

Failure of neutrophil migration toward infectious focus in severe sepsis: a critical event for the outcome of this syndrome

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Sepsis is a systemic inflammatory response commonly caused by bacterial infection. We demonstrated that the outcome of sepsis induced by cecal ligation and puncture (CLP) correlates with the severity of the neutrophil migration failure towards infectious focus. Failure appears to be due to a decrease in the rolling and adhesion of neutrophil to endothelium cells. It seems that neutrophil migration impairment is mediated by the circulating inflammatory cytokines, such as TNF- α and IL-8, which induce the nitric oxide (NO) production systemically. It is supported by the fact that intravenous administration of these cytokines reduces the neutrophil migration induced by different inflammatory stimuli, and in severe sepsis the circulating concentrations of the cytokines and chemokines are significantly increased. Moreover, the neutrophil migration failure and the reduction in the rolling/adhesion were not observed in iNOS-/- mice and, aminoguanidine prevented this event. We also demonstrated that the failure of neutrophil migration is a Toll-4 receptor (TLR4) dependent mechanism, since it was not observed in TLR4 deficient mice. Furthermore, it was also observed that circulating neutrophils obtained from septic patients present failure of neutrophil chemotaxis toward fMLP, IL-8, and LTB_4 and an increased in sera concentrations of NO_3 and cytokines. In conclusion, we demonstrated that, in sepsis, failure of neutrophil migration is critical for the outcome and that NO is involved in the process.

Key words: sepsis - neutrophil migration - nitric oxide

Sepsis continues to be one of the most important challenges of modern medicine. Despite many efforts of surgical management, modern antibiotic therapy and intensive care, this disease is the major cause of morbidity and mortality in critically ill patients. In the United States more than 500,000 patients per year develop sepsis with mortality reported between 30 and 70%. Moreover, the costs of treatment have been calculated to amount of more than US\$15 billion per year (Angus & Wax 2001, Riedemann et al. 2003).

Sepsis describes a host inability to limit bacterial spread during an ongoing infection, consequently developing a systemic inflammatory response. It is a complex clinical syndrome characterized by severe hypotension and hyporeactivity to vasoconstrictor agents, often associated with multiple organ failure. Significant complications from sepsis include central nervous system dysfunction, adult respiratory distress syndrome, liver failure, acute renal failure and disseminated intravascular coagulation (Bone 1996, Schrier & Wang 2004). For this reason, the host response toward the pathogens must be under strict regulation because the consequences of uncontrolled inflammation can be more fatal than the original inciting pathogens.

During the onset of infection, a complex cascade of events is initiated after the invasion of the host by pathogenic microorganisms (Medzhitov & Janeway 2000). In this context, the neutrophils display a crucial role in orchestrating the host defense. They are the first cells that migrate to an infectious site and are able to kill microorganisms by releasing bactericidal agents like reactive oxygen and nitrogen species. In addition, these cells also release cytokines and chemokines which enhance the recruitment and activation of themselves and other immune cells (Yamashiro et al. 2001). Therefore, neutrophil recruitment to the infection focus is extremely important for the local control of bacterial growth and consequently for the prevention of bacterial dissemination. In fact, the importance of this phenomenon in the evolution of sepsis has been clearly demonstrated in experimental sepsis in our laboratory. As shown in Fig. 1, mice subjected to lethal sepsis induced by cecal ligation and puncture (CLP) model present impaired neutrophil migration to sites of infection. This impaired neutrophil migration was associated with fail of bacterial clearance in the infectious focus, since we observed increased number of bacteria in peritoneal exudate and blood and high mortality. Conversely, in mice subjected to sub-lethal sepsis, the neutrophil migration was not suppressed and the bacterial infection was restricted to the peritoneal cavity, consequently no mortality was observed (Benjamim et al. 2000). In subsequent study, similar impairment of neutrophil migration with consequent fail to clear bacteria from the infectious focus was observed in lethal sepsis induced by *Staphylococcus aureus* inoculation (Crosara-Alberto et al. 2002). Altogether, these data provide evidence that the outcome

