HMGB1 induces neutrophil dysfunction in experimental sepsis and in patients who survive septic shock
Murielle Grégoire, Jean-Marc Tadié, Fabrice Uhel, Arnaud Gacouin, Caroline Piau, Nathaniel Bone, Yves Le Tulzo, Edward Abraham, Karin Tarte, Jaroslaw W. Zmijewski

To cite this version:

HAL Id: hal-01533343
https://hal-univ-rennes1.archives-ouvertes.fr/hal-01533343
Submitted on 18 Jul 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
HMGB1 induces neutrophil dysfunction in experimental sepsis and in patients who survive septic shock

Murielle Grégoire*,†,‡§1; Jean-Marc Tadié *;†,‡,¶,||1; Fabrice Uhel *;†,‡,¶,||; Arnaud Gacouin ¶,||; Caroline Piau #; Nathaniel Bone **; Yves Le Tulzo*;†,‡,¶,||; Edward Abraham ††; Karin Tarte*;†,‡,§; Jaroslaw W Zmijewski **

* . INSERM, UMR U917, Rennes, F-35043, France
†. Université Rennes 1, UMR U917, Rennes, F-35043, France
‡. Etablissement Français du sang, UMRU917, Rennes, F-35043, France
§. CHU Rennes, SITI, Pole de Biologie, F-35043, France
¶. CHU Rennes, Maladies infectieuses et réanimation médicale, F-35043, Rennes, France
||. INSERM, CIC 1414, F-35043, France
#. CHU Rennes, Service de Bactériologie, F-35033, France
**. Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294-0012, USA
††. Office of the Dean, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA
1. These authors contributed equally to the work

Address for reprint requests and other correspondence: JM Tadié, Service des Maladies Infectieuses et Réanimation Médicale, Hôpital Pontchaillou, 2 rue Henri Le Guilloux, 35033 Rennes Cedex 9, France. (E-mail: jeanmarc.tadie@chu-rennes.fr).

Key words: nicotinamide adenine dinucleotide phosphate oxidase; peritonitis; sepsis; inflammation, HMGB1, neutrophils, immunosuppression

Summary Sentence: HMGB1 in the late phase of sepsis plays a specific role in the development of post-sepsis immunosuppression, and specifically neutrophil dependent antibacterial defense mechanisms

Presented in part as an abstract at the 43rd SRLF Annual Congress, Paris 2016
Abbreviations:

CLP = cecal ligation and puncture
HMGB1 = high mobility group box 1
NADPH = nicotinamide adenine dinucleotide phosphate
PMN = polymorphonuclear neutrophil
ICU = intensive care unit
Abstract

Sepsis is accompanied by the initial activation of pro-inflammatory pathways and long-lasting immunosuppression that appears to contribute to late onset mortality. Although high mobility group box 1 (HMGB1) is involved in many aspects of inflammation, its role in sepsis-induced immune suppression remains unclear. In these studies, we examined HMGB1’s contribution to neutrophil NADPH oxidase activity dysfunction and associated neutrophil dependent bacterial clearance in mice subjected to sepsis and in patients that survive septic shock. Using a murine model of polymicrobial septic peritonitis, we demonstrated that treatment with anti-HMGB1 antibody significantly diminished sepsis-induced dysfunction of neutrophil NADPH oxidase activity. In a subsequent set of experiments, we found that blocking HMGB1 preserved the ability of neutrophils from patients recovering from septic shock to activate NADPH oxidase. Taken together, our data suggest that HMGB1 accumulation in the late phase of sepsis plays a specific role in the development of post-sepsis immunosuppression, and specifically affected neutrophil dependent antibacterial defense mechanisms. Thus, blocking HMGB1 may be a promising therapeutic intervention to diminish adverse effects of sepsis-induced immunosuppression.
Introduction

