α-Chemokine receptor blockade reduces high mobility group box 1 protein-induced lung inflammation and injury and improves survival in sepsis

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METHODS

Synthesis and use of Antileukinate. The hexapeptide receptor inhibitor Ac-RRWWCR-NH2 (Antileukinate) was synthesized as previously described (10). The peptide was dissolved in sterile saline (0.9% wt/vol) immediately before use and administered subcutaneously.

All animal experiments were approved by the Institutional Animal Care and Use Committee at the Medical Research Institute at North Shore-LIJ.

Induction of polymicrobial bacterial peritonitis. Peritonitis was induced in female BALB/c mice (Taconic, Germantown, NY) by the intraperitoneal injection of a mixture of E. coli (5 × 108 CFU), S. aureus (5 × 108 CFU), and P. aeruginosa (5 × 108 CFU) in physiological saline (15 mL/kg body weight).

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cecal ligation and puncture (CLP). Mice (20–25 g) were anesthetized [ketamine (75–80 mg/kg) + medetomidine (1.0–1.2 mg/kg) in 200 μl ip], and a 1- to 2-cm incision was made on the lower left abdomen. The cecum was exposed and ligated below the ileocecal valve. The cecum was punctured once with a 23-gauge needle, and stool (~1 mm) extruded from the hole. After replacing the cecum, we closed the abdomen, and the mice were resuscitated with 0.5 ml of warm saline (0.9% wt/vol). In some experiments, starting 24 h after the induction of sepsis, groups of animals received either Antileukinate (250 μg in 100 μl sterile pyrogen-free saline, 0.9% wt/vol) or saline alone (control) twice daily for 4 days.

BAL. The mice were anesthetized as before. Blood was collected via cardiac puncture. The trachea was directly visualized, and a 20-gauge × 1/2-in iv catheter was inserted and secured in place. The lungs were gently lavaged (two times each aliquot) with two aliquots of saline (total 2.5 ml), which were pooled. The recovery ratio of the lavage fluid ranged from 70% to 80% and did not differ significantly among the groups. The total cell count (using a Neubauer hemocytometer) and cell differential (performed on hematoxylin and eosin-stained cytocentrifuge preparations, counting at least 400 cells per condition) were assessed.

Analysis of BAL fluid. MIP-2 and macrophage migration inhibitory factor (MIF) concentrations were measured by ELISA (R&D Systems, Minneapolis, MN and Chemicon International, Temecula, CA, respectively). Total protein concentration was measured using Coomassie Plus assay (Pierce Chemical, Rockford, IL). Reactive nitrogen species (RNS), including NO, nitrate, and nitrite, were measured using a Sievers NO analyzer (model 280; Boulder, CO).

Western blot analysis. Proteins within the BAL fluid were separated on SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and located with specific rabbit anti-HMGB1 IgG as previously described (42, 43).

MIP-2 reverse transcriptase polymerase chain reaction. After CLP or sham surgery, the lungs were removed and rapidly frozen in liquid nitrogen. Total mRNA was isolated from the tissues using RNeasy (Qiagen, Valencia, CA), and reverse transcriptase polymerase chain reaction was performed using the method and primers outlined by Mancardi et al. (18). The resultant bands were scanned, and the density of each band was expressed as a ratio of β-actin expression in the same samples (22).

Production and purification of recombinant HMGB1. Recombinant rat HMGB1 (rHMGB1) was expressed in Escherichia coli and purified as described previously (42). The protein was dialyzed extensively against phosphate-buffered saline (PBS), passed over a polymixin B column, lyophilized, and redissolved in sterile water before use. rHMGB1 preparations were shown to be free of residual LPS contamination using a colorimetric limulus assay. In some experiments, the protein was digested with trypsin before instillation.

Instillation of rHMGB1. Mice were anesthetized as described above. An incision (2–3 mm) was made, and the trachea was directly visualized. A 25-gauge needle was used to make a 1-mm hole in the trachea, and the HMGB1 was instilled via a 50-μl Hamilton syringe. Each group contained between 5 and 11 animals, and experiments were performed independently at least twice.

Bacterial counts. Bacterial counts in septic animals were performed as previously described (40). In brief, the spleen was removed by a sterile technique and homogenized in PBS (0.05 M, pH 7.4). After serial dilutions with PBS, the homogenate was plated on 0.15-ml aliquots on tryptic soy agar plates (Difco, Detroit, MI), and colony forming units (CFU) were counted after overnight incubation at 37°C. Bacterial load was expressed as CFU/g tissue.

