytoskeleton and signaling pathways, being able to respond to other stimuli than chemoattractants.

Aiming to reproduce, in part, the endogenous septic environment, human neutrophils obtained from healthy individuals were treated with cytokines and LPS, and further stimulated with chemoattractants. Interestingly, this in vitro treatment also inhibited chemotactic response, actin polymerization and the increase of phosphotyrosine content in response to IL-8 or LTB₄ stimulation, suggesting that endogenous mediators produced during sepsis might activate circulating neutrophils, impairing these cells to respond to further chemoattractant stimulation.

In similarity with other GPCRs, chemotactic receptor activation by agonists can lead to receptor desensitization, a phenomenon modulated by GRKs, which phosphorylates GPCR and often results in a rapid attenuation of receptor responsiveness.¹⁵,²⁹ Desensitization of migratory responses is an important determinant to regulate neutrophil influx in pathological conditions. Continuous and excessive chemokine receptor activation can induce an increase in the expression of GRKs, which phosphorylate GPCRs to signal receptor desensitization.¹⁶ Prolonged LTB₄ stimulation was reported to desensitize human neutrophils for chemotaxis and
calcium influx, through the phosphorylation, by GRK6, of the LTB$_4$ receptor.$^{30}$ Macrophage inflammatory protein-2 was shown to induce, in vitro, expression of GRK2 and GRK5 in blood neutrophils.$^{17}$ Studies on CXCR1 and CXCR2 functionality showed that high concentrations of IL-8 (CXCL8) decrease migratory responses.$^{31}$ This effect seems to be related to GRK2-induced phosphorylation of CXCR1, desensitizing this receptor and probably allowing its internalization.$^{32}$

Since CXCR1 expression was maintained on neutrophils during severe sepsis, it was conceivable to assume that desensitization of chemoattractant receptors may account for the reduced chemotactic responses. Supporting this idea we demonstrate that neutrophils from septic patients show a significant increase in GRK2 and GRK5 expression. Furthermore, treatment of control neutrophils with cytokines plus LPS and further stimulation with IL-8 (CXCL8) or LTB$_4$ also up-regulated GRK2 and GRK5, indicating a key role for those mediators in neutrophil desensitization.

In summary, we present evidence that the systemic septic environment can highly activate circulating neutrophils, inducing GRK expression and desensitization of migratory activity. Increase in the GRK expression is associated with impairment of chemoattractant-induced tyrosine kinase activity and subsequent rearrangement of actin network, hence compromising the ability of neutrophils from septic patients to migrate towards the infectious focus. The shift in chemotaxis from activation to desensitization provides a delicate interplay between many cellular factors that tightly regulate the neutrophil migration in sepsis.
Acknowledgements

We thank Fabiola L. Mestriner and Giuliana Bertozi Francisco (USP) and Marcos B. Batista (UERJ) for technical assistance.

The authors have no conflicting financial interests.
References


Table 1. Septic Patients´ Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>52</td>
</tr>
<tr>
<td>Average age (range, years)</td>
<td>52.1 (17 - 89)</td>
</tr>
<tr>
<td>Male/female ratio</td>
<td>51.9/48.1</td>
</tr>
<tr>
<td>APACHE II score</td>
<td>26.66 ± 7.68</td>
</tr>
</tbody>
</table>

Micro-organism isolates

- Gram-negative rods: 5
- Gram-positive rods: 1
- Gram-positive coccus: 5
- Levenings: 3
- Miscellaneous: 12
- Negative culture: 24
- Not determined: 2
Table 2. Concentrations of cytokines detected in sera from controls and septic patients

<table>
<thead>
<tr>
<th></th>
<th>TNF-α</th>
<th>IFN-γ</th>
<th>IL-8</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>0 (0-71.7)</td>
<td>170.8 (0-283.6)</td>
<td>0 (0-18.4)</td>
<td>155.5 (29.1-233.8)</td>
</tr>
<tr>
<td>(n=9) (n=5) (n=9) (n=9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td>*202.4 (147.1-334.6)</td>
<td>*528.1 (176-815)</td>
<td>*155.7 (122.8-257.5)</td>
<td>**343.2 (256-465.9)</td>
</tr>
<tr>
<td>(n=9) (n=26) (n=12) (n=12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are medians (25th and 75th percentiles in parenthesis) of the levels (pg/ mL) of cytokines detected in serum samples obtained 1 to 5 days after the onset of sepsis. *P < 0.05, **P < 0.01 compared to respective controls (Mann-Whitney U test). The number of subjects is presented inside of parenthesis.
FIGURE LEGENDS