Sepsis is one of the most frequent causes of morbidity and mortality in intensive care unit (ICU) and affects more than one million critically ill patients each year in the United States alone (1). Although activation of the innate immune system is an essential step in bacterial clearance, the initial pro-inflammatory response that accompanies sepsis subsequently evolves into immune immuno-paralysis, also known as immunosuppression (2-4). In particular, critically ill patients who develop chronic critical illness for longer than two weeks often progress to persistent immunosuppression that is characterized by enhanced apoptosis of lymphocytes and immune cell dysfunction (4-6). It has been suggested that preservation of immune function in septic patients as well as recovery from immunosuppression will result in improved outcomes (2, 7). In spite of advances in clinical and translational research, therapeutic interventions for sepsis are limited to use of antibiotics and fluid resuscitation, as specific pharmacological treatment is not available for this detrimental condition. Similarly, there are no effective pharmacologic approaches to accelerate the recovery of dysfunctional neutrophils, monocytes, dendritic cells, and lymphocytes from sepsis-induced immunosuppression (1, 8-11). Of note, loss of phagocytic function and diminished microbial killing during severe infection are associated with deficient production of reactive oxygen species (ROS), which has adverse effects on dissemination of existing infection and increased susceptibility to nosocomial infections and viral reactivation (12-14).

Neutrophils and macrophages are essential cell populations responsible for bacterial eradication, primarily through production of anti-bacterial peptides, cytokines,
and reactive oxygen/nitrogen species (ROS/RNS) (13, 15). Among antimicrobial mediators, activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a key anti-microbial mechanism linked to high superoxide output, known as the respiratory burst (16, 17). NADPH oxidase consists of several subunits that form an active membrane-bound or soluble complex, assembled in response to specific signals triggered by microbial or fungal products (18). Dysfunction in NADPH oxidase has serious adverse effects on neutrophil-dependent bacterial clearance in experimental models of intra-abdominal polymicrobial sepsis and in patients with chronic granulomatous disease (16, 19, 20). It is important to note that while NADPH oxidase is activated during the initial phase of polymicrobial sepsis, this event is followed by impairment of polymorphonuclear neutrophil (PMN)-dependent ROS production at later time points (12). The exact mechanism responsible for development of such neutrophil dysfunction, which also affects newly produced neutrophils, is not well understood (21), but is implicated in delayed or poor recovery of immune homeostasis in sepsis survivors.

We have recently shown that appearance of damage-associated molecular pattern (DAMP) proteins, in particular high-mobility group box 1 protein (HMGB1), diminished neutrophil-dependent bacterial killing in models of sepsis (17). HMGB1, originally described as a nuclear non-histone DNA-binding protein, has subsequently been shown to be an alarmin, and involved in the inflammatory response (22-24). HMGB1 released from dying cells promotes pro-inflammatory activation of immune cells contributing to organ injury in polymicrobial sepsis and sterile inflammatory conditions associated with trauma and hemorrhage (23, 25-28). Although plasma levels of HMGB1
are increased during sepsis, substantial amounts of HMGB1 are also present in the circulation of patients following severe infection for extended periods (23, 27). We hypothesize that such prolonged accumulation of extracellular HMGB1 promotes development of immunosuppression related to dysfunction of neutrophil NADPH oxidase and diminished bacterial clearance in mice subjected to sepsis and in patients that survive septic shock.
Material and methods

Patients and control participants

This study was performed in the infectious diseases department and ICU at Rennes University Hospital. The study design was approved by our ethics committee (CHU Rennes, n°13-8 and n°15.44-2) and informed consent was obtained. Patients admitted in our ICU for septic shock (septic patients) or for other types of shock (control patients) were included. Pregnant women, patients who were younger than 18 years old, patients with malignancy, HIV-infected patients, and patients receiving immunosuppressive agents were excluded. Standard criteria were used for diagnosis of septic shock (a clinical construct of infection with persisting hypotension requiring vasopressors to maintain mean arterial pressure higher than 65 mm Hg) (1). Patients under vasopressor therapy admitted for other reasons than septic shock were also included as control. The following data were recorded: age, reason for admission, length of stay, mortality and occurrence of nosocomial infection. Nosocomial infections were defined as already described (29). To investigate the late phase of sepsis, patients were included when vasopressor therapy (mainly norepinephrine) was stopped and blood samples were collected. Thus, post-sepsis is defined as weaning from vasopressor therapy. The decision to include patients after weaning of vasopressor therapy is based on clinical data that demonstrated that patients admitted for septic shock could be divided in two groups regarding mortality: early mortality (till day 5) and late mortality (30). Furthermore, ROS/RNS generation in neutrophils is significantly higher in septic patients at admission and decreased dramatically after one week (12, 31).
**Human PMNs isolation and culture**

