ORIGINAL ARTICLE

Norepinephrine Dysregulates the Immune Response and Compromises Host Defense during Sepsis

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Abstract

Rationale: Sepsis is characterized by a dysregulated immune response to infection. Norepinephrine, the cornerstone vasopressor used in septic shock, may contribute to immune dysregulation and impact host defense.

Objectives: To investigate effects of norepinephrine and the alternative vasopressor vasopressin on the immune response and host defense.

Methods: Leukocytes from six to nine donors were stimulated in the presence or absence of norepinephrine and vasopressin. A total of 190 C57BL/6J mice received a continuous infusion of norepinephrine or vasopressin via microosmotic pumps and were challenged with LPS or underwent cecal ligation and puncture. Thirty healthy volunteers were randomized to a 5-hour infusion of norepinephrine, vasopressin, or saline and intravenously challenged with LPS. The relationship between the norepinephrine infusion rate and the use of β-blockers and plasma cytokines was assessed in 195 patients with septic shock.

Measurements and Main Results: Norepinephrine attenuated the production of proinflammatory mediators and reactive oxygen species and augmented antiinflammatory IL-10 production both *in vitro* and in LPS-challenged mice. Norepinephrine infusion during cecal ligation and puncture resulted in increased bacterial dissemination to the spleen, liver, and blood. In LPS-challenged volunteers, norepinephrine enhanced plasma IL-10 concentrations and attenuated the release of the proinflammatory cytokine IFN-γ–induced protein 10. Vasopressin exerted no immunomodulatory effects across these experimental setups. In patients, higher norepinephrine infusion rates were correlated with a more antiinflammatory cytokine balance, whereas β-blocker use was associated with a more proinflammatory cytokine balance.

Conclusions: Norepinephrine dysregulates the immune response in mice and humans and compromises host defense. Therefore, it may significantly contribute to sepsis-induced immunoparalysis, whereas vasopressin does not have untoward immunologic effects.

Keywords: norepinephrine; sepsis; vasopressin; immunoparalysis

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At a Glance Commentary

Scientific Knowledge on the Subject: Immunoparalysis in patients with sepsis results in ineffective clearance of infectious foci and increased susceptibility toward secondary infections, thereby contributing to morbidity and mortality. *In vitro* data suggest that norepinephrine, the mainstay vasopressor for the treatment of septic shock patients, has antiinflammatory effects. The impact of norepinephrine on the immune response and host defense *in vivo* is unknown.

What This Study Adds to the Field: Norepinephrine attenuates the production of proinflammatory mediators and reactive oxygen species and augments antiinflammatory IL-10 production both in human leukocytes stimulated with various inflammatory ligands and in LPS-challenged mice. Furthermore, norepinephrine infusion increases bacterial dissemination in a murine polymicrobial sepsis model. Also, in healthy volunteers intravenously challenged with LPS, norepinephrine infusion enhances the IL-10 response and attenuates concentrations of the proinflammatory cytokine IFN-γ–induced protein 10. The alternative vasopressor vasopressin exerts no effects across these experimental setups. Finally, in patients with septic shock, higher norepinephrine infusion rates are associated with a more antiinflammatory than proinflammatory plasma cytokine balance, whereas the use of β-blockers is associated with a more proinflammatory cytokine balance. Together, our results show that norepinephrine dysregulates the immune response and compromises host defense. Therefore, it may contribute to immunoparalysis observed in patients with septic shock.

Sepsis, defined as life-threatening organ dysfunction caused by a dysregulated host response to infection (1), is the leading cause of death worldwide, accounting for 20% of global mortality (2). This has prompted the World Health Organization to designate sepsis as a global health priority (3). Sepsis can comprise both hyperinflammatory and immunosuppressive traits (4). Although hyperinflammation, for instance observed in patients with the macrophage activation syndrome (5), can have clear detrimental effects, immunosuppressive interventions have failed to improve outcomes in sepsis. Therefore, attention has shifted toward the detrimental role of sepsis-induced immunosuppression (6, 7). This "immunoparalysis" is characterized by various aberrations in the immune system, including decreased HLA-DR isotype expression on antigen-presenting cells, diminished proinflammatory cytokine production by *ex vivo*–stimulated leukocytes, and imbalanced cytokine profiles, with higher levels of the archetypal antiinflammatory cytokine IL-10 and lower levels of proinflammatory mediators such as TNF-α (tumor necrosis factor α) and IL-6 (6, 8). This results in ineffective clearance of infectious foci and increased susceptibility toward secondary infections, often with opportunistic pathogens, and accounts for high mortality and morbidity (7, 9). The importance of sepsis-induced immunoparalysis is further illustrated by recent reports showing that patients with sepsis can be classified to different endotypes based on their leukocyte transcriptome, with immunosuppressed endotypes consistently showing the worst clinical outcome (10, 11). Therefore, many current efforts are focused on reconstituting immunocompetence with immunostimulatory treatments (6, 8). However, supportive treatments, which are currently indiscriminately applied, could significantly contribute to the dysregulation of the host response and may require reevaluation in light of the emerging concept of immunoparalysis.

Norepinephrine is the current mainstay therapy for hemodynamic stabilization and is administered to practically all patients with septic shock and other severe systemic inflammatory conditions worldwide (1). *In vitro* evidence points toward the overriding antiinflammatory effects of norepinephrine (12–14). However, animal *in vivo* data on the putative immunomodulatory effects of norepinephrine are very scarce, and human *in vivo* data are nonexistent.

We previously hypothesized that norepinephrine may contribute to the dysregulation of the host response and sepsis-induced immunoparalysis, whereas other vasopressors, such as vasopressin, may not (15). In the present work, we first evaluated the immunological effects of norepinephrine and vasopressin *in vitro*, using primary human leukocytes. Subsequent experiments in murine models of inflammation and sepsis were performed to assess the functional relevance of these findings. Furthermore, we made the translation to the human setting, using an *in vivo* model of systemic inflammation (experimental human endotoxemia) and observations in patients with sepsis.

Methods

Detailed study procedures and analysis methods are described in the online supplement.

In Vitro Studies

After approval from the local ethics committee of the Radboud University Medical Center (registration no. 2010/10), blood was obtained from healthy adult volunteers ($n = 6$–9 donors per experiment) who provided written informed consent. Whole blood and isolated monocytes were used in various experiments to assess the immunological and metabolic effects of norepinephrine and vasopressin.

Murine Studies

All procedures were approved by the Dutch Council for Animal Care (AVD103002016447). A total of 190 C57Bl/6J male mice (Charles Rivers) aged 6–9 weeks were used for all experiments. For the endotoxemia experiments ($n = 29$–41 per experiment), microosmotic pumps with connected jugular vein catheters were filled with norepinephrine, vasopressin, or phosphate-buffered saline (PBS) and implanted 3 or 24 hours before LPS challenge, and animals were killed 90 minutes after LPS. For the sepsis experiments, cecal ligation and puncture (CLP) (60% ligation, 21-gauge needle, $n = 30$) or a sham operation ($n = 3$) was performed, followed by placement of microosmotic pumps with connected jugular vein catheters, filled with norepinephrine, vasopressin, or PBS, and animals were killed 48 hours later.

Human Studies

Thirty healthy male volunteers provided written informed consent to participate in a randomized, double-blind, placebo-controlled study performed in accordance with the declaration of Helsinki. The study was approved by the local ethics committee (CMO 2015–2079) and was registered at Clinicaltrials.gov (identifier NCT02675868). Under continuous monitoring of vital parameters, subjects ($n = 10$ per group) received a 5-hour infusion of either low-dose norepinephrine (0.05 μg/kg/min), vasopressin (0.04 IU/min), or placebo (NaCl 0.9%) starting 1 hour before intravenous LPS challenge (2 ng/kg). Patient data were collected from a prospective observational cohort study performed between April 2012 and January 2017, which included 195 adult patients with newly developed septic shock who were admitted to the ICU. The study was performed in accordance with the applicable rules concerning the review of research ethics committees and informed consent in the Netherlands. All patients or legal representatives were informed about the study details and could refuse to participate. Medication use was retrieved from the electronic patient data management system. Patients assigned to the norepinephrine + β-blocker group were chronic users who received a β-blocker on the day of study inclusion and before blood withdrawal, which was performed within 24 hours after inclusion.