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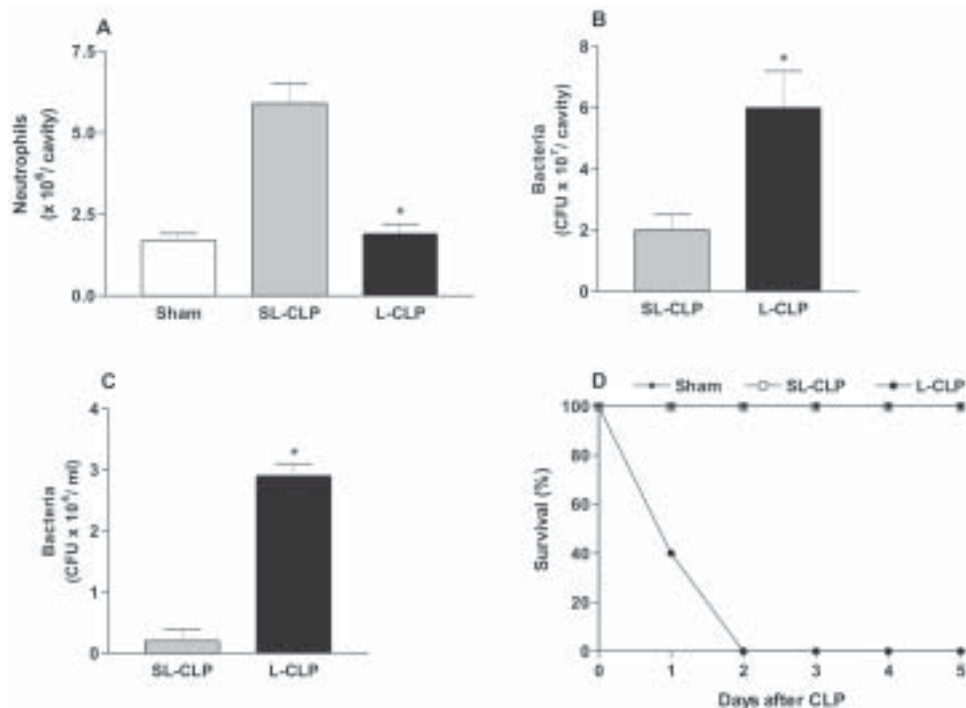


Fig. 1: mice subjected to lethal sepsis by cecal ligation and puncture (CLP) present impairment of neutrophil migration which is associated with fail of bacterial clearance and increased mortality. A: neutrophil migration into the peritoneal cavity; B: bacterial counts in peritoneal exudates; C: bacterial counts in blood. These parameters were evaluated 6 h after sub-lethal CLP (SL-CLP) and lethal CLP (L-CLP) surgery. Results are expressed as mean \pm SEM; * $P < 0.05$ compared with SL-CLP group (analysis of variance, followed by Bonferroni's test); D: survival curves of mice after sepsis induced by CLP, determined daily up to 5 after surgery. The L-CLP mice were significantly different from sham-operated and SL-CLP mice; $P < 0.05$, Mantel-Cox log rank test. Results are expressed as percent survival (reproduced from Benjamim et al. 2000).

of severely septic animals is correlated with failure of neutrophil migration to the infection site.

The mechanism involved in the impairment of neutrophil migration is still elusive, but it may be due to excessive release of proinflammatory chemokines/cytokines and a concomitant increase in nitric oxide (NO) derived from inducible NO synthase (iNOS). This notion is favored by the following observations that the concentrations of circulating cytokines and chemokines are significantly increased in mice subjected to lethal sepsis when compared with animals subjected to sub-lethal sepsis (Benjamim et al. 2000, 2002, Crosara-Alberto et al. 2002) and intravenous administration of TNF- α and IL-8 inhibited neutrophil migration induced by different inflammatory stimuli (Otsuka et al. 1990, Hechtman et al. 1991, Tavares-Murta et al. 1998). Moreover, the neutrophil migration impairment and reduction of rolling/adhesion found in lethal sepsis induced by CLP were not observed in iNOS-deficient mice or in animals treated with aminoguanidine, a selective iNOS inhibitor (Fig. 2). Thus, overproduction of cytokines, chemokines and NO might be critical events that result in impaired neutrophil migration to sites of infection observed during lethal sepsis induced by microbial infections.

It seems that during an infection, the reaction of immune cells toward pathogens is initiated by toll-like receptors (TLR) which act as sensors of the pathogen-associated molecular patterns (PAMPs), recognizing them

as danger signals and triggering the host defense (Medzhitov & Janeway 2000). To date, the TLR family consists of 10 members (TLR1-TLR10). The TLR4 recognizes endotoxin (LPS) of gram-negative bacteria, whereas the TLR2 recognizes different toxins from gram-positive bacteria such as lipoteichoic acid (LTA) and macrophage-activating lipopeptide-2 (MALP-2) (Barton & Medzhitov 2002, Takeda et al. 2003). The role of TLR in response to infection is complex, they are essential components of the innate immune response to infection, but a growing body of evidence indicates that these receptors also may play a role in the pathophysiology of sepsis (Williams et al. 2003, Ishii & Akira 2004, Meng et al. 2004). In this context, recently we investigated the potential role of TLR4 on development of neutrophil migration impairment in polymicrobial sepsis induced by CLP. We observed that TLR4-deficient mice (C3H/HeJ) subjected to lethal sepsis did not present failure of neutrophil migration and this deficiency renders mice more resistant to the lethal effects of sepsis (Fig. 3). This observation suggests that TLR4 signaling is involved in the impairment of neutrophil migration to the infectious focus during lethal sepsis induced by CLP, leading to high mortality.