PMNs were purified using whole blood CD15 microbeads (Miltenyi Biotech) as previously described (32). The purified fraction, ≥95% cell purity as evaluated by flow cytometry (Galios, Beckman Coulter), was used for further experiments. PMNs were cultured in RPMI 1640 containing 10% FBS. To investigate the late phase of sepsis, patients were included when vasopressor therapy was stopped and blood samples were collected.

**Measurement of ROS**

PMNs were incubated with or without plasma followed by stimulation with PMA (10 µM, Sigma-Aldrich) for 15 minutes. To detect ROS production, PMNs were incubated with H$_2$DCFDA probes (10µM, Invitrogen) for 30 minutes. Quantification of ROS production was performed by flow cytometry. Results were expressed as the ratio of mean fluorescence intensity (rMFI) obtained from PMA-treated and untreated PMNs. HMGB1 neutralizing antibody used was purchased from IBL International GmbH (Hamburg Germany).

**Expression of RAGE and TLR4 receptors on PMNs change during the course of sepsis**

Expression of Receptor for Advanced Glycation End Products (RAGE) and Toll-Like Receptor 4 (TLR4) was measured at admission and after resolution of sepsis using Real-time quantitative PCR. cDNA synthesis was performed with the Superscript II reverse transcriptase and random hexamers (Life Technologies). For quantitative RQ-
PCR, we used assay-on-demand primers and probes, and Taqman Universal Master Mix (Life Technologies). Gene expression was measured using the StepOnePlus (Life Technologies) based on the $\Delta Ct$ calculation method. 18S was determined as the appropriate internal standard gene using TaqMan Endogenous Control Assays (Life Technologies). Quantification of AGER and TLR4 was performed using appropriate assay-on-demand primers and probes. For each sample, the Ct value for the gene of interest was determined, normalized to its respective value for 18S, and results were then standardized by comparison to gene expression of PMNs at admission.

**PMNs Bacterial killing assay**

PMNs ($10^6$ cells) were incubated with *E. coli* ($2 \times 10^6$) or *methicillin-susceptible Staphylococcus aureus* (MSSA, $2 \times 10^6$) for 16 hours. Cells were lysed using triton X-100 (0.1%) and serial dilutions were incubated on agar plates overnight at 37°C. The amounts of viable bacteria colonies were calculated as colony forming units (CFU).

**Mice**

Male C57BL/6 mice were purchased from the National Cancer Institute–Frederick (Frederick, MD, USA). Male mice, 8 to 10 weeks of age, were used for experiments. The mice were kept on a 12-hours light-dark cycle with free access to food and water. All experiments were conducted in accordance with protocols approved by the University of Alabama at Birmingham Animal Care and Use Committee.

