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Noradrenergic Neurons Regulate Monocyte Trafficking and Mortality during Gram-Negative Peritonitis in Mice

Eric J. Seeley, Sophia S. Barry, Saisindhu Narala, Michael A. Matthey, and Paul J. Wolters

Effective host defense requires a robust, yet self-limited response to pathogens. A poorly calibrated response can lead to either bacterial dissemination due to insufficient inflammation or organ injury due to excessive inflammation. Recent evidence suggests that the cholinergic anti-inflammatory reflex helps calibrate the immune response. However, the influence of peripheral noradrenergic neurons, which are primarily sympathetic neurons, in regulating immunity remains incompletely characterized. Using a model of 6-hydroxydopamine–mediated noradrenergic nerve ablation, we show that elimination of noradrenergic neurons improves survival during Klebsiella pneumoniae peritonitis (67 versus 23%, \( p < 0.005 \)) in mice. The survival benefit results from enhanced MCP-1–dependent monocyte recruitment and a subsequent decrease in bacterial loads. Splenectomy eliminated both the survival benefit of 6-hydroxydopamine and monocyte recruitment, suggesting that monocytes recruited to the peritoneum originate in the spleen. These results suggest that noradrenergic neurons regulate the immune response through two pathways. First, sympathetic nerve-derived norepinephrine directly restrains MCP-1 production by peritoneal macrophages during infection. Second, norepinephrine derived from the vagally innervated splenic nerve regulates splenic monocyte egress. Removal of these two modulators of the immune response enhances antibacterial immunity and improves survival. These results may have implications for how states of catecholamine excess influence the host response to bacterial infections. The Journal of Immunology, 2013, 190: 4717–4724.

Sepsis is a major medical problem that is associated with a high mortality and escalating hospital costs (1). In the United States, there are >700,000 cases of sepsis each year, leading to >200,000 deaths, and the incidence of sepsis is on the rise (1). Since the advent of antibiotics, no pathogen-targeted or immunomodulatory therapy has decreased sepsis-related mortality. Although organ injury during sepsis is thought to result from a poorly calibrated immune response to infection, clinical therapies aimed at dampening this inflammatory response have been unsuccessful (2, 3). Thus, treatment strategies based on new biologic paradigms are needed.

There is an extensive literature describing interactions between the parasympathetic nervous system and immunity during infection and inflammation. This research has culminated in the recognition of a parasympathetic anti-inflammatory reflex that helps to maintain immunologic homeostasis after acute inflammation (4, 5). According to this reflex, regional inflammation induces action potentials in afferent neurons that travel to the CNS, where they are integrated and then descend via the parasympathetic vagus nerve to the periphery, where neurotransmitters act through neuroimmune synapses to limit the production of inflammatory mediators by splenic macrophages (4). These neural pathways have been dissected to the level of neurotransmitters and their receptors, largely using the LPS model of acute inflammation (6, 7). Less is known about interactions between noradrenergic nerves and host defense mechanisms during in vivo bacterial infections.

The primary goal of this study was to determine the influence of noradrenergic neurons on the immune system during Gram-negative septic peritonitis in mice. Peripheral noradrenergic nerves (8) were ablated using 6-hydroxydopamine (6-OHDA), a commonly employed method of peripheral noradrenergic nerve destruction (9, 10). Key elements of the immune response to infection were then measured in vivo and in vitro, which collectively demonstrate that norepinephrine (NE) regulates chemokine secretion, splenic monocyte trafficking, and mortality during Gram-negative peritonitis in mice.

Materials and Methods

Materials

All chemicals were obtained from Sigma-Aldrich, unless otherwise noted.

Experimental animals

C57BL/6, C57BL/6 MCP1 \(^{-/-}\), Kit \(^{W-sh}\)/Kit \(^{W-sh}\) and C57BL/6 CX3CR1 \(^{GFP}\) mice were either purchased from The Jackson Laboratory or were gifts from other laboratories. C57BL/6 nude/nude were purchased from the National Cancer Institute. All experimental procedures were performed on 8- to 12-wk-old mice and were approved by the University of California, San Francisco, Committee on Animal Research.

Ablation of noradrenergic nerves

Mice were injected i.p. with a single-dose 250 mg/kg 6-OHDA dissolved in PBS. The 6-OHDA solutions were prepared freshly from 6-OHDA powder for each experiment. Mice were used for experimental studies 4 d after injection. For blocking studies, desipramine (DES; 10 mg/kg) was dissolved in PBS and delivered i.p. 30 min before 6-OHDA injection. Sterile PBS without 6-OHDA was used as a vehicle control.