In humans impairment of neutrophil migration has also been described in cancer (Lejeune et al. 1996), diabetes (Pereira et al. 1987) and AIDS (Mastroianni et al. 1999), all of which are diseases associated with a high susceptibility to infection. In patients infected with human immuno-

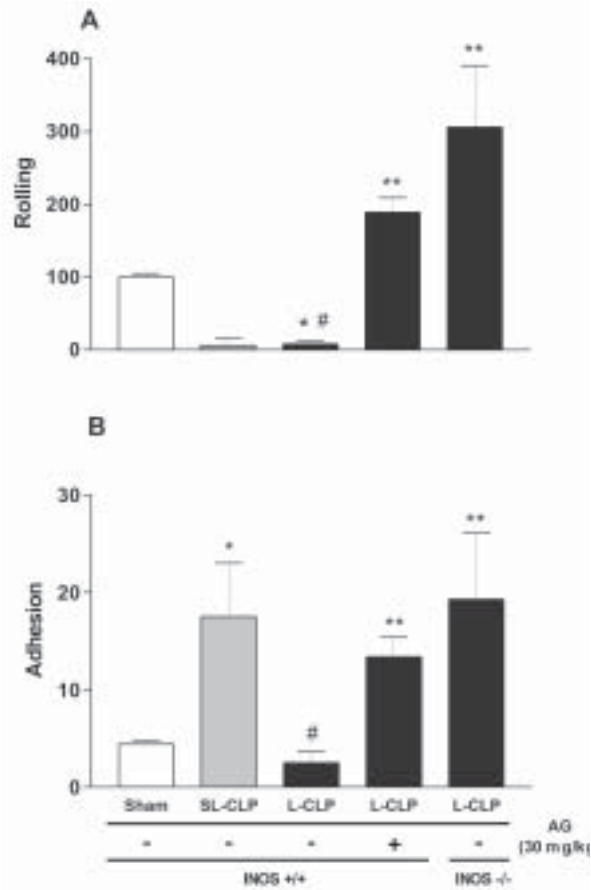


Fig. 2: leukocyte rolling and adhesion to mesentery in wild-type and *iNOS*^{-/-} mice subjected to sub-lethal (SL) and lethal (L)-cecal ligation and puncture (CLP). Bars show the number of rolling and adherent leukocytes in postcapillary venules of mesentery, using an *in vivo* intravital microscopy assay. One group of L-CLP mice received 30 mg of AG kg⁻¹ subcutaneously 30 min before surgery. Sham-operated animals served as controls. The parameters were evaluated 3 h after the surgery. The results are expressed as mean numbers of leukocytes ± SEM, and each group had 15 mice; *: *P* < 0.05 compared with sham-operated animals; #: *P* < 0.05 compared with wild SL-CLP group; **: *P* < 0.05 compared with wild L-CLP group (analysis of variance, followed by Bonferroni's test) (reproduced from Benjamin et al. 2000).

deficiency virus-1, the functional improvement of neutrophil chemotaxis has been found to reduce the incidence and ameliorate the severity of opportunistic infections (Mastroianni et al. 1999). Considering that marked impairment of neutrophil migration is observed in experimental models of sepsis, which is crucial to disease outcome, we also investigated the *in vitro* chemotactic function of neutrophils from septic patients. We found that the chemotactic responses to FMLP or LTB₄ stimuli were suppressed in neutrophils from septic patients compared with healthy controls (Fig. 4). Furthermore, we found that the impairment of neutrophil chemotaxis occurred mainly in neutrophils obtained from nonsurvivor patients (Tavares-Murta et al. 2002). These results, at least to our knowledge, were the first to demonstrate that the outcome of sepsis could be related to the grade of neutrophil chemotactic capacity, pointing to a potential mechanism for the observed reduced host response in septic patients.