Results

Norepinephrine Exerts Antiinflammatory Effects in Primary Human Leukocytes

In the initial experiments, whole blood was stimulated with the TLR4 (Toll-like receptor 4) ligand LPS to elicit an inflammatory response in the presence or absence of increasing concentrations of norepinephrine and vasopressin. Norepinephrine dose-dependently attenuated the production of the proinflammatory mediators TNF-α ($P < 0.0001$), IP-10 (IFN-γ–induced protein 10) ($P < 0.0001$), and IL-1β ($P = 0.01$), whereas release of the antiinflammatory cytokine IL-10 was enhanced ($P < 0.0001$, Figure 1A). Vasopressin did not influence cytokine production ($P = 0.58$, $P = 0.98$, $P = 0.84$, and $P = 0.87$ for TNF-α, IP-10, IL-1β, and IL-10, respectively; Figure 1A). No effects of norepinephrine on cell survival were observed (Figure E1 in the online supplement). To assess whether norepinephrine's effects are confined to TLR4 stimulation, whole blood was stimulated with other TLR ligands, namely Pam3Cys (TLR1/2), Poly I:C (TLR3), flagellin (TLR5), R848 (TLR7/8), and CpG (TLR9), as well as heat-killed pathogens *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, *Pseudomonas aeruginosa*, and *Aspergillus fumigatus* in the presence or absence of norepinephrine. In general, norepinephrine caused a dose-dependent shift toward an antiinflammatory phenotype, which was exemplified by attenuated production of proinflammatory cytokines IP-10 and TNF-α, and enhanced IL-10 responses (Figure 1B). LPS stimulation experiments using isolated primary human monocytes yielded identical effects for norepinephrine and vasopressin on TNF-α and IL-10 production as those observed in the whole-blood stimulation experiments (Figure E2). We subsequently assessed the effects of both vasopressors on the production of reactive oxygen species (ROS) by monocytes. As depicted in Figures 2A and 2B, norepinephrine inhibited ROS production by phorbol myristol acetate–stimulated monocytes ($P = 0.004$), whereas vasopressin exerted no such effect ($P = 0.10$) (Figures 2C and 2D).

Antiinflammatory Effects of Norepinephrine Involve β_2-Adrenergic Stimulation and Protein Kinase A

Because norepinephrine has both α- and β-adrenergic receptor (AR) affinity, we set out to determine which receptor is involved in the observed immunomodulatory effects. Preincubation with the nonselective β-AR antagonist propranolol nullified the effects of norepinephrine on both LPS-induced TNF-α ($P = 0.001$) and IL-10 ($P = 0.006$) production in whole-blood cultures (Figure E3A). The selective α_1-AR antagonist prasozin and the α_2-AR antagonist yohimbine exerted no effects on the norepinephrine-mediated attenuated TNF-α release ($P = 0.12$ and $P = 0.13$, respectively), although yohimbine reversed the IL-10-enhancing effects of norepinephrine ($P = 0.01$; Figure E3A). These findings indicate the predominant involvement of β-ARs, and we therefore examined the effects of selective β_1-AR (atenolol) and β_2-AR (ICI-118,551) antagonists in a follow-up whole-blood LPS stimulation experiment. ICI-118,551 reversed the effects of norepinephrine on TNF-α ($P = 0.04$) but on not IL-10 production ($P = 0.22$), whereas atenolol did not modulate LPS-induced cytokine responses ($P = 0.75$ and $P = 0.31$ for TNF-α and IL-10, respectively; Figure E3B). In isolated primary human monocytes, ICI-118,551 nullified norepinephrine's effects on both TNF-α ($P = 0.003$) and IL-10 ($P = 0.005$) (Figure E3C), supporting an important role for β_2-ARs. β-ARs are G protein–coupled receptors and signal intracellularly via PKA (protein kinase A) (14). As illustrated in Figure E3D, preincubation with PKA inhibitor H89 dose-dependently reversed the effects of norepinephrine on both TNF-α ($P = 0.02$, $P = 0.06$, and $P = 0.007$ for 300 nM, 1 μM, and 3 μM H89, respectively) and IL-10 production ($P = 0.04$, $P = 0.007$, and $P = 0.001$) in LPS-stimulated monocytes.

Norepinephrine Suppresses Immunometabolism

Because metabolic defects were recently shown to play a crucial role in impaired cytokine responses in general and in sepsis-induced immunoparalysis in particular (16), this could represent a mechanism through which norepinephrine exerts its immunosuppressive effects. Therefore, we evaluated the effects of norepinephrine on energy metabolism in isolated monocytes. Norepinephrine attenuated the LPS-induced increase in lactate production ($P = 0.003$; Figure 3A), indicating inhibition of the glycolytic shift, and this effect was mitigated by pretreatment with either ICI-118,551 ($P = 0.06$) or H89 ($P = 0.03$; Figures 3B and 3C). Next, the extracellular acidification rate (reflecting glycolytic capacity) and $\dot{V}O_2$ rate (reflecting oxidative phosphorylation capacity) of monocytes were assessed in a live-cell metabolic assay. As depicted in Figures 3D–3G, norepinephrine attenuated both $\dot{V}O_2$ rate (expressed by spare respiratory capacity; $P = 0.002$) and extracellular acidification rate ($P = 0.004$) in these experiments.

Norepinephrine Infusion Exerts Antiinflammatory Effects in Mice

To investigate whether the immunosuppressive effects of norepinephrine also apply *in vivo*, we