Statistical analysis. The data are expressed as means ± SD and were analyzed for significance by Student’s t-test (for two groups) or for multiple comparisons by analysis of variance with a post hoc Dunn’s or Bonferroni analysis. A P value of <0.05 was considered statistically significant. Differences in the survival curves were assessed using the Mann-Whitney U-test.

RESULTS

HMGB1 accumulates within the alveolar spaces following CLP. HMGB1 accumulated within the lungs of mice following the induction of sepsis by ligation and puncture of the cecum. Although there were detectable amounts of HMGB1 within the BAL fluid in the control mice (those having undergone anesthesia and laparotomy without ligation and puncture of the cecum), there was a steady accumulation of HMGB1 within the alveoli over the 36 h of evaluation following CLP (Fig. 1).

MIP-2 accumulates within the alveolar spaces during sepsis. mRNA encoding MIP-2 was elevated in the lung tissues 6 h after the induction of sepsis and was further increased at 24 h (Fig. 2A). This was associated with an increase in MIP-2 protein within the BAL fluid at 12 h post-CLP. The level remained elevated at 48 h postsurgery (Fig. 2B).

Inflammatory neutrophils within the alveoli following direct instillation of HMGB1. Sepsis is often associated with acute lung inflammation and injury. Therefore, we examined whether HMGB1 alone was sufficient to induce lung inflammation and injury. rHMGB1 was instilled directly into the lungs, and the alveolar spaces were lavaged 18 h postinstillation. Leukocyte accumulation occurred within the alveoli in a dose-dependent manner with respect to rHMGB1 (Fig. 3). Even modest instillations of as little as 0.5 μg of rHMGB1 (~20 pmol) increased the number of lavaged leukocytes to 8- to 10-fold higher than those animals instilled with saline alone. At 18 h after the instillation of HMGB1, a differential cell count revealed that the percentage of neutrophils had increased to 72.6 ± 14.9% compared with the saline-instilled controls that contained 2.3 ± 1.3% neutrophils within the lavage fluid. Furthermore, the neutrophil accumulation induced by 0.5 μg of HMGB1 was comparable to that induced by 5 μg of LPS. The leukocyte accumulation time dependent with significant increases occurring at 6 h postinstillation (Fig. 4A) and due predominantly to an influx of neutrophils. The ability to induce accumulation of inflammatory cells was lost when the HMGB1 was digested with trypsin before instillation (Fig. 4B), indicating a specific effect of the rHMGB1 protein.

Neutrophil chemoattractant MIP-2 accumulates within the alveoli following instillation of rHMGB1. Neutrophil accumulation within the lung during periods of acute inflammation, particularly associated with sepsis, are associated with α-chemokines (8, 23, 24). Therefore, we examined the role of α-chemokines in HMGB1-induced neutrophil accumulation within the lung. After instillation of HMGB1 into the alveoli,
there was a time-dependent increase in the \( \alpha \)-chemokine MIP-2 (Fig. 5). This increase, which was evident 1 h after rHMGB1 instillation and peaked at around 3 h, preceded the neutrophil accumulation. Additionally, 6 h after instillation of rHMGB1 (0.5 \( \mu \)g) into the lungs, there was an accumulation of MIP-2 (26.5 \( \pm \) 5.1 pg/ml) in the plasma that was significantly higher than the plasma concentration of MIP-2 (11.0 \( \pm \) 2.7 pg/ml) following instillation of trypsin-degraded rHMGB1 (\( P \leq 0.05 \)).

\( \alpha \)-Chemokine receptor blockade reduces rHMGB1-induced acute lung injury. Because neutrophil accumulation following the instillation of rHMGB1 occurred in association with an increase in \( \alpha \)-chemokine, we evaluated the effect of specific receptor blockade on the inflammatory response and acute lung injury. \( \alpha \)-Chemokine receptor blockade was achieved with the use of Antileukinate, which we have shown previously effectively binds both \( \alpha \)-chemokine receptors 1 and 2 (10) and suppresses acute lung injury in mice (11, 17). Antileukinate administered subcutaneously following intratracheal instillation of rHMGB1 (0.5 \( \mu \)g) significantly decreased, in a dose-dependent manner, the total leukocyte count within the BAL (Fig. 6A) by reducing the neutrophil trafficking to the alveolar spaces (Fig. 6B). Concurrent with the decreased influx of inflammatory cells in the lung, following \( \alpha \)-chemokine receptor blockade, there was a decrease in oxidative stress as assessed by total RNS (Fig. 7A), injury evaluated by increased total protein (Fig. 7B), and inflammatory potential measured by the accumulation of other inflammatory mediators, such as MIF (Fig. 7C), which has also been shown to play a key role in the pathogenesis of sepsis (5, 6). These results indicate that there were decreases both in the injury and the potential for further damage to the epithelium. However, blockade of \( \alpha \)-chemokine receptors had no significant effect on MIP-2 accumulation within the plasma (rHMGB1-treated animals = 26.5 \( \pm \) 5.1 pg/ml vs. rHMGB1 + Antileukinate animals = 20.5 \( \pm \) 2.9 pg/ml) of rHMGB1-treated animals.