Induction of Klebsiella pneumoniae peritonitis

K. pneumoniae (strain 43816, serotype 2; American Type Culture Collection) was resuspended in 5 ml Nutrient Broth (Difco) and cultured...
overnight at 37°C. Forty microliters of this suspension was added to 20 ml Nutrient Broth and grown for 3–4 h to log phase when CFUs were determined by OD600 readings and confirmed by culture. K. pneumoniae septic peritonitis was induced by injecting 150 CFU K. pneumoniae bacteria suspended in 200 μl sterile PBS into the peritoneum of mice. Mice were monitored for morbidity or death three times daily. Moribund mice were euthanized by CO2 inhalation and cervical dislocation.

Induction of LPS-mediated shock
For survival experiments, LPS (Escherichia coli 0111:B4) was solubilized in PBS and sonicated for 30 min before injection. Mice were injected i.p. with 30 mg/kg LPS and monitored for morbidity or death three times daily. For subthalamic point experiments, mice were injected with 6 mg/kg LPS and mice were sacrificed 90 or 240 min later.

Quantification of the cellular response to infection
To recover peritoneal inflammatory cells for enumeration, mouse abdomens were cleaned with 70% ethanol. Four milliliters of sterile PBS was then instilled into the peritoneum. The abdomen was massaged gently for 1 min and opened with sterile scissors, and lavage fluid was reclained. Lavage fluid was centrifuged at 600 × g for 5 min at 4°C, and the supernatants were saved for cytokine analysis. Cell pellets were resuspended in red cell lysis buffer for 3 min and then washed, and the cell pellet was resuspended in PBS. Cell numbers were enumerated with a hemocytometer, and cell differentials were determined on cytospun cells stained with Diff-Quik (American Scientific Products).

Quantification of peritoneal and serum bacterial CFUs
Ten microliters of peritoneal lavage fluid (obtained as above) was cultured on nutrient agar plates, and serial dilutions for later time points were performed. Serum was obtained aseptically by direct cardiac puncture, and serial dilutions of infected blood were performed.

Cytokine analysis
Cytokine concentrations were measured in peritoneal lavage fluid or serum using ELISA kits, as follows: MCP-1 (R&D Systems), IL-6 (R&D Systems), IFN-γ (R&D Systems), IL-1β (R&D Systems), and TNF (R&D Systems), according to the manufacturers’ protocols.

Flow cytometry
Single-cell suspensions were obtained from blood and peritoneal lavage fluid and were filtered using a 40-μm filter. RBCs were lysed, and, after washing, cells were incubated with anti-mouse FcRIII (2.4G2) for 20 min at 4°C in FACS buffer (BD Biosciences) and then stained with anti-mouse Abs specific for the anti-CD11b (M1/70, Alex 647 or biotin; University of California, San Francisco, hybridoma core), Ly-6C (AL-21 PE-Cy7; BD Biosciences), anti-CD3 (145-2C11 allophycocyanin-eFluor780; eBiosciences), anti-NK1.1 (PK136 allophycocyanin-eFluor780; eBiosciences), anti-CD220 (RA3-6B2 allophycocyanin-eFluor780; eBiosciences), anti-CD3 (145-2C11 allophycocyanin-eFluor780; BD Biosciences), anti-CD11b (M1/70, Alex 647 or biotin; University of California, San Francisco, hybridoma core), and anti-F4/80 (C1;39; eFluor450; eBiosciences). Cells were analyzed on a nine-color BD LSRII, using the following antibodies: anti-CD11b (R73; BD Biosciences), anti-CD11c (N418; eBiosciences), anti-CD220 (RA3-6B2 allophycocyanin-eFluor780; eBiosciences), anti-NK1.1 (PK136 allophycocyanin-eFluor780; eBiosciences), and anti-F4/80 (C1;39; eFluor450; eBiosciences). Data were analyzed using Flowjo 8.7 software. Monocytes were identified as GFP+CD11b+ (NK1.1 CD3 CD11c f4/80−). These monocytes were then divided into Ly-6C high (+) or low (−).

Catecholamine analysis
NE levels were measured in the sera of mice after 6-OHDA treatment and during infection using an ELISA kit (Rocky Mountain Diagnostics), according to the manufacturer’s protocol.