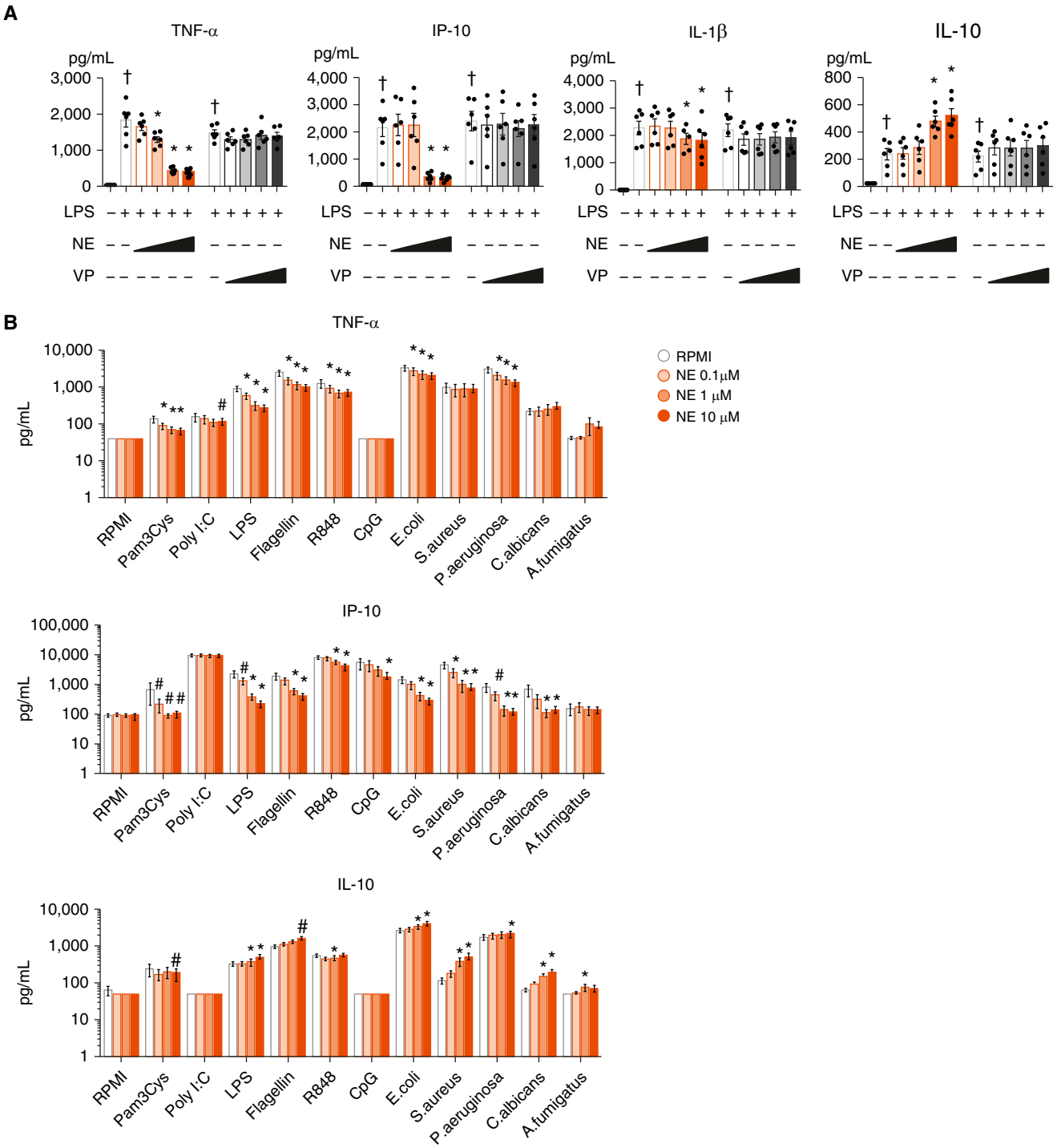

Figure 1. Norepinephrine (NE) modulates cytokine production by human whole blood. (*A*) Concentrations of TNF-α (tumor necrosis factor α), IP-10 (IFN-γ–induced protein 10), IL-1β, and IL-10 in supernatants of human whole-blood cultures preincubated with RPMI (medium control), NE (0.01, 0.1, 1, and 10 μM), or vasopressin (VP) (0.01, 0.1, 1, and 10 μM) for 1 hour and subsequently stimulated with LPS (10 ng/ml) or RPMI for 24 hours. (*B*) Concentrations of TNF-α, IP-10, and IL-10 in supernatants of human whole-blood cultures that were preincubated with either RPMI or NE (0.1, 1, and 10 μM) for 1 hour and subsequently stimulated with several TLR (Toll-like receptor) ligands and heat-killed pathogens (Pam3Cys [TLR1/2 agonist], 10 μg/ml; Poly I:C [TLR3 agonist], 50 μg/ml; LPS [TLR4 agonist], 10 ng/ml; flagellin [TLR5 agonist], 10 μg/ml; R848 [TLR7/8 agonist], 1 μM; CpG [TLR9 agonist], 10 μg/ml; *Escherichia coli*, 1×10^7/ml; *Staphylococcus aureus*, 1×10^7/ml; *Pseudomonas aeruginosa*, 1×10^7/ml; *Candida albicans*, 1×10^6/ml; and *Aspergillus fumigatus*, 1×10^7/ml) or RPMI for 24 hours. Data are expressed as individual data points and/or mean ± SEM of six individual donors. $^{\dagger}P < 0.05$ compared with RPMI and $^{*}P < 0.05$ and $^{\#}P = 0.05$–0.10 compared with LPS or other stimuli (calculated using one-way ANOVA with Dunnett's *post hoc* tests). *A. fumigatus* = *Aspergillus fumigatus*; *C. albicans* = *Candida albicans; E. coli* = *Escherichia coli*; *P. aeruginosa* = *Pseudomonas aeruginosa*; *S. aureus* = *Staphylococcus aureus*.

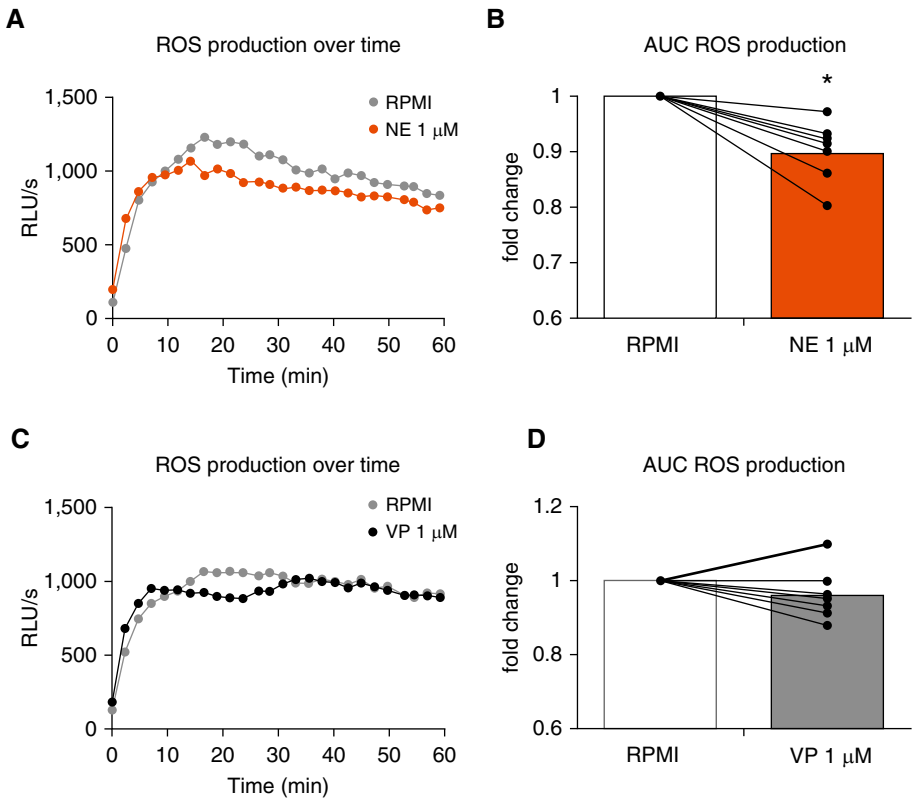

Figure 2. Norepinephrine (NE) attenuates phorbol myristol acetate–induced reactive oxygen species (ROS) production by primary human monocytes. (*A–D*) Primary human monocytes were incubated with RPMI (medium control) NE (1 μM) or vasopressin (VP) (1 μM) for 1 hour and subsequently stimulated with phorbol myristol acetate (50 ng/ml) in the presence of luminol, and luminescence was measured for 1 hour. Modulation of ROS production by NE expressed as (*A*) relative light units (RLUs) per second and (*B*) area under the curve (AUC) depicted under *A*. Modulation of ROS production by VP expressed as (*C*) RLUs per second and (*D*) AUC depicted under *C*. (*A–D*) Data are expressed as means (*A* and *C*), and individual data points and mean fold change compared with RPMI-incubated cells (*B* and *D*) of six individual donors. *$P < 0.05$ compared with RPMI calculated using a *t* test.

investigated cytokine levels in endotoxemic mice that received continuous intravenous norepinephrine treatment delivered by microosmotic pumps. First, mice were infused with norepinephrine or PBS for 3 hours followed by an intravenous injection with LPS or saline. Continuous delivery by the microosmotic pumps was verified by dose-dependent increases in plasma norepinephrine concentrations (Figure E4A). Furthermore, norepinephrine infusion dose-dependently attenuated LPS-induced TNF-α ($P = 0.33$ and $P = 0.04$ for 1 and 5 μg/kg/min, respectively), IP-10 ($P = 0.34$ and $P = 0.0004$), MIP-1α (macrophage inflammatory protein 1α) ($P = 0.12$ and $P = 0.01$) and MCP-1 (macrophage chemoattractant protein 1) ($P = 0.33$ and $P = 0.001$) responses, whereas plasma concentrations of IL-10 were enhanced ($P = 0.34$ and $P = 0.0004$; Figure 4A), indicating broad antiinflammatory effects. A similar experiment using 24-hour instead of 3-hour infusion yielded similar immunosuppressive effects of norepinephrine infusion (Figures 4B, E4B, and E5A). Furthermore, norepinephrine infusion dose-dependently attenuated basal neutrophilic ROS production in LPS-challenged mice ($P = 0.38$, $P = 0.03$, and $P = 0.02$ for 1, 3, and 5 μg/kg/min, respectively) and suppressed the maximal neutrophilic respiratory burst in non–LPS-challenged mice ($P = 0.06$, $P = 0.006$, and $P = 0.02$; Figure 4C). Finally, we compared the effects of 3-hour infusion of norepinephrine and vasopressin (Figure 5). As expected, increased plasma norepinephrine concentrations were only observed in norepinephrine-infused animals (Figure E4C), and no effects of either vasopressor were found in non–LPS-challenged mice (Figure E5B). Norepinephrine infusion again significantly suppressed LPS-induced proinflammatory cytokine responses ($P = 0.03$ for TNF-α, IP-10, MIP-1α, and MCP-1) while enhancing IL-10 concentrations ($P = 0.03$). Vasopressin infusion exerted no immunomodulatory effects (all P values > 0.30), Figure 5). Furthermore, pulmonary myeloperoxidase levels, reflecting neutrophil influx, were attenuated in norepinephrine-infused ($P = 0.02$) but not vasopressin-infused ($P = 0.65$) mice challenged with LPS (Figure E6).