Figure 1. Chemotactic activity and CXCR1 receptor expression on neutrophils.
(A) Chemotactic response of septic neutrophils (gray bars, n=20) or control cells (open bars, n=15) towards FMLP (10^{-7} M), LTB_4 (10^{-8} M) or IL-8 (10^{-9} M), or medium alone (C: controls) in a microchemotaxis chamber. Data show means ± SD. *P < 0.01 compared to respective control. (B) IL-8 receptor gene expression: Expression of CXCR1 mRNA in neutrophils from controls (open bars, n=7) and septic patients (gray bars, n=16) evaluated by RT-PCR as described in Methods. Results are shown as units of β-Actin mRNA. (Insert) Two representative experiments showing CXCR1 and β-Actin mRNA expression in neutrophils from controls (C1, C2) and septic patients (P1, P2). (C) Flow cytometric analysis of CXCR1 receptor expression on neutrophils from controls (open bars, n=8) and septic patients (gray bars, n=13). Results are shown as median fluorescence intensity (MFI).
Figure 2. IL-8 and LTB\textsubscript{4} did not increase actin polymerization and protein tyrosine phosphorylation from septic neutrophils. The contents of F-actin (A, C) and phosphotyrosine (B, D) were analyzed by cytofluorescence in neutrophils from volunteers (a, b, c; open bars) or septic patients (d, e, f; closed bars), treated with medium alone (a, d), IL-8 (10\textsuperscript{-9} M; b, c) or LTB\textsubscript{4} (10\textsuperscript{-8} M; e, f). Actin filaments were stained with TRITC-phalloidin (A) and tyrosine phosphorylated proteins were immunolabeled with FITC conjugated anti-phosphotyrosine ab (B). Panels show images representative of at least five independent experiments. Additionally, cells were imaged (1000x) as described, and the fluorescence intensity of F-actin (C) or phosphotyrosine (D) was quantified. Data are means ± SD from five independent experiments. *\textit{P} < 0.05 compared to cells incubated with medium alone (ANOVA followed by Bonferroni), #\textit{P} < 0.05 compared to control neutrophils (Unpaired \textit{t}-test). Magnification: 1000x.
Figure 3. Effect of phagocytosis on actin polymerization and protein tyrosine phosphorylation in septic neutrophils. (A) Phagocytic index of neutrophils from controls (open bars) or septic patients (gray bars), treated (+) or not (-) with CyB (15 µg/mL) or with Gen (80 µM). Data are means ± SD. *P < 0.05 compared to controls, (ANOVA followed by Bonferroni). Fluorescence microscopy (Magnification: 1000x) for F-actin (B) and phoshotyrosine (C) of neutrophils from controls (a; open bars) and septic patients (b; gray bars), after phagocytosis of opsonized zymosan, performed as described in Methods.
Figure 4. Effects of LTB₄ and IL-8 on actin assembling and protein tyrosine phosphorylation of neutrophils treated in vitro with cytokines and LPS. Neutrophils from healthy individuals were incubated with medium alone (a, b and c) or with cytokines+LPS (d, e and f) and further stimulated with IL-8 (10⁻⁹ M; b, e) or LTB₄ (10⁻⁸ M; c, f), as described in Methods. F-Actin, stained with TRITC-phalloidin (A and C left panels), and tyrosine phosphorylated proteins, immunolabeled with FITC-conjugated anti-phosphotyrosine ab (B and D right panels), were analyzed by fluorescence microscopy (Magnification: 1000x) and quantified as described. Data are means ± SD from ten independent experiments. *P < 0.05 compared to control neutrophils; †P < 0.05 compared to cells incubated with medium alone (ANOVA followed by Bonferroni).
Figure 5. Increase of GRK2 and GRK5 expression in septic neutrophils. (A) Neutrophils from controls (a, c) or septic patients (b, d) were immunostained for GRK2 (a, b) and GRK5 (c, d). The panels show a representative experiment out of at least 7 independent experiments. (B) Immunoblotting for GRK2 (upper panel) and GRK5 (lower panel) in neutrophils from septic patients (gray bars) or healthy volunteers (open bars). The blots, obtained from two representative experiments, are shown. GRK2 and GRK5 expression in neutrophils from controls (C1, C2) and septic patients (P1, P2). (C) Immunoblotting for GRK2 (left) and GRK5 (right) in neutrophils from healthy volunteers incubated with cytokines and LPS, plus IL-8 (10^{-9} M) or LTB_{4} (10^{-8} M), as described in Methods. For immunoblotting, the densitometry of each band, expressing the content of GRK2 and GRK5, was analyzed and expressed in arbitrary units (AU). Data show means ± SD of at least six experiments. *P < 0.05 compared to no-treated cells (ANOVA followed by Bonferroni).
Figure 1

A

Neutrophils/field

Controls (n=15)
Patients (n=20)

C fMLP LTB₄ IL-8

*B

CXCR1/β−Actin (IOD/µg)

B

CXCR1

P1 P2 C1 C2

Controls (n=7)
Patients (n=16)

C

CXCR1 expression (MFI)

Controls (n=8)
Patients (n=13)
Figure 3

A

Phagocytic Index

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>150</th>
<th>300</th>
<th>450</th>
<th>600</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gen</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CyB</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

C

For personal use only.
Figure 4

A

B

C

D

Figure 4

A

B

C

D

Figure 4

A

B

C

D

Figure 4

A

B

C

D

Figure 4

A

B

C

D

Figure 4

A

B

C

D

Figure 4

A

B

C

D
Figure 5

A

B

C

IL-1+IFN+LPS IL-1+IFN+LPS

GRK-2 Expression (AU)

GRK-5 Expression (AU)

GRK-2 (80 KDa)

GRK-5 (67 KDa)

IL-1+IFN+LPS

IL-1+IFN+LPS

C1 C2 P1 P2

C1 C2 P1 P2

GRK-2 Expression (AU)

GRK-5 Expression (AU)

C

IL-8

LTB4

C

IL-8

LTB4

# #

# #

# #

# #

# #

For personal use only.
Impaired neutrophil chemotaxis in sepsis associates with GRK expression and inhibition of actin assembly and tyrosine phosphorylation

Sandra Mara A Arraes, Marta S Freitas, Simone V da Silva, Heitor A de Paula Neto, Jose Carlos Alves-Filho, Maria A Martins, Anibal Basile-Filho, Beatriz M Tavares-Murta, Christina Barja-Fidalgo and Fernando Q Cunha