Splenectomy
Mice were anesthetized with ketamine/xylazine. Local anesthesia was provided with butricaine injection and analgesia with buprenorphine. Ongoing anesthesia was provided with continuous isoflurane delivered via inhalational mask. Using sterile forceps and scissors, a midline incision was made and the spleen was mobilized. The blood vessels at the distal and proximal pole of the spleen were ligated with 2-0 suture, and the spleen was removed from the peritoneum. The peritoneal membrane and skin were sutured in two layers, and betadine was applied to the incision site. Mice were subjected to i.p. K. pneumoniae peritonitis 2 wk later.

Immunofluorescence of spleen sections
Spleens were harvested as above, immediately deposited in optimum cutting temperature compound, and then flash frozen in a dry ice and isopropanol slurry. Tissues were stored at −80°C prior to use. Five-micron sections of spleen were fixed in acetone at −20°C for 10 min and then stained overnight at 4°C with rat anti-mouse CD11b Ab (M1/70; University of California, San Francisco, hybridoma core). Sections were washed and then stained with a fluorophore-conjugated secondary Ab for 1 h at room temperature. Images of spleen sections immunostained with anti-CD11b were obtained using a Nikon Eclipse TE300 microscope at a high power magnification of ×200. CD11b+ cells in the subcapsular red pulp (SRP) were counted, and the number per unit area was quantified using ImageJ software.

NE suppression assays
Primary peritoneal macrophages (obtained as above) were plated at 100,000 cells per 200 μl in RPMI 1640 supplemented with penicillin/streptomycin and 5% FCS. Cells were rested overnight, and the following morning titrated doses of NE were added to culture media. Thirty minutes following the addition of NE, heat-killed K. pneumoniae were added to culture media. After 12 h of heat-killed K. pneumoniae stimulation, supernatants were harvested and levels of MCP-1 were quantified by ELISA.

RT-PCR for adrenergic receptor expression
Expression of all nine adrenergic receptors was detected by RT-PCR. The following primers were used: α1A, forward, 5′-CCAGTTGCTTC-GCAGAAGG-3′, and reverse, 5′-TGTTTATGCTGATGTCGCGG-3′; α1B, forward, 5′-CGAGGCAGGCAACCACTATTG-3′, and reverse, 5′-GCCAGGGAATACCGCAAT-3′; α2A, forward, 5′-GGTGGTTGCACTTTTCATTGTC-3′, and reverse, 5′-GGTGGTTGTAAGCTCACTTGTC-3′, and reverse, 5′-CAGTACACCCTGAGG-3′, and reverse, 5′-AGAGTACCCAGTTAGAGTGC-3′; α2B, forward, 5′-CTGTGGTTGCTTCTCATGTC-3′, and reverse, 5′-GCCTAAGAACCCTGTAAGCT-3′, and reverse, 5′-GAACCTCGAACTCGTCTG-3′, and reverse, 5′-GCCAGGGAATACCGCAAT-3′; β1 AR, forward, 5′-AACAGCAGGACCTCAGCTGTGG-3′, and reverse, 5′-GGGGAAGCAGACGCTCTTCTT-3′, and reverse, 5′-AACAGCAGGACCTCAGCTGTGG-3′; β1 AR, forward, 5′-AACAGCAGGACCTCAGCTGTGG-3′, and reverse, 5′-AGAAAAAGGCTCTCGGTTTG-3′, and reverse, 5′-CAGGACGACGCTCGCAAAGG-3′; β2 AR, forward, 5′-TTGGAATCTCTGGGCATCCATAC-3′, and reverse, 5′-TAAAGGCAGCTCAGTAAACAGTCCG-3′.

Peritoneal macrophages were obtained as above, and RNA was extracted from macrophages and tissue homogenates by TRIzol extraction.

Statistical analysis
Survival curves were analyzed using the log-rank (Mantel–Cox) test. Normally distributed data were compared using the Student t test. Non-normally distributed data were compared using the Mann–Whitney U test. All statistical tests were performed using GraphPad Prism 5.0 software (GraphPad, La Jolla, CA). Data are displayed as mean ± SEM. Significance was assigned *p < 0.05.