Norepinephrine Infusion Increases Bacterial Dissemination in Experimental Sepsis

To investigate if norepinephrine functionally impairs host defense, mice were implanted with microosmotic pumps containing norepinephrine, vasopressin, or PBS, followed by induction of experimental sepsis using CLP. Sham-operated mice were used as a negative control. CLP led to profound bacterial dissemination, as evidenced by increased colony-forming unit (cfu) counts in the spleen ($P = 0.04$), liver ($P = 0.04$), and blood ($P = 0.046$) of PBS-infused CLP mice compared with sham-operated animals (Figure 6). Compared with PBS-infused CLP mice, cfu counts were significantly higher in the spleen ($P = 0.04$) and liver ($P = 0.03$) of norepinephrine-infused, but not vasopressin-infused ($P = 0.37$ and $P = 0.75$), CLP mice (Figure 6). Furthermore, a trend toward higher blood cfus was observed in norepinephrine-infused versus PBS-infused CLP mice ($P = 0.06$), whereas no difference was observed in vasopressin-infused CLP mice ($P = 0.95$, Figure 6). Plasma cytokine concentrations, determined 4 hours after the induction of CLP, did not reveal statistically significant differences between groups (Figure E7; TNF-α concentrations were below the detection limit [3.2 pg/ml] in all animals). Nevertheless, several proinflammatory/antiinflammatory cytokine ratios, providing an indication of the proinflammatory/antiinflammatory balance, were lower in norepinephrine-infused CLP mice compared with PBS-infused CLP mice ($P = 0.04$ for keratinocyte chemoattractant/IL-10, $P = 0.06$ for IP-10/IL-10, and $P = 0.03$ for MIP-1α/IL-10; Figure E8).

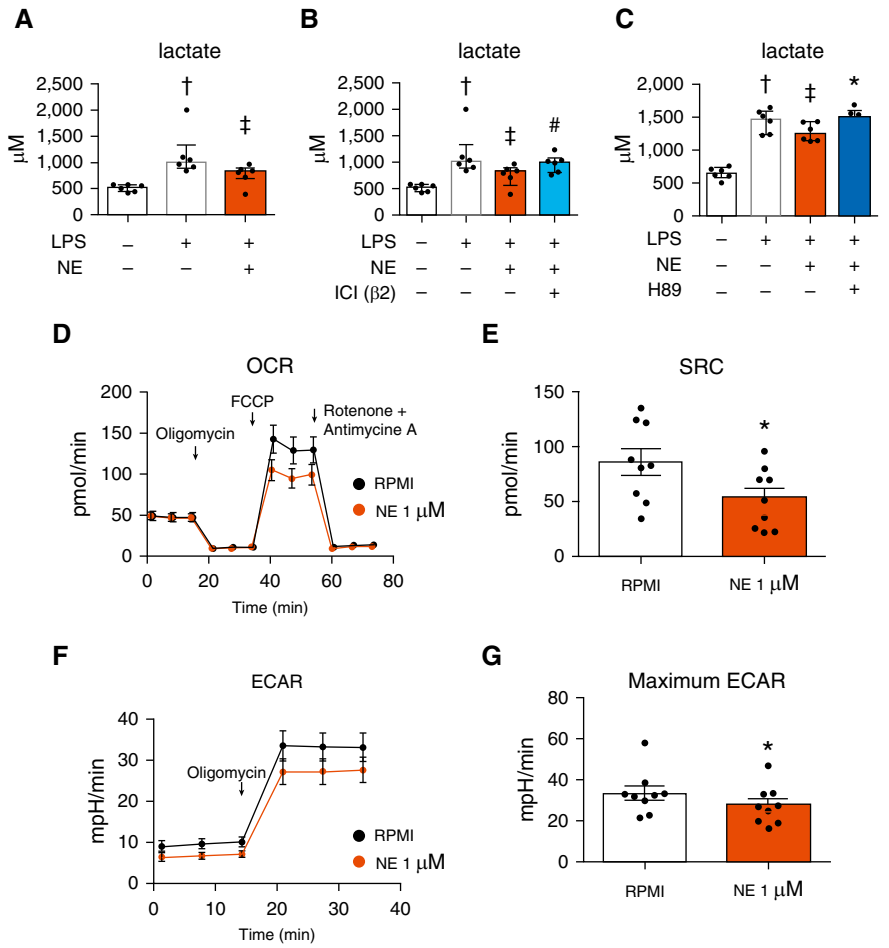

Figure 3. Norepinephrine (NE) suppresses immunometabolism in primary human monocytes. (*A–C*) Lactate concentrations in supernatants of primary human monocyte cultures that were preincubated with either RPMI or the β_2 antagonist ICI-118,551 (0.1 μM) or the PKA inhibitor H89 (3 μM) for 30 minutes, followed by incubation with NE (1 μM) for 1 hour and subsequent stimulation with LPS (10 ng/ml) or RPMI for 24 hours. (*D–G*) $\dot{V}O_2$ rate (OCR) (*D*) and spare respiratory capacity (calculated as the difference between basal and maximal OCR, *E*), extracellular acidification rate (*F* and *G*) of primary human monocytes in the absence (RPMI) or presence of NE (1 μM). *D* and *F* depict real-time changes in OCR and extracellular acidification rate during the Mito-stress test, consisting of sequential treatment with oligomycin (ATPase inhibitor) or for *D* only, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (proton uncoupler), and rotenone together with antimycin A (electron transport chain complex I and III inhibitors). (*A–G*) Data are expressed as median and interquartile range (*A–C*) or mean ± SEM (*D–G*) of six to nine individual donors. $^{\dagger}P < 0.05$ compared with RPMI, $^{\ddagger}P < 0.05$ compared with LPS, and $^{*}P < 0.05$ and $^{\#}P = 0.05$–0.10 compared with NE + LPS calculated using (*A–C*) Wilcoxon matched pairs tests or (*E* and *G*) *t* tests. ECAR = extracellular acidification rate; FCCP = carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; ICI = ICI-118,551; SRC = spare respiratory capacity.