\( \alpha \)-Chemokine receptor blockade increases survival in sepsis. HMGB1 is a late mediator of septic mortality that, as we have shown above, can cause neutrophil-mediated acute lung injury. Because acute lung injury in sepsis is associated with increased mortality (23), we examined whether \( \alpha \)-chemokine receptor blockade would improve survival during sepsis. Because our data indicate that HMGB1 levels in the lung do not significantly increase earlier than 24 h postinduction of sepsis, we delayed initiation of \( \alpha \)-chemokine receptor blockade until 24 h after the CLP procedure. Figure 8 shows that 2 days following induction of polymicrobial sepsis, there was an increase in the...
tissue bacterial load and that the number of bacteria had subsided by day 4. Administration of Antileukinate in these animals had no significant effect on these numbers. However, H9251-chemokine receptor blockade by administration of Antileukinate, starting 24 h after the induction of sepsis, significantly improved the survival rate of these animals from 58% in controls to 89% (Fig. 9).

DISCUSSION

Severe sepsis, a lethal systemic inflammatory reaction to infection, kills more than 215,000 people annually in the United States, with over $16.7 billion nationally in costs (1). Severe sepsis frequently precipitates acute lung injury, and even those individuals that survive the septic episode commonly suffer from chronic pulmonary fibrosis, reduced pulmonary function, and a reduced health-related quality of life (7, 12, 27, 32). A better understanding of the mechanisms involved in the injury is essential to a reduction in the morbidity and mortality of acute lung injury associated with sepsis.

Recently, rHMGB1, a lethal mediator of sepsis (44), has been shown to induce acute lung inflammation (1). However, before the current study it remained unclear whether the injury resulted from the direct effects of HMGB1 or from subsequent neutrophil activity. Neutrophils are thought to be a critical component of the pathogenesis of acute lung injury. One of the earliest events in acute lung injury is the sequestration of neutrophils in the lung microvasculature (37). However, sequestration alone is insufficient to cause severe injury to the lung tissue (19). To cause tissue damage, activation of the neutrophils is required. Adherence of the neutrophils to endothelium, epithelium, or to interstitial matrix can trigger the uncontrolled release of proteolytic and oxidative enzymes, as well as reactive oxygen and nitrogen species. This can lead to tissue damage and the release of growth factors, chemokines, and other cytokines that can enhance the inflammatory response (9, 15). Neutrophil adhesion to endothelial cells is mediated via β-integrin molecules on the neutrophil cell surface, with expression and increased avidity of these adhesion molecules being augmented by chemoattractants such as IL-8 (45, 46). Adhesion primes neutrophils, greatly increasing their ability to degranulate and activate the respiratory burst in response to soluble mediators (4, 35), with the amount of oxidant production increasing by up to 1,000-fold over that of nonadherent neutrophils (9). The release of reactive oxygen and nitrogen species from neutrophils can directly injure the lung (33). Tsujimoto et al. (38) have shown the importance of the α-chemokine MIP-2 in acute lung injury following peritonitis. They demonstrated an early accumulation of the α-chemokine within the lungs associated with marked interstitial edema and inflammatory infiltration at 6–12 h after the induction of peritonitis. The model was lethal starting at ∼24 h, and anti-MIP-2 antibody improved survival at 72 h from 0% in sham-treated animals to 50% in the group receiving anti-MIP-2 antibody. The absolute timing and peak of the MIP-2 genera-

![Graph showing Total Cells in BAL](image1)

**A**

**B**

Fig. 4. HMGB1 induces neutrophil accumulation in the lung. Purified recombinant HMGB1 free from measurable LPS (0.5 μg in 50 μl of sterile pyrogen-free saline, 0.9% wt/vol) instilled directly into the lungs induced significant increases in cell accumulation in a time-dependent manner. A: *significantly different from instillation of saline alone (P < 0.05). This increase was predominantly due to an increase in neutrophils. The inflammatory affects of HMGB1 were abolished following trypsin digestion (n = 9 per group). B: there was a significant reduction in cell accumulation following the instillation of trypsized HMGB1 [0.5 μg in 50 μl of sterile pyrogen-free saline, 0.9% wt/vol (n = 11)] vs. instillation of an equal concentration of native protein (n = 14). *P < 0.05.