**Reagents**
RPMI 1640 was purchased from BioWhittaker (Walkersville, MD, USA). FBS and penicillin-streptomycin were obtained from Gemini Bioproducts (Calabasas, CA, USA). Hanks balanced salt solution (HBSS) was purchased from Invitrogen (Grand Island, NY, USA). Custom antibody mixtures (Abs) and negative selection columns for PMNs isolation were from StemCell Technologies (Vancouver, BC, Canada). Anti-phospho-p40phox (Thr154) antibody was purchased from Cell Signaling (Danvers, MA, USA). Polyclonal antibodies to neutralize HMGB1 were prepared as previously described (33). Phorbol myristate acetate (PMA), Escherichia coli 0111:B4 endotoxin (LPS), and IgG were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cecal ligation and puncture (CLP) -induced sepsis

Animals underwent CLP or sham laparotomy (sham). In brief, a midline abdominal incision was made under isoflurane anesthesia. For animals that received CLP, the cecum was exposed and ligated in the middle of cecum below the ileocecal valve, punctured once using a 21-G needle and a small amount of fecal matter was squeezed out of the cecum to induce polymicrobial peritonitis (34). The cecum was then replaced into the abdominal cavity, and the abdominal wall was closed in layers. Antibiotic therapy (25 mg/kg imipenem) was initiated 4 hours after CLP or sham and administered by subcutaneous injection every 12 hours for 5 days. Saline for fluid resuscitation was administered to create a more clinically relevant sepsis model as this is standard care for humans. Mice were then sacrificed 7 days after surgery and bone marrow PMNs were isolated (Figure 1A). In a second set of experiments, mice underwent CLP and neutralizing anti-hmgb1 antibody (125 µg, 500 µl PBS) or IgG was administrated with
antibiotics for five days. Mice were then sacrificed 7 days after surgery and bone marrow PMNs were isolated.

PMNs isolation and culture

Bone marrow PMNs were isolated as described previously (17). PMNs were cultured in RPMI 1640 medium containing 5% FBS and treated as indicated in the figure legends. PMNs viability under experimental conditions was determined using trypan blue staining and was consistently greater than 95%.

Assay for NADPH activity.

NADPH oxidase activity was measured using a standard cytochrome c reduction assay as previously described (17). Briefly, PMNs (5 x 10^5/ml) were incubated with cytochrome c (10 µM) in the presence or absence of *E. coli* (10^6/ml) or HMGB1 (300 ng/ml) in 1 ml of HBSS. The rate of cytochrome c reduction was recorded using a spectrophotometer (UV-2501PC Shimadzu; Shimadzu, Japan) for 15 minutes.

In vitro killing activity assay.

PMNs (0.5 x 10^6) were incubated with ampicillin-resistant *E. coli* DH5α (1 x10^6) in RPMI 1640 medium (1 ml) without serum for 2, 6 and 16 hours at 37°C. Next, 20 µl of cell/bacterial suspension was incubated with 480 µl Triton X-100 (0.1%) for 10 minutes to lyse PMNs. Serial dilutions were then plated on agar plates with ampicillin and incubated overnight at 37°C. The number of bacterial colonies on agar plates was determined using colony counter software (Bio-Rad, Hercules, CA, USA).
Western blot analysis

Western blot analysis was performed as previously described (33). Briefly, equal amounts of protein were resolved using 8 or 12% SDS-PAGE and transferred onto PVDF membranes (polyvinylidenedifluoride membrane, Immobilon-P; Millipore, Billerica, MA). To measure the amount of total and phosphorylated proteins, membranes were probed with specific antibodies followed by detection with horseradish peroxidase-conjugated goat anti-rabbit IgG. Bands were visualized by enhanced chemiluminescence (ECL Plus, Amersham) and quantified by AlphaEase FC software (Alpha Innotech, San Leandro, CA, USA). Each experiment was carried out two or more times.

Statistical analyses

Statistical analyses were performed with GraphPad Prism 6.0 software using the non-parametric Wilcoxon test for matched pairs or the Mann-Whitney nonparametric U test as appropriate for continuous variables. Proportions were compared between groups using Chi-2 test or Fisher exact test when required.
Results

Deficient oxidative burst and diminished bacterial killing is associated with neutrophil dysfunction in experimental sepsis