Norepinephrine Exerts Antiinflammatory Effects in LPS-challenged Healthy Volunteers and in Patients with Septic Shock

To translate our findings to the human *in vivo* setting, we first performed an experimental endotoxemia study in which healthy subjects were randomized to a 5-hour intravenous infusion of either low-dose norepinephrine (0.05 μg/kg/min), vasopressin (0.04 IU/min), or placebo (saline) starting 1 hour before intravenous administration of 2 ng/kg LPS. Baseline characteristics of the three groups were comparable (Table E1). Norepinephrine, but not vasopressin, treatment resulted in a swift increase in mean arterial pressure (+13 ± 1 mm Hg; Figure E9A) and a reduction in heart rate (−5 ± 2 beats/min; Figure E9B). None of the vasopressors influenced the LPS-induced increase in body temperature ($P = 0.64$ and $P = 0.74$ for norepinephrine and vasopressin, respectively; Figure E9C). Plasma norepinephrine concentrations increased to a maximum of 10.2 ± 0.4 nmol/L in the norepinephrine group (Figure E9D). Circulating numbers of monocytes, neutrophils, or lymphocytes were not affected by norepinephrine ($P = 0.27$, $P = 0.21$, and $P = 0.45$, respectively) or vasopressin ($P = 0.78$, $P = 0.08$, and $P = 0.22$, respectively) treatment (Figure 7A). Plasma concentrations of TNF-α, IL-6, IL-8, IP-10, MCP-1, G-CSF (granulocyte colony–stimulating factor), and IL-10 increased profoundly in all subjects after LPS administration (Figure 7B). Low-dose norepinephrine infusion resulted in a significantly enhanced IL-10 response ($P = 0.007$) compared with that of the placebo group. Furthermore, LPS-induced plasma concentrations of IP-10 were attenuated by norepinephrine ($P = 0.04$; Figure 7B), whereas the other cytokines were not influenced. Vasopressin treatment did not modulate levels of any of the measured cytokines (all P values > 0.76; Figure 7B).

Finally, we explored the immunomodulatory effects of norepinephrine in a cohort of 195 patients admitted to the ICU with septic shock who were all treated with norepinephrine and 66 of whom chronically used β-blockers. Blood for TNF-α and IL-10 analysis was obtained at study inclusion, which took place within 24 hours after the start of norepinephrine infusion (median [interquartile range] of 8.8 [4.2–14.2] and 7.6 [4.4–15.9] hours in the norepinephrine-only and norepinephrine + β-blocker groups, respectively; $P = 0.91$). In the 129 patients not treated with β-blockers, norepinephrine infusion rates at inclusion correlated with lower TNF-α/IL-10 ratios ($r = -0.38$; $P < 0.0001$; Figure 8A), illustrating the immunosuppressive effects of norepinephrine. We hypothesized that concomitant use of β-blockers would mitigate norepinephrine's antiinflammatory effects, which we previously showed to be primarily mediated via the β_2-AR. Although most of the clinically used β-blockers, such as metoprolol and atenolol, are regarded as β1-selective, they actually have significant β_2-affinity (17). Patients in the norepinephrine + β-blocker group

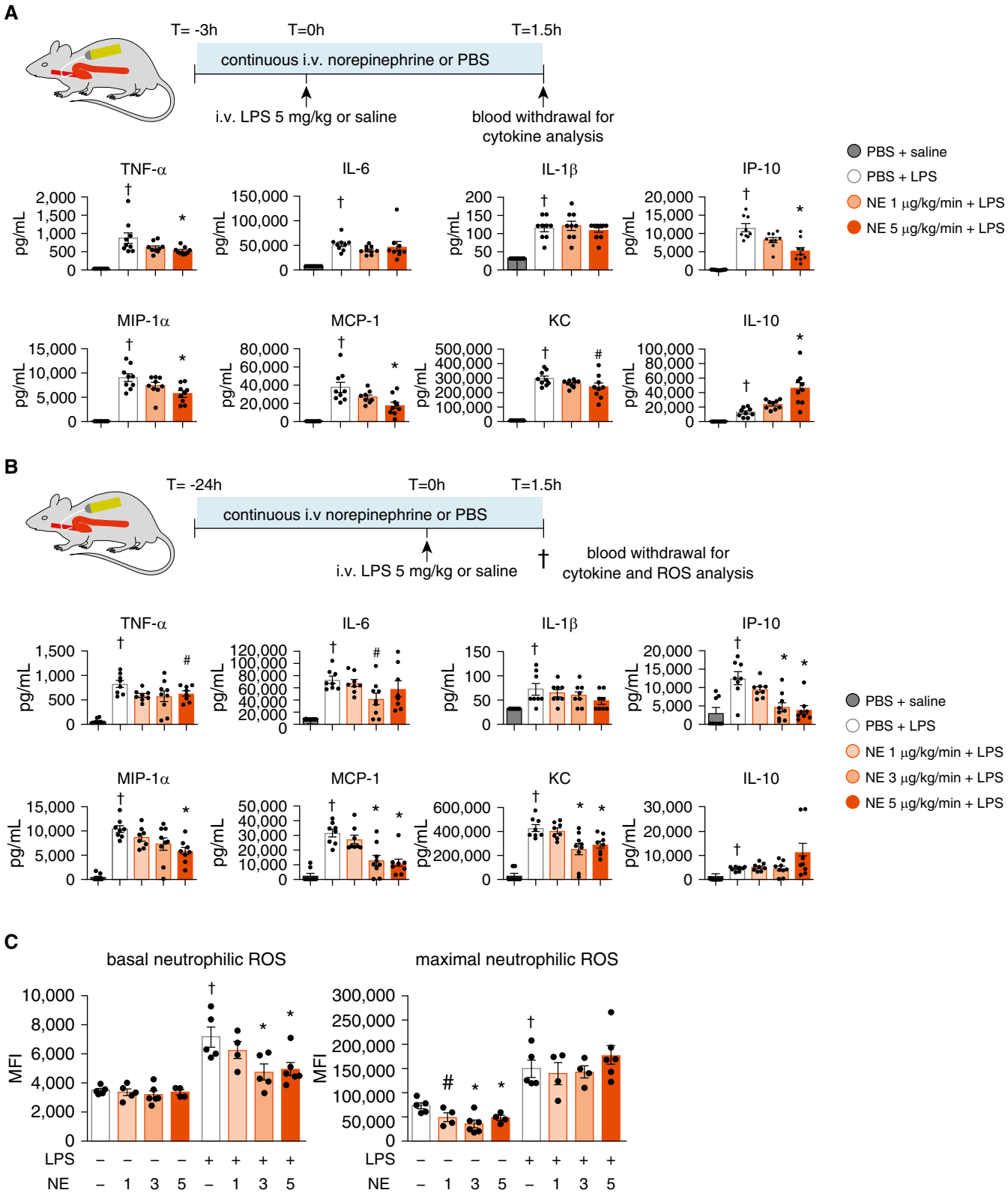

Figure 4. Norepinephrine (NE) infusion modulates *in vivo* cytokine responses and *ex vivo* reactive oxygen species (ROS) production in mice. (*A* and *B*) Plasma concentrations of TNF-α (tumor necrosis factor α), IL-6, IL-β, IP-10 (IFN-γ–induced protein 10), MIP-1α (macrophage inflammatory protein 1α), MCP-1 (macrophage chemoattractant protein 1), KC (keratinocyte chemoattractant), and IL-10 in mice intravenously infused with NE (1, 3 or 5 μg/kg/min) or phosphate-buffered saline (PBS) via microosmotic pumps connected to a jugular vein catheter for 4.5 (*A*) or 25.5 (*B*) hours and challenged intravenously