![Graph showing MIP2 in BAL](image2)

**Fig. 5.** HMGB1 induces MIP-2 accumulation in the lung. Purified recombinant human (rh) HMGB1 free from measurable LPS (0.5 μg in 50 μl of sterile pyrogen-free saline, 0.9% wt/vol) instilled directly into the lungs induced significant increases in MIP-2 measured by ELISA (n = 6 per group). The concentration of MIP-2 in the BAL fluid was at a maximum at 3 h postinstillation and returned to control levels after 18 h. *Significantly different from instillation of saline alone (P < 0.05).
tion in our study differ from that of the earlier study. This may be due in part to the use by Tsujimoto et al. of the inhaled anesthetic diethyl ether before the induction of sepsis. Compared with injectable anesthetics, volatile anesthetics have been shown to alter the course of the acute inflammatory response in the lung (26, 29). Our data show that the concentration of MIP-2 within the BAL is significantly increased by 12 h post-CLP. This is consistent with the findings of Walley et al. (41), who found MIP-2 raised in the lung at both 8 and 24 h post-CLP, and consistent with the release of HMGB1 into the alveolar space, which occurs within the first 12 h of sepsis.

From our studies with rHMGB1 instilled directly into the lungs, it might be expected that during sepsis, the release of HMGB1 and subsequent MIP-2 would attract neutrophils to the lungs, and the studies of Tsujimoto et al. (38) showed a marked infiltration of inflammatory cells within the alveolar walls. However, consistent with that study and that of Yin et al. (47), we do not see a large increase of intra-alveolar neutrophils during the first 48 h of sepsis. Although we have shown that in otherwise normal mice, the presence of HMGB1 in the alveolar spaces induces a neutrophil influx, during sepsis the build up of HMGB1 within the alveoli occurs in the setting of a severe systemic inflammatory response. Although neutrophils are sequestered in the lung, particularly 24 h post-CLP (21, 48), Yin et al. (47) have shown that the neutrophils stay mainly within the pulmonary vasculature and are predomi-

**Fig. 6.** α-Chemokine receptor blockade abrogates HMGB1-induced leukocytosis. Antileukinate (in 200 μl of sterile pyrogen-free saline, 0.9% wt/vol) was administered subcutaneously, along the dorsum, as a component of the resuscitation fluid, ~30 min after the intratracheal (it) instillation of purified recombinant HMGB1 (0.5 μg in 50 μl of sterile pyrogen-free saline, 0.9% wt/vol). Antileukinate administration significantly reduced, in a dose-dependent manner, the total numbers of white cells (A) and neutrophils (B) lavaged from the lungs after 6 h (n = 5 per group). PMN, polymorphonuclear neutrophil. *Significantly different from administration of subcutaneous (s.q.) saline (P < 0.05).

**Fig. 7.** α-Chemokine receptor inhibitor blocks rHMGB1-induced oxidative stress and injury. After the intratracheal instillation of purified rat rHMGB1 (0.5 μg in 50 μl of sterile pyrogen-free saline, 0.9% wt/vol), the animals received a single, subcutaneous bolus of Antileukinate (200 μg in 200 μl of sterile pyrogen-free saline, 0.9% wt/vol) administered subcutaneously. Antileukinate significantly reduced the concentrations of total reactive nitrogen species (A), total protein (B), and macrophage migration inhibitory factor (MIF, C) in the BAL fluid at 6 h post-rHMGB1 administration (n = 6 per group). *Significantly different from administration of subcutaneous saline (P < 0.05).
nantsly immature band cells. Such cells have abnormal surface
receptors, cytoskeletal elements, and chemotactic activity that
restrict their transendothelial migration. Although sequestra-
tion alone is insufficient to cause acute lung injury (19), the
potential for injury remains since neutrophils are primed by
interaction with the endothelium, greatly increasing their abil-
ity to degranulate and activate the respiratory burst in response
to soluble mediators (4, 35).