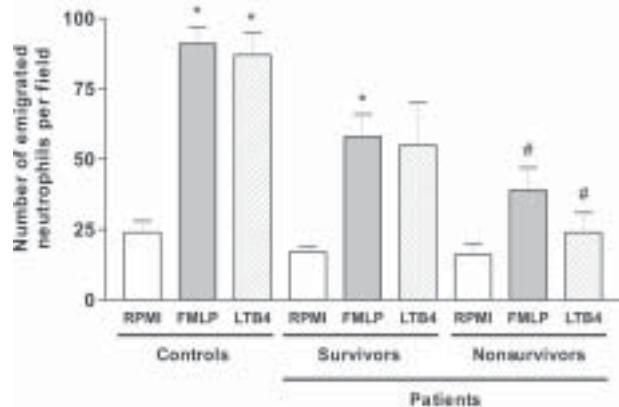


Fig. 4: septic patients present suppressed neutrophil chemotactic responses *in vitro*. The bars indicate the mean ± SEM of number of emigrated neutrophil obtained from controls or septic patients (survivor and nonsurvivor), which migrated in response to RPMI (random migration) or the chemotactic stimuli *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) or leukotriene B₄ (LTB₄); *: *P* < 0.01 compared with respective control RPMI; #: *P* < 0.05 compared with respective control FMLP or LTB₄ (analysis of variance, followed by Bonferroni's test) (reproduced from Tavares-Murta et al. 2002).

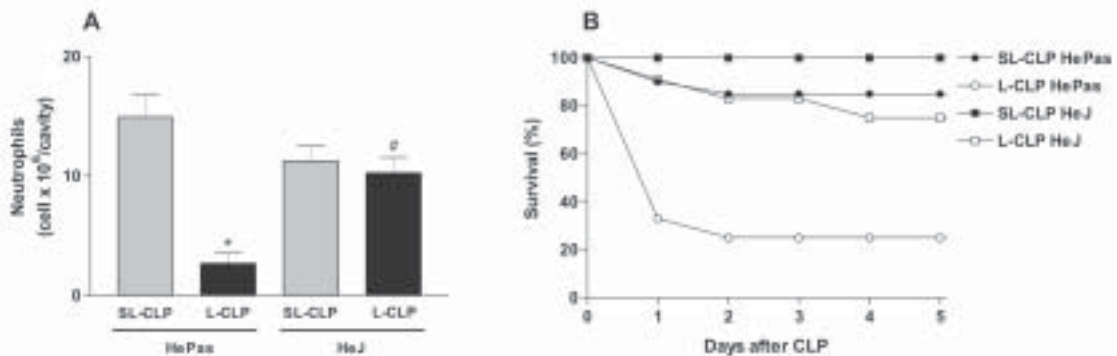


Fig. 3: toll-like receptors-4 signaling is involved in the impairment of neutrophil migration to the infectious focus during lethal sepsis induced by cell ligation and puncture (CLP). A: neutrophil migration into the peritoneal cavity in C3H/HePas (control) and C3H/HeJ (TLR4-deficient) mice subjected to sub-lethal (SL)- and lethal (L)-CLP. Assessment of neutrophil migration into the peritoneal cavity was performed 6 h after surgery. Results are expressed as mean numbers of neutrophils per cavity ± SEM. *: *P* < 0.05 compared with HePas (control) SL-CLP group; #: *P* < 0.05 compared with HePas (control) L-CLP group (analysis of variance, followed by Bonferroni's test); B: survival curves of mice after sepsis induced by CLP, determined daily up to 5 after surgery. The HePas L-CLP mice were significantly different from HeJ L-CLP mice. *P* < 0.05; Mantel-Cox log rank test. Results are expressed as percent survival.

The role of the inflammatory response in the pathogenesis of sepsis appears at first contradictory, since both deficient and excessive immune responses appear to be involved (Volk et al. 1996). Inflammatory responses are advantageous for the eradication of bacteria, as long as they are under control. However, once out of control, deregulated inflammation leads to massive production of proinflammatory cytokines. Exaggerated production of cytokines leads to coagulation disorder, tissue injury and finally multiple organ failure, the clinical hallmark of sepsis. Many efforts have been made to improve the understanding of the deregulation of the host response resulting in sepsis. Here, we have provided compelling evidence that the outcome for severity of sepsis is correlated with failure of neutrophil migration to the infection site. We have demonstrated that high concentration of cytokines and chemokines in the circulation with consequent production of NO might be critical events that result in this impaired neutrophil migration. However, it is still unclear the source of these circulating cytokines and chemokines, i.e., if they are produced into the local infectious focus and leak to the circulation, or the bacteria and/or their byproduct leak from the infectious focus to the circulation and stimulate the production of the cytokines systemically.

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