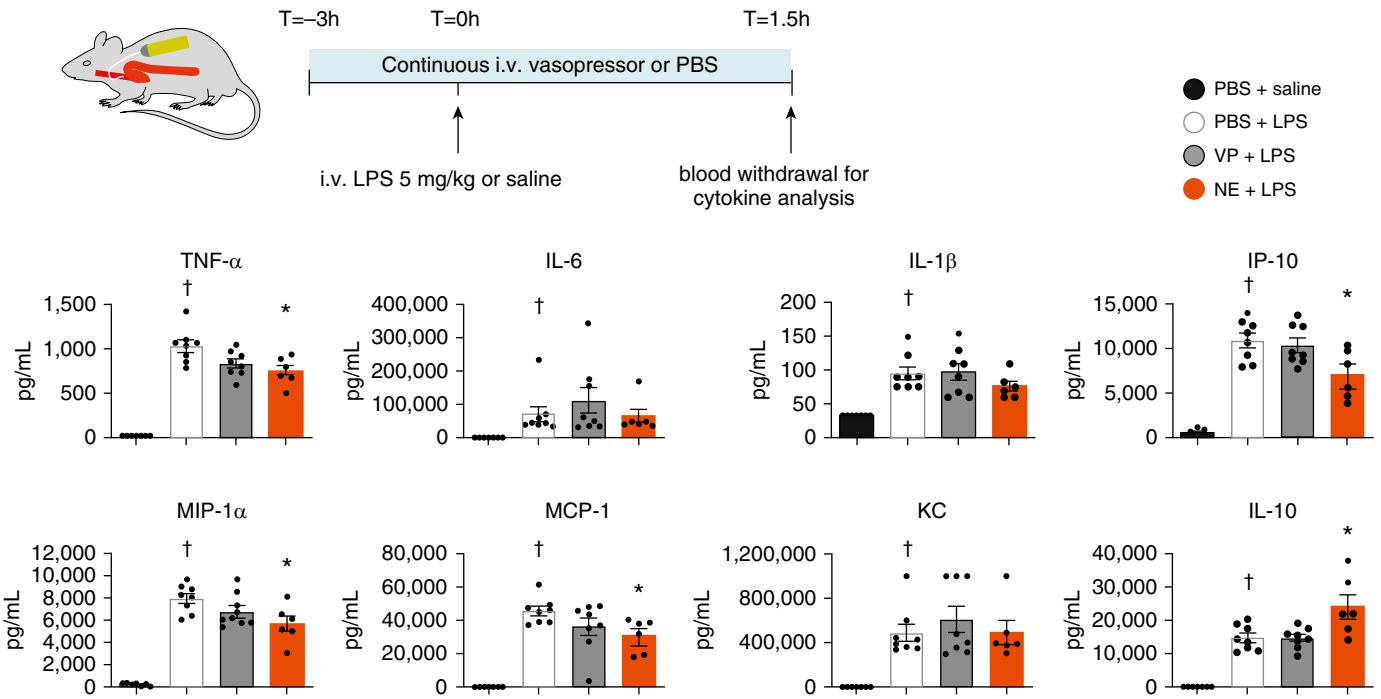

Figure 5. Vasopressin (VP) infusion does not exert immunomodulatory effects in mice. Plasma concentrations of TNF-α (tumor necrosis factor α), IL-6, IL-β, IP-10 (IFN-γ–induced protein 10), MIP-1α (macrophage inflammatory protein 1α), MCP-1 (macrophage chemoattractant protein 1), KC (keratinocyte chemoattractant), and IL-10 in mice intravenously infused with norepinephrine (NE) (5 μg/kg/min), VP (0.00057 IU/kg/min, equivalent to 0.04 IU/min in a 70 kg human), or phosphate-buffered saline (PBS) via microosmotic pumps connected to a jugular vein catheter for 4.5 hours and challenged intravenously with LPS (5 mg/kg) or saline 3 hours after start of infusion. Data are expressed as mean ± SEM of six to eight animals per group. $^{\dagger}P < 0.05$ compared with PBS + saline and $^{*}P < 0.05$ compared with PBS + LPS calculated using *t* tests followed by false discovery rate correction (Benjamini-Hochberg). i.v. = intravenous; T = time.

used metoprolol ($n = 57$), atenolol ($n = 3$), bisoprolol ($n = 3$), labetalol ($n = 1$), sotalol ($n = 1$), and propranolol ($n = 1$) and had received a dose on the day of study inclusion before blood was obtained for cytokine analysis. There were no differences in sex, age, disease severity (Acute Physiology and Chronic Health Evaluation II score), use of corticosteroids, requirement of mechanical ventilation, or norepinephrine infusion rate between patients with or without concomitant β-blocker use (Table E2). The plasma TNF-α/IL-10 ratio, reflecting the proinflammatory/antiinflammatory balance, was significantly higher in patients with β-blockers (median [interquartile range], 0.74 [0.24–1.97]) than in those without them (0.51 [0.16–1.18]; $P = 0.03$; Figure 8B).

Discussion

We demonstrate that norepinephrine enhances antiinflammatory cytokine production and attenuates proinflammatory cytokine release and ROS generation by human leukocytes in response to stimulation with a wide variety of inflammatory ligands. The underlying mechanism involves the activation of the β_2-AR, and immunometabolism is suppressed as well. In endotoxemic mice, continuous norepinephrine infusion caused a dose-dependent shift toward an antiinflammatory cytokine response pattern and compromised neutrophilic ROS production. Furthermore, norepinephrine infusion resulted in increased bacterial dissemination during experimental sepsis. In healthy volunteers, norepinephrine infused at a very low dosage exhibited antiinflammatory effects during experimental endotoxemia. In sharp contrast, vasopressin exerted no immunomodulatory effects across all our experimental setups. Finally, higher norepinephrine infusion rates were related to a more pronounced antiinflammatory cytokine balance in patients with septic shock, whereas the use of β-blockers was associated with a more proinflammatory cytokine balance.

Norepinephrine's immunomodulatory effects were counteracted by the nonselective β-blocker propranolol and the selective β_2-blocker ICI-118,551. These findings are in line with earlier reports showing that β-blockers reverse (nor)epinephrine-induced inhibition of proinflammatory cytokine release after LPS stimulation (13, 18, 19) and with previous work demonstrating that selective β_2-agonists attenuate proinflammatory cytokine

Figure 4. (*Continued*). with LPS (5 mg/kg) or saline at 3 (*A*) or 24 (*B*) hours after start of infusion. (*C*) ROS content and phorbol myristol acetate–induced maximal respiratory burst of neutrophils obtained at the end of the experiment depicted in *B*. Data are expressed as mean ± SEM of seven to nine (cytokines) or four to six (ROS) animals per group. $^{\dagger}P < 0.05$ compared with PBS + saline and $^{*}P < 0.05$ and $^{\#}P = 0.05$–0.10 compared with PBS + LPS calculated using *t* tests followed by false discovery rate correction (Benjamini-Hochberg). i.v. = intravenous; MFI = mean fluorescence intensity; T = time.

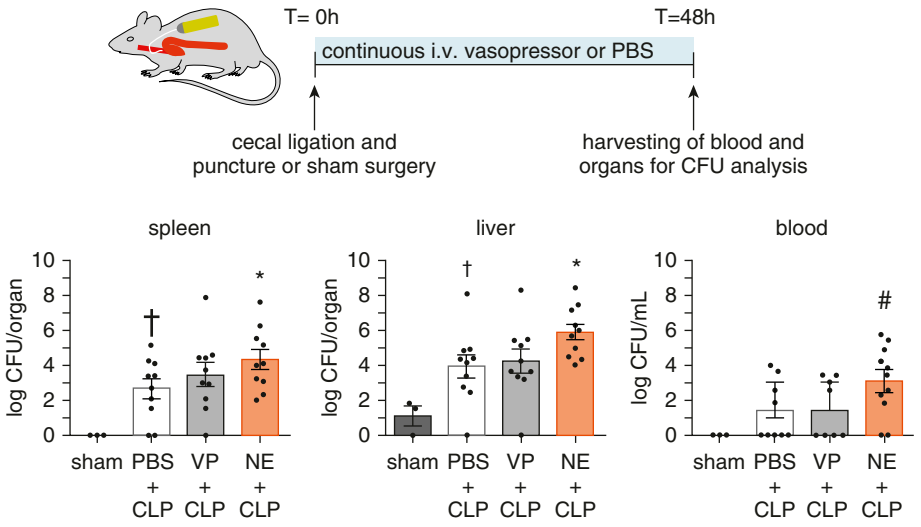

Figure 6. Norepinephrine (NE) infusion increases bacterial dissemination in experimental sepsis. Bacterial load (expressed as colony-forming units [cfu]) in the spleen, liver, and blood of mice intravenously infused with NE (3 μg/kg/min), vasopressin (VP) (0.00057 IU/kg/min [equivalent to 0.04 IU/min in a 70 kg human]), or phosphate-buffered saline (PBS) for 2 days via microosmotic pumps connected to a jugular vein catheter and subjected to cecal ligation and puncture (CLP) to induce sepsis or sham surgery. Data are expressed as mean ± SEM of three (sham group) or 10 (other groups) animals per group (it was not possible to obtain sufficient amounts of blood for cfu counts from three animals because of severity of illness resulting in inadequate blood flow.) $^{\dagger}P < 0.05$ compared with sham surgery and $^{*}P < 0.05$ and $^{\#}P = 0.05$–0.10 compared with PBS + CLP calculated using *t* tests. i.v. = intravenous; T = time.

production (20). In this respect, it is also noteworthy that dobutamine, which primarily has β1-affinity, did not influence the innate immune response during human endotoxemia (21), whereas epinephrine infusion enhanced the IL-10 response and attenuated TNF-α concentrations in the same model (18). Our data further reveal that the effects of norepinephrine are nullified by the PKA inhibitor H89, supporting the notion that β_2-receptor–mediated immunologic actions are cyclic adenosine monophosphate dependent (22, 23).