To examine the nature of neutrophil dysfunction during sepsis, mice were subjected to CLP or sham surgery. Similar to antibiotic regimes administered in critically ill patients, mice were treated with imipenem (25 mg/kg), each day for total of 5 days (Figure 1A). Early administration of antibiotics and fluid resuscitation resulted in marked improvement of viability as 100% of mice on imipenem survived at least 7 days after CLP, whereas those without antibiotics incurred 80% mortality within 48 hours (34) (data not shown). The extent of immunosuppression was determined by measurement of neutrophil oxidative burst, e.g. NADPH oxidase-derived superoxide formation, in PMA-stimulated bone marrow neutrophils that were isolated from mice 7 days after sham procedures or CLP. As shown in Figure 1B, significant reduction in NADPH oxidase activation was found in post-sepsis neutrophils, as compared to those obtained from the sham group. Along with dysfunctional oxidative burst, post-sepsis neutrophils also had diminished capacity to kill bacteria (Figure 1C).

HMGB1 inhibits neutrophil NADPH oxidase activation and bacterial killing

As shown in Figure 2A, development of post-sepsis immunosuppression is associated with prolonged accumulation of HMGB1 in plasma. We further examined if HMGB1 affects oxidative burst activity in the presence of bacteria. As shown in Figure 2B, inclusion of HMGB1 effectively reduced NADPH oxidase activation in the presence of *E. coli*. Next, to establish the importance of HMGB1 in regulating sepsis-mediated
neutrophil dysfunction, septic mice were injected with HMGB1 neutralizing antibody or isotype specific (control) IgG. We found that HMGB1 neutralizing antibody effectively prevented inactivation of NADPH oxidase in mice subjected to sepsis (Figure 2C).

NADPH oxidase requires phosphorylation and assembly of several subunits, including activatory phosphorylation of p40phox (19). A robust increase of p40phox phosphorylation occurs promptly after stimulation of neutrophils (control) with PMA. However, there was little to no p40phox phosphorylation in neutrophils isolated 7 days after CLP (Figure 2C). Of note, PMA-induced p40phox phosphorylation was preserved in neutrophils isolated from mice treated with CLP and anti-HMGB1 antibody, but not in neutrophils obtained from mice subjected to sepsis and control IgG (Figure 2C). These results suggest that HMGB1 plays an important role in acquisition of the neutrophil immunosuppressive phenotype that is characterized by diminished oxidative burst.

**Deficient oxidative burst and bacterial killing persist in PMNs of patients who survive septic shock**

To establish whether loss of bacterial killing is associated with dysfunctional NADPH oxidase, oxidative burst was measured in PMNs obtained from patients after septic shock or from either healthy donors or critically ill patients without sepsis (control patient) (Figure 3A). A total of 33 patients, including 21 patients with septic shock (Infective endocarditis = 6 (29%), pneumonia = 5 (23%), Urinary tract infection =3 (14%), intra-abdominal infection = 3 (14%), bacteremia = 2 (10%) and meningitis = 2 (10%)) and 12 critically ill non-septic patients (control patients) (ARDS = 5 (42%), cardiogenic shock = 2 (17%), status epilepticus = 2 (17%), cardiac arrest = 2 (17%),...
stroke = 1 (8%), were prospectively enrolled and compared with 16 healthy control participants. Duration of vasopressor therapy in septic and control patients was (median days [IQR]) 6 days [5-10] in septic patients and 4 days [3.75-8.75] in control patients. Characteristics of patients included in the study are shown in Table 1.

Circulating levels of HMGB1 are elevated for prolonged periods in both survivors of sepsis and in preclinical models of polymicrobial infection (23, 35) and as shown in our study (Figure 3B). Expression of RAGE products and TLR4, two receptors for HMGB1 on neutrophils were increased during the course of sepsis (Figure 3C). PMNs loaded with redox sensitive H$_2$DCF-DA fluorogenic probe (36, 37) were treated with PMA to stimulate NADPH oxidase and then DCF fluorescence measured using flow cytometry. As shown in Figures 3D, neutrophils of healthy or critically ill patients without sepsis had robust activation of NADPH oxidase. However, this response was reduced in neutrophils from patients after septic shock. Decrease in NADPH oxidase activity was also associated with diminished bacterial killing; this was shown using neutrophils from patients who had sepsis or a non-septic critical illness, cultured with *E. coli* or *MSSA* (Figures 3E).