Results
Ablation of noradrenergic neurons improves survival during Gram-negative septic peritonitis
To test the contribution of noradrenergic neurons to host defense during K. pneumoniae septic peritonitis, noradrenergic neurons were ablated using 6-OHDA. This compound, which is structurally similar to dopamine, is taken up through the NE and dopamine reuptake transporters expressed on peripheral noradrenergic nerves and leads to neuronal death through free radical formation and mitochondrial toxicity (11). Our data (Supplemental Fig. 1) and others (12) indicate that 6-OHDA treatment leads to an 80–90% reduction in circulating and tissue NE content 3–4 d after injection (13). Thus, 4 d after 6-OHDA administration, mice were infected by i.p. injection with 150 CFU K. pneumoniae and then monitored for morbidity and mortality. The ablation of noradrenergic neurons prior to infection dramatically improved survival from 27 to 67% (Fig. 1A). This survival benefit was not due to indirect effects of 6-OHDA because the survival benefit was blocked by pretreatment with DES, a compound that blocks the uptake of 6-OHDA into noradrenergic nerves (Fig. 1B). In contrast, noradrenergic nerve ablation, performed 4 d prior to LPS.
administration, did not reduce LPS-induced mortality (Fig. 1C). These results show that the ablation of noradrenergic neurons improves survival following *K. pneumoniae* infection, but not following LPS-mediated sterile inflammation, suggesting that ablation of noradrenergic neurons improves antibacterial host defense mechanisms.

**Ablation of noradrenergic neurons improves peritoneal monocyte recruitment and enhances bacterial clearance**

Rapid leukocyte recruitment to sites of infection is essential for bacterial containment and eradication. Thus, leukocyte recruitment to the peritoneum and peritoneal and serum bacterial loads were quantified at multiple time points after *K. pneumoniae* infection. Prior to infection, there was no difference in total peritoneal cell counts or differentials in 6-OHDA–treated mice compared with controls (Fig. 2A–C). However, 4 h after i.p. injection of *K. pneumoniae*, noradrenergically ablated mice had greater numbers of peritoneal mononuclear cells, which persisted until 10 h postinfection (Fig. 2B). The peritoneal bacterial loads in control and noradrenergically ablated mice (Fig. 2D) were equivalent 4 and 10 h postinfection. However, by 24 h postinfection, noradrenergically ablated mice had significantly lower i.p. bacterial loads than control mice (Fig. 2D). Serum bacterial loads were equivalent 10 and 24 h postinfection, but subsequently decreased in noradrenergically ablated mice (Fig. 2E). In contrast, serum bacterial counts continued to increase in control mice (Fig. 2E). These data suggest that noradrenergically ablated mice have improved survival because they more effectively recruit monocytes to the peritoneum during bacterial infection.

**Ablation of noradrenergic neurons enhances inflammatory cytokine secretion postinfection or after sterile inflammation**

Prior studies suggest that NE can either increase or decrease inflammatory cytokine secretion depending on the immunologic context and cell of origin (14, 15). To assess how the absence of NE affects cytokine production in response to infection, IL-6, TNF, IL-1β, and IFN-γ were measured in the serum and peritoneal fluid 4 and 24 h after *K. pneumoniae* peritonitis. There were no statistical differences between TNF, IL-1β, and IFN-γ levels in 6-OHDA–treated mice compared with controls (Supplemental Fig. 2). However, IL-6 levels were elevated in the serum of 6-OHDA–treated mice 4 h after i.p. infection with *K. pneumoniae* (Fig. 3B). By 24 h following i.p. infection, the i.p. levels of IL-6 were lower in 6-OHDA–treated mice, most likely because 6-OHDA–treated mice had lower bacterial loads 24 h postinfection (Fig. 2D, 2E).

To avoid the confounding effects of asymmetric bacterial growth on inflammatory cytokine production at latter time points in our infection model (Fig. 2D, 2E), we measured IL-6 levels after administration of sublethal LPS. Serum and peritoneal IL-6 levels were higher in mice lacking noradrenergic neurons (Fig. 3C, 3D). These data suggest that noradrenergic...
neurons restrain IL-6 production during infection and sterile inflammation.

The survival advantage of noradrenergically ablated mice is not mast cell dependent

Mast cells express adrenergic receptors, can be regulated by catecholamines, and can act as central regulators of the i.p. response to infection (16, 17). Thus, we considered the possibility that mast cells regulate monocyte recruitment and survival in noradrenergically ablated mice. To test this hypothesis, *K. pneumoniae* peritonitis was induced in 6-OHDA–treated or control mast cell–deficient *Kit*<sup>W-sh/W-sh</sup> mice. The ablation of noradrenergic neurons in *Kit*<sup>W-sh/W-sh</sup> mice led to improved survival (Supplemental Fig. 3), indicating the survival advantage provided by noradrenergic nerve ablation was not due to interrupting interactions between mast cells and noradrenergic neurons.