Disturbances in the energy metabolism have been associated with worse outcomes in patients with sepsis (24), and recently, defective immune cell metabolism was identified as a mechanism underlying sepsis-induced immunoparalysis (16). More specifically, suppressed glycolysis and oxidative phosphorylation in the leukocytes of patients with sepsis was associated with impaired proinflammatory cytokine production (16). Regarding the specific roles of glycolysis and oxidative phosphorylation, previous work showed that the inhibition of glycolysis with 2-deoxyglucose decreased the production of both proinflammatory and antiinflammatory mediators in LPS-stimulated primary human monocytes (25). Data on inhibition of oxidative phosphorylation are ambiguous; although LPS-induced production of proinflammatory cytokines was shown to be attenuated by the oxidative phosphorylation inhibitor rotenone in both human monocytes and murine bone marrow–derived macrophages (25, 26), LPS-induced IL-10 release was enhanced in murine bone marrow–derived macrophages (26) but decreased in human monocytes (25). We show that norepinephrine suppresses both glycolysis and oxidative phosphorylation in human monocytes, which potentially contributes to its modulatory effects on cytokine production. Norepinephrine also impaired ROS production by monocytes and neutrophils. The generation of ROS is an essential mechanism for bacteria killing, for instance, exemplified by the fact that genetic disorders characterized by defective ROS production are associated with greatly increased susceptibility toward bacterial and fungal infections (27, 28). Furthermore, increased ROS generation by macrophages was shown to confer resistance toward *Listeria monocytogenes* infection (29).

A distinctive feature of the present work is the use of microosmotic pumps connected to a jugular vein catheter to allow for the continuous intravenous administration of vasopressors in conscious mice. This setup was chosen to mimic the human situation as closely as possible and to minimize possible confounding effects of sedatives (30). For vasopressin, we used a clinically relevant dosage that has been used in various septic shock trials (31–33). It could be argued that the norepinephrine dosages used are relatively high compared with those used in humans. Nevertheless, previous murine experiments employed similar dosages, which were infused subcutaneously for 14 days, suggesting that mice are less sensitive to norepinephrine (34). Moreover, the plasma norepinephrine levels attained in our murine experiments were comparable to those in patients with septic shock and trauma treated with norepinephrine, in whom median concentrations of approximately 100 nmol/L were found (range, 6–1,000 nmol/L) (35). Norepinephrine infusion resulted in increased bacterial dissemination in the liver, spleen, and blood in mice subjected to CLP, indicating functional impairment of host defense. No statistically significant effects of norepinephrine infusion on cytokine concentrations were observed in CLP mice, which may be related to the early sampling time point at which cytokine concentrations were still relatively low. Because of the limited amount of blood that can be obtained from mice, we did not assess cytokine profiles at other time points, which represents a limitation of our work. Nevertheless, in line with the immunomodulatory effects observed in our other experiments and patient data, norepinephrine infusion resulted in significantly lower proinflammatory/antiinflammatory cytokine ratios in CLP mice.

To assess the immunomodulatory effects of norepinephrine and vasopressin in humans, healthy volunteers were challenged with LPS. Similar to our *in vitro* and murine endotoxemia results, norepinephrine infusion resulted in an enhanced IL-10 response, whereas concentrations of the proinflammatory cytokine IP-10 were attenuated. The fact that significant effects were not attained for other proinflammatory cytokines may be explained by the low dose of norepinephrine used (for safety reasons)

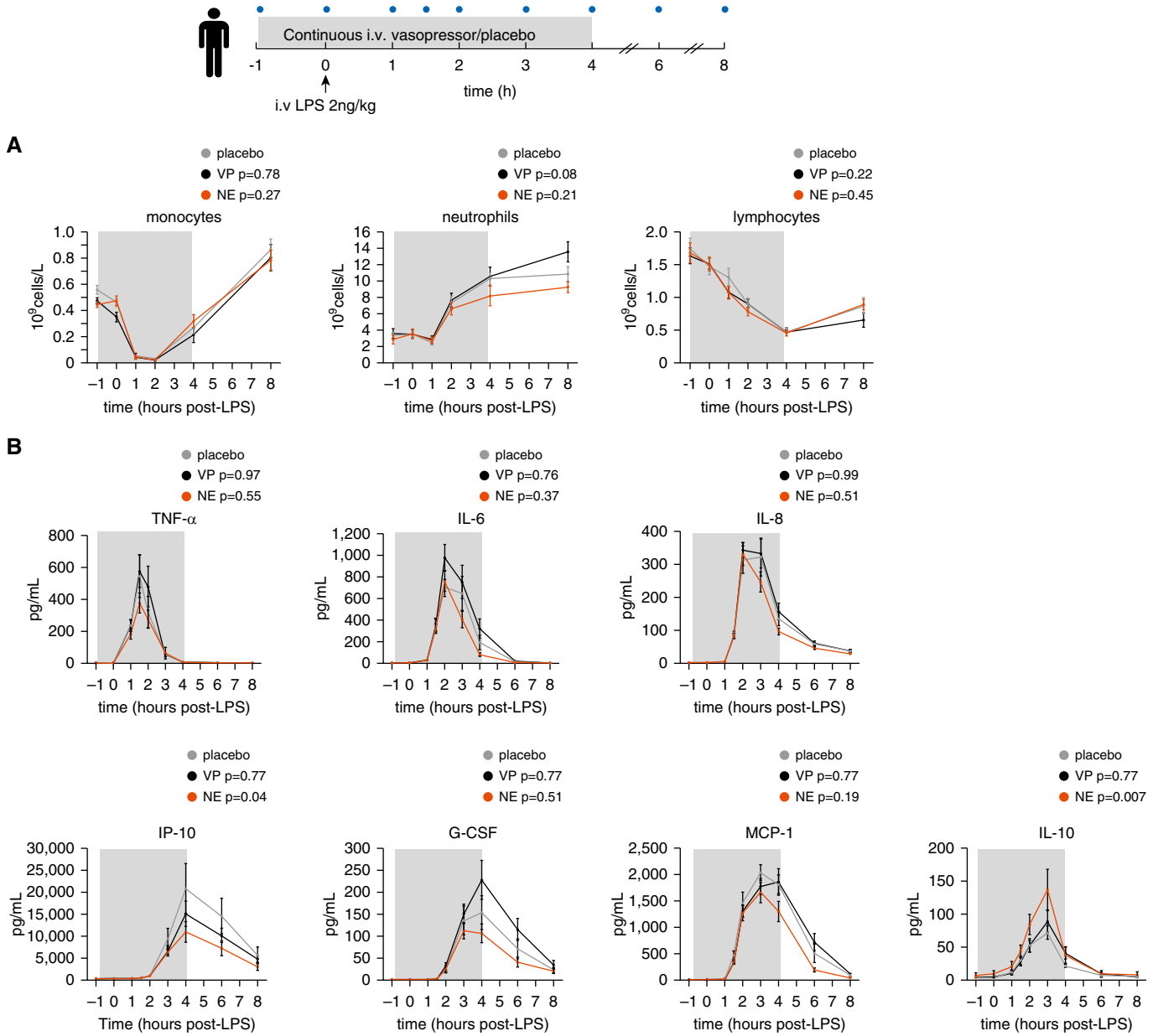

Figure 7. Low-dose norepinephrine (NE) infusion enhances the IL-10 response and attenuates IP-10 (IFN-γ–induced protein 10) concentrations during experimental human endotoxemia. (*A*) Circulating monocyte, neutrophil, and lymphocyte numbers, (*B*) plasma concentrations of TNF-α (tumor necrosis factor α), IL-6, IL-8, IP-10, G-CSF (granulocyte colony–stimulating factor), MCP-1 (macrophage chemoattractant protein 1), and IL-10 in healthy volunteers randomized to 5 hours of intravenous infusion with placebo (saline), low-dose NE (0.05 μg/kg/min), or vasopressin (VP) (0.04 IU/min) and challenged intravenously with 2 ng/kg LPS 1 hour after start of infusion. Data are expressed as mean ± SEM of 10 subjects per group. The gray area represents the period during which NE, VP, or saline was infused. The blue dots in the study design indicate blood withdrawal time points for cytokine and leukocyte count analysis. *P* values were calculated using repeated measures two-way ANOVA (time × treatment interaction term) versus the placebo group followed by false discovery rate correction (Benjamini-Hochberg). i.v. = intravenous.