**HMGB1 promotes dysfunction of neutrophils following sepsis**

It is not known whether prolonged accumulation of extracellular HMGB1 contributes to complications after sepsis, including increased susceptibility to secondary infections. As shown in Figure 4A, neutrophils isolated from healthy donors had a substantial loss of oxidative burst when cultured with plasma from post-sepsis patients, as compared to exposure to plasma from healthy donors or critically ill patients without
sepsis. Because HMGB1 effectively diminished NADPH oxidase activation in mice subjected to polymicrobial sepsis, we also examined if similar immunosuppressive effects mediated by HMGB1 are found in patients that survive septic shock. To test this possibility, neutrophils of healthy donors were cultured with plasma obtained from post-sepsis patients in the presence or absence of HMGB1 neutralizing antibody or control isotype specific IgY. As shown in Figure 4B, anti-HMGB1 antibody increased the ability of neutrophils to activate NADPH oxidase when compared to control IgY. These results suggest that circulating levels of HMGB1 in post-sepsis patients are implicated in the development of immunosuppression characterized by diminished activity of neutrophil NADPH oxidase.
Discussion

In the present studies, increased amounts of circulating HMGB1 have substantial impact on the development of neutrophil immunosuppressive phenotypes in a mouse model of intra-abdominal polymicrobial sepsis and in patients that survive septic shock. In particular, we found that exposure of neutrophils to HMGB1 directly suppressed PMA or *E. coli*-induced oxidative burst and the ability of neutrophils to eradicate bacteria. In turn, neutralization of HMGB1 effectively prevented neutrophil dysfunction and preserved their capacity for bacterial clearance. We also demonstrated that circulating amounts of HMGB1 are sufficient to diminish neutrophil oxidative burst in patients that survive septic shock. Moreover, our data indicate that neutrophils obtained from healthy or critically ill patients without sepsis developed a phenotype consistent with immunosuppression, as defined by diminished oxidative burst and bacterial killing, after culture with plasma from patients recovering from sepsis, despite an increase in the expression of RAGE and TLR4 in PMNs of patients that survive septic shock. The inhibitory effects of septic plasma on neutrophil activation were preventable upon inclusion of anti-HMGB1 neutralizing antibody.

Although recent studies have shown improved survival of patients in the first several days following sepsis (38), there remain multiple long term complications that accompany this condition, including frailty, cognitive impairment, and diminished ability to contain ongoing infections as well as an increased risk of acquiring nosocomial bacterial, viral, and fungal infections (39, 40). While most studies have focused on the detrimental effects of activated PMNs during the pro-inflammatory phase of sepsis, less
is known about mechanisms linked to loss of anti-bacterial action by existing and newly produced PMNs at later times following severe infection. Of note, experimental studies have shown impairment of innate immune responses, especially dysfunction of neutrophil oxidative burst and increased susceptibility to secondary infections (e.g. *P. aeruginosa*) following the induction of severe infection (41).

Our previous study suggested that HMGB1 interferes with NADPH oxidase functional assembly, including phosphorylation of p40phox and show that interaction between HMGB1 and RAGE effectively suppressed NADPH oxidase activation in neutrophils (17). Such findings are consistent with results obtained in our current study that showed the ability of HMGB1 to diminish oxidative burst and bacterial clearance by neutrophils isolated from mice with sepsis or from patients recovering from septic shock. Of note, we found that RAGE expression was increased in neutrophils from patients that survive septic shock which validates a previously published study (42). In addition to prompting a decrease in oxidative burst, a possible mechanism by which HMGB1 affects neutrophil function is related to impairment of neutrophil chemotaxis and failure to target infectious foci for bacterial eradication. In particular, modulation of neutrophil mitochondrial membrane potential is directly linked to dysfunctional neutrophil motility and diminished NADPH oxidase activation (43). This concept is supported by distal localization of mitochondria in polarized neutrophils that was recently linked to ATP extracellular flux and enhanced directional motility by neutrophils (44).