The studies by Talwar et al. (36) have also shown elevated
concentrations of HMGB1 (86 ± 12 ng/ml) in the BAL fluid
from patients with the severest form of acute lung injury, acute
respiratory distress syndrome. Normal lavage procedures dilute
the epithelial lining fluid ~100-fold (24). Therefore, the local
concentration of HMGB1 within the alveolar spaces may
exceed 8 μg/ml. We found that instillation of rHMGB1 di-
rectly into the lungs of normal, healthy mice induced a sig-
ificant dose-dependent increase in total leukocytes, even at doses
as low as 0.5 μg. Because this dose was comparable with that
found during acute lung injury, we instilled this amount to
assess the effects of α-chemokine receptor blockade on
HMGB1-induced lung injury. Instillation of HMGB1 induced
an increase in leukocytes primarily due to a neutrophil influx.
The cell influx was specific to the intact rHMGB1 molecule (as
it was ablated by trypsin degradation) and preceded by an in-
crease in α-chemokine MIP-2. We used our specific receptor
inhibitor, Antileukinate, to block the α-chemokine receptors.
The blockade of α-chemokine receptors with Antileukinate
reduced the neutrophil influx, total RNS, and injury as assessed
by the BAL protein content. The blockade also reduced the
accumulation in the air spaces of MIF, another late-acting
chemokine important in the pathogenesis of sepsis (16), sug-
gesting a further benefit of the α-chemokine receptor block-
ade strategies. In addition, rHMGB1 instillation into the lung
initiated a systemic inflammatory response shown by the in-
crease in plasma MIP-2 concentration; this effect was abol-
ished by trypsin digestion of the HMGB1 but was not reduced
by the administration of Antileukinate. It remains to be deter-
mined whether the accumulation of the chemokines in the
plasma were due to leakage from the air spaces or whether
rHMGB1, intratracheally instilled, initiates the release of
α-chemokines from extrapulmonary sources. However, in ac-
cordance with HMGB1 being a late mediator of the lethality
associated with sepsis, the concentration of HMGB1 in the
epithelial lining fluid (sampled in the BAL) increased dramat-
ically between 24 and 36 h after the onset of sepsis. In addition,
α-chemokine receptor blockade did not affect overall bacterial
counts in the animals but increased survival during sepsis even
when administration was delayed until 24 h after the induction
of sepsis. Santos et al. (30) showed that following the induction
of polymicrobial sepsis in mice (by CLP), the bacterial burden
in the lung is similar to that of other body compartments and,
consistent with our data, the CFU peak within the lung 24–48
h postinduction of sepsis and then decrease over the next 48 h.
This suggests that the animals are not dying from an over-
whelming growth of bacteria but from the inflammatory re-
sponse set in motion by the original infection.

We conclude that HMGB1 is potentially an important me-
diator of acute lung injury during severe sepsis and that its
effects, which are due predominantly to the recruitment of
neutrophils, can be reduced by α-chemokine receptor inhibi-
tion. We have demonstrated that substantial increase in sur-

![Fig. 9. α-Chemokine receptor inhibitor improves survival in sepsis. From 24 h after the induction of sepsis, Antileukinate (250 μg in 100 μl of sterile
pyrogen-free saline, 0.9% wt/vol) or saline alone (control) was administered
twice daily for 4 days. Survival in each group was assessed daily, and overall
survival was evaluated 7 days after the induction of sepsis. Initial numbers
of animals in each group were Antileukinate n = 23; control n = 22. The survival
curves were significantly different by Mann-Whitney U-analysis (P < 0.05).
](image)

![Fig. 8. Bacterial load following induction of polymicrobial sepsis. From 24 h
after the induction of sepsis, Antileukinate (250 μg in 100 μl of sterile
pyrogen-free saline, 0.9% wt/vol) or saline alone (control) was administered
twice daily for 4 days. At days 2, 4, and 7, animals were killed, and the spleens
were assessed for viable bacteria. There were no significant differences noted
between treated and control groups (n = 5 per group). cfu, Colony forming
unit.](image)
DISCLOSURES

K. J. Tracey is a coinventor on a patent describing anti-HMGB1 antibodies, which were not used in the work described here. North Shore LIJ licensed this technology to Critical Therapeutics, Inc., which has a sponsored research agreement with Dr. Tracey.

E. J. Miller and S. Hayashi are coinventors on patents describing Antileukinate; these patents are held by The University of Texas Board of Regents and not by the inventors.

REFERENCES