and the subsequently low plasma concentrations reached (∼10 nmol/L). Because our data illustrate that the immunosuppressive effects of norepinephrine are clearly dose dependent, stronger antiinflammatory effects are expected in patients with sepsis, who exhibit much higher norepinephrine concentrations (35). Of note, we used a clinically relevant dose of vasopressin (which was also similar to that used in our murine experiments), because this compound does not exert vasopressor effects in healthy volunteers at this infusion rate.

Clinical studies have established that high norepinephrine concentrations are an independent risk factor for mortality in patients with sepsis (36). However, to date, no clinical studies have specifically investigated whether or not these detrimental effects may be due to norepinephrine-

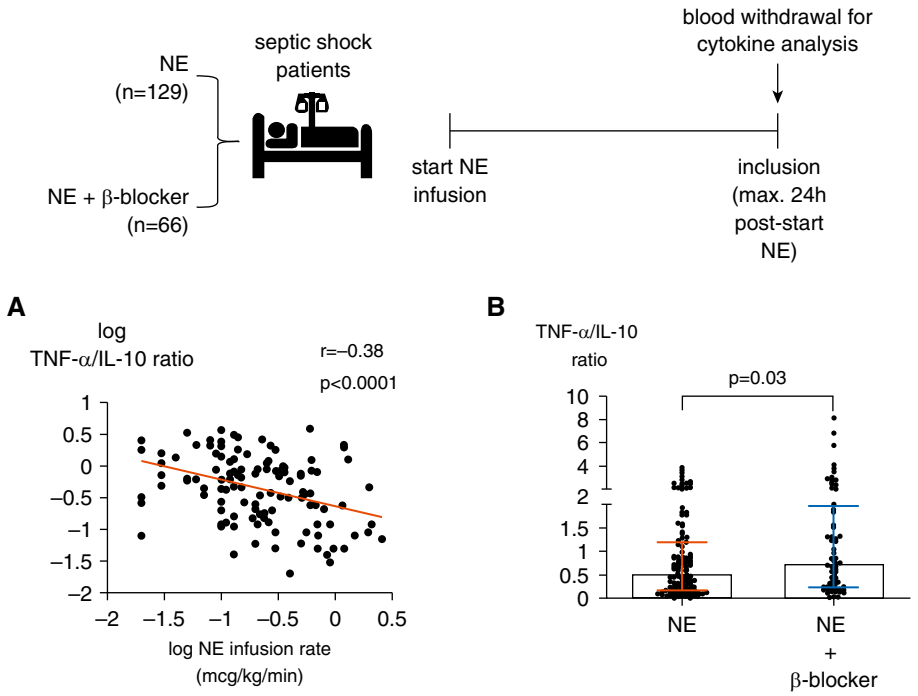

Figure 8. Relationship between norepinephrine (NE) infusion rate, β-blocker use, and the proinflammatory/antiinflammatory balance in patients with septic shock. (*A*) Correlation between NE infusion rate at the time of inclusion and the plasma TNF-α (tumor necrosis factor α)/IL-10 ratio at the same time point in septic shock patients. (*B*) Plasma TNF-α/IL-10 ratio at the time of inclusion in septic shock patients treated with NE with or without concomitant use of β-blockers. (*B*) Data are expressed as median and interquartile range. *P* values were calculated using (*A*) Pearson correlation and (*B*) a Mann-Whitney *U* test.

induced immunosuppression. Clear assessment of the immunologic effects of norepinephrine in patients with sepsis is precluded by the fact that it is the recommended vasopressor specified by the surviving sepsis guidelines and is therefore used in virtually all patients. To gain insight into the possible immunomodulatory effects of norepinephrine in the clinical setting, we therefore analyzed the relationship between norepinephrine infusion rates and inflammatory parameters as well as the influence of β-blockers in a relatively large cohort of patients with septic shock treated with norepinephrine. Our data reveal that the norepinephrine infusion rate is associated with lower plasma TNF-α/IL-10 ratios, indicating a shift toward a more antiinflammatory phenotype, and use of β-blockers was associated with an increased plasma TNF-α/IL-10 ratio, signifying a more proinflammatory phenotype. Collectively, these results are indicative of antiinflammatory effects of norepinephrine in patients with septic shock. Therefore, the clinical use of norepinephrine appears to dysregulate the host response, and β-blockers might have beneficial immunological effects in patients with sepsis. In this context, it is noteworthy that a higher TNF-α/IL-10 ratio was previously shown to be associated with improved survival in patients with sepsis (37). Furthermore, the β-blocker esmolol dramatically increased survival in a randomized trial in patients with septic shock treated with norepinephrine (38). Although these promising results were ascribed to esmolol's cardiac effects and no immunological parameters were assessed, immunomodulation may have played an important role as well. In addition, our findings may shed a new light on recent studies that identified different sepsis endotypes based on transcriptomic signatures already alluded to. Interestingly, compared with the other endotypes, a higher proportion of patients classified as having an immune-suppressed endotype were in shock and required (high dosages of) norepinephrine (10, 11). As such, it may be speculated that the endotypes are not only driven by pathogen and host factors but also by iatrogenic factors such as norepinephrine administration.

In all large clinical trials to date, vasopressin and, more recently, the selective V1-receptor agonist selepressin, were investigated as adjunctive therapies to norepinephrine (32, 33, 39). Therefore, virtually all patients in the vasopressin/selepressin treatment groups also received norepinephrine, and in most cases, this is in much higher dosages than those we used in our human endotoxemia study, which already significantly dysregulated the host response. As an add-on therapy, vasopressin (analogs) exerted no overall effects on mortality (32, 33, 39), although a survival benefit was observed in a subset of vasopressin-treated patients (33), and vasopressin reduced the need for renal replacement therapy (32). Differences in infectious complications were unfortunately not assessed in these trials. Nevertheless, a secondary analysis of the VASST (Vasopressin and Septic Shock Trial) assessed cytokine concentrations, which were similar between the vasopressin and norepinephrine groups after correction for baseline characteristics such as sex, age, and shock severity (40). This might appear to contradict our findings, but its use as an adjunctive therapy to norepinephrine in this trial precludes clear interpretation. Because compounds like vasopressin and selepressin appear to be suitable as first-line vasopressors as well (31, 41), it would be interesting to evaluate the immunologic effects in a norepinephrine versus alternative vasopressor monotherapy study. If our results could be recapitulated in such a setting, reappraisal of the current vasopressor therapy for sepsis is warranted. With the emergence of the era of "precision medicine" for sepsis, future use of different vasopressors may be tailored to specific subgroups of patients with sepsis based on their (molecular) immunological endotype.

Conclusions

Norepinephrine, the first-choice vasopressor administered to patients with septic shock worldwide, significantly dysregulates the host response. Because of its potent antiinflammatory and host defense–compromising effects, it may therefore importantly contribute to the development and propagation of sepsis-induced immunoparalysis.

Vasopressin, a viable alternative vasopressor, does not exert these untoward side effects. These findings may prompt a reappraisal of the current clinical management of patients with sepsis. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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