Recently, HMGB1 was implicated in mitochondrial depolarization (45). Although HMGB1 is released from dying cells, activated macrophages and other cell populations during inflammation, the pathophysiologic impact of late extracellular
HMGB1 accumulation is not well understood. In particular, elevated levels of HMGB1 in the circulation appear to be similar between survivors and non-survivors of severe infection and do not predict hospital mortality (27). However, neutralization of HMGB1 with anti-HMGB1 antibody had favorable outcomes in experimental sepsis (23, 25, 46). Furthermore, an improved clinical outcome was observed in sepsis patients that produced substantial amounts of anti-HMGB1 antibody (47). Previous studies have shown that besides sepsis, trauma/hemorrhage is also associated with development of immunosuppression accompanied by the release of HMGB1 (48, 49). Elevated amounts of HMGB1 in circulation were also associated with reduced immune system function in diabetes, patients undergoing therapies for cancer, and chronic inflammatory conditions resulting from atherosclerosis or rheumatoid arthritis (23, 48). It is important to note that acute organ injury associated with trauma and hemorrhage causes a rapid release of HMGB1 from necrotic cell. HMGB1 is a late mediator of morbidity and mortality among patients that initially survive severe infection. Similarly, we observed the only modest increase of HMGB1 in plasma of mice 24 hours after CLP, as compared to sham, whereas significant increases were found 7 days after sepsis.

While HMGB1 was initially described as a nuclear protein with important regulatory functions associated with gene expression, more recent studies have shown that HMGB1 has potent proinflammatory actions and can be classified as a DAMP mediator. Therapeutic approaches aimed at inhibiting the actions of HMGB1 could be of interest in diminishing tissue injury and organ dysfunction. Our data suggest that HMGB1 accumulation in the late phase of sepsis contributes to the development of post-sepsis immunosuppression through inhibiting neutrophil dependent antimicrobial
defense mechanisms. Blocking HMGB1 may be a promising therapeutic intervention to diminish adverse effects of sepsis-induced immunosuppression and improve recovery of immune homeostasis in this setting.
Authorship: M.G., J-M.T., C.P., N.B., J-W.Z. performed the experiments. M.G., J-
M.T., F.U., A.G., J-W.Z. wrote the report and designed the experiments. J-M.T and
and discussed the results and read and discussed the report. M.G., A.G. and J-
W.Z. performed the statistical analyses. M.G., J-M.T., F.U., A.G., C.P., N.B., Y.L-T.,
E.A., K.T. and J-W.Z. read and approved the final report.

Acknowledgements: This work was supported by grants from CORECT 2013 (Comité
de la Recherche Clinique et Translationnelle), CHU Rennes, France. MG was a
recipient from the Association pour la Recherche contre le Cancer (ARC). Funding was
provided by National Institutes of Health Grant HL107585 to JW Zmijewski.

Disclosures: The authors declare no conflicts of interest.
REFERENCES


**Figure 1.** Mice subjected to intra-abdominal polymicrobial sepsis developed neutrophil dysfunction. (A) Mice subjected to CLP or sham surgery were treated with antibiotics for 5 days followed by measurement of neutrophil function at day 7. Panel (B) shows the extent of NADPH oxidase activation in PMA-stimulated bone marrow neutrophils obtained from control (sham) or CLP mice. (C) Bacterial clearance was determined after culture of neutrophils (CLP or control) with *E. coli* for 16 hours. Means ±SD, n = 6, ** P < 0.01, NS -not significant.
Figure 2. HMGB1 diminished neutrophil oxidative burst. (A) Western blot and quantitative analysis show the amount of HMGB1 in mouse sera obtained before, 24 hours, or 7 days after CLP or sham. Means ±SD, n = 5, ** P < 0.01. (B) Representative rates of cytochrome c reduction show NADPH oxidase inactivation in neutrophils treated with HMGB1 (0 or 300 ng/ml) for 15 minutes. Oxidative burst was determined after inclusion of E. coli (10⁶/ml) for 15 minutes. (C) Sham and CLP-induced sepsis mice were treated with HMGB1-neutralizing antibody (125 µg) or control isotype specific IgG for (5 days). NADPH oxidase subunit phospho-phox40 (left panel) or oxidative burst (right panel) was than determined in bone marrow neutrophil stimulated using PMA (0 or 10 nM) for 15 minutes. (Means ±SD, n = 3, ** P < 0.01).
Figure 3. Human PMNs have diminished NADPH oxidase activation and ability to kill bacteria in patients that survived septic shock. (A) PMNs purified from blood of normal, ICU non-sepsis, or 7 day after septic shock. (B) The amount of HMGB1 was determined in plasma of healthy donors, non-sepsis patients, or after septic shock (Means ±SD, n = 6-8, * P < 0.05, ns- not significant). (C) The extent of RAGE and TLR4 mRNA expression is shown.
expression was determined in PMNs of control and post-septic patients. RQ-PCR was performed on neutrophils at admission (T0) and when vasopressor therapy was stopped (T1) to analyze RAGE and TLR4 expression. Each sample was normalized to 18S expression and compared to expression levels on T0 neutrophils. (D) PMNs purified from blood of normal, ICU non-sepsis, or 7 day after septic shock were loaded with H$_2$DCF-DA and treated with PMA (0 or 10 μM) for 30 minutes. Measurement of ROS production occurred before and after stimulation with PMA (10 μM) for 15 minutes. Representative FACS analyses (left panel) show the extent of ROS production in unaltered (white) on stimulated (gray) PMNs. (E) Measurement of ROS production after stimulation with PMA (10 nM) for 15 minutes: PMA-stimulated NADPH oxidase activation was determined in PMNs isolated form patients with septic shock, non-septic patients and healthy donors (right panel) (Means ±SD, n = 6-7, * P < 0.05). (F) PMNs (10$^6$ cells) obtained from healthy or post-sepsis patients were incubated with E. coli ormethicillin susceptible Staphylococcus aureus (MSSA) for 16 hours and the amount of viable bacteria determined using CFU assay (Means ±SD, n = 6-7, * P < 0.05).
Figure 4. HMGB1 in plasma of post sepsis patients promoted dysfunction of PMNs oxidative burst. (A) Healthy donor PMNs were incubated with plasma of healthy, non-sepsis, or sepsis patients for 30 minutes and then oxidative burst determined after exposure to PMA (Means ±SD, n = 6-8, * P < 0.05, NS- not significant). (B). Plasma obtained from post septic patients was treated with HMGB1-neutralizing antibody (50µg) or IgG (isotype control) for two hours and then treated plasma was used in culture with healthy donor PMNs for an additional 30 minutes. Oxidative burst was measured after inclusion of PMA (0 or 10 µM) for 15 minutes. Means ±SD, n = 6, * P < 0.05.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Postsepsis patients (n = 21)</th>
<th>Critically ill nonseptic patients (n = 12)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median years [IQR]</td>
<td>63 [49–69]</td>
<td>62 [57.8–71.8]</td>
<td>0.79</td>
</tr>
<tr>
<td>Outcome, n (%)</td>
<td></td>
<td></td>
<td>0.37</td>
</tr>
<tr>
<td>Survivor</td>
<td>15 (71)</td>
<td>10 (83)</td>
<td></td>
</tr>
<tr>
<td>Nonsurvivor</td>
<td>6 (29)</td>
<td>2 (17)</td>
<td></td>
</tr>
<tr>
<td>Nosocomial infections, n (%)</td>
<td>6 (29)</td>
<td>3 (25)</td>
<td>0.58</td>
</tr>
</tbody>
</table>