Monocyte recruitment and improved survival in noradrenergically ablated mice are MCP-1 dependent

As an explanation for the accelerated recruitment of monocytes to the peritoneum of noradrenergically ablated mice, MCP-1 levels in peritoneal fluid were determined postinfection or after LPS administration. MCP-1 levels were higher 4 h after *K. pneumoniae* peritonitis and 1.5 h after LPS in 6-OHDA–treated mice (Fig. 4A, 4B). These results suggested that NE inhibits the production of MCP-1 during infectious or inflammatory stimuli.

To assess whether NE could be acting directly on peritoneal macrophages to suppress MCP-1 secretion, we first assessed the expression of adrenergic receptors on primary peritoneal macrophages. Primary peritoneal macrophages express the α<sub>1A</sub>, α<sub>2A</sub>, β<sub>1</sub>, β<sub>2</sub>, and β<sub>3</sub> adrenergic receptors (Supplemental Fig. 4). Second, MCP-1 production by primary peritoneal macrophages was measured after stimulation with heat-killed *K. pneumoniae* in the presence of titrated doses of NE. The addition of NE to primary peritoneal macrophages stimulated in vitro with *K. pneumoniae* led to a dose-dependent decrease in MCP-1 production (Fig. 4C). These data confirm that NE directly suppresses MCP-1 secretion from peritoneal macrophages after stimulation with heat-killed *K. pneumoniae*.

To test the in vivo importance of noradrenergic modulation of MCP-1 secretion, *K. pneumoniae* peritonitis was induced in 6-OHDA–treated or control MCP-1<sup>−/−</sup> mice. Ablation of noradren-
ergic neurons prior to infection in MCP-1\(^{-/-}\) mice did not lead to improved survival, illustrating that MCP-1 is essential for the survival benefit of 6-OHDA treatment (Fig. 4D). Further examination of the immune response at key time points postinfection in MCP-1\(^{-/-}\) mice showed that noradrenergically ablated MCP-1\(^{-/-}\) mice failed to recruit monocytes to the infected peritoneum (Fig. 4E) and failed to clear serum or peritoneal bacterial burdens as effectively as wild-type mice in which noradrenergic neurons were ablated (Fig. 4F). Collectively, these data suggest that the ablation of noradrenergic neurons removes inhibitory noradrenergic signals to MCP-1–producing peritoneal macrophages. Removal of this inhibitory signal is associated with increased MCP-1 production and accelerated monocyte recruitment to the infected peritoneum.

**The spleen is required for improved survival and enhanced monocyte recruitment in noradrenergically ablated mice**

Recent studies have characterized the autonomic innervation and functional significance of the splenic nerve (6, 18). These studies show that signals originating from the vagus nerve synapse at the celiac mesenteric ganglion and then travel to the spleen via the splenic nerve where acetylcholine-producing T cells act as intermediaries between splenic nerve-derived NE and \(\alpha_7\) acetylcholine receptor–bearing macrophages (18, 19). The splenic nerve, which is under vagal control, secretes NE, traditionally considered a sympathetic neurotransmitter. Of relevance to our model system, the splenic nerve is destroyed by 6-OHDA treatment (8). To isolate the role of splenic nerve-derived catecholamines, splenectomies were performed on mice prior to administration of 6-OHDA. Survival postinfection was then assessed in four groups, as follows: control mice, 6-OHDA–treated mice, as well as control and 6-OHDA–treated mice without spleens. Splenectomy prior to 6-OHDA treatment abolished the survival benefit of noradrenergic nerve ablation, indicating that the spleen is required for the improved survival of noradrenergically ablated mice (Fig. 5A, 5B). Notably, NE levels were not lower in splenectomized mice 4 h postinfection when compared with control mice (Supplemental Fig. 1). These data suggest that, although NE is released into the spleen by the splenic nerve, this NE does not significantly contribute to circulating NE levels during infection. To functionally characterize why splenectomized mice did not show improved survival after noradrenergic nerve ablation, peritoneal cell counts and differentials were performed 4 h postinfection in each of the four groups of mice (Fig. 5C, 5D). Mice treated with 6-OHDA after splenectomy failed to recruit monocytes to the peritoneum 4 h postinfection (Fig. 5C, 5D), suggesting that the spleen contains a population of monocytes that are recruited to the peritoneum during infection.

T cells have been shown to transduce signals via the splenic nerve during inflammation (7, 20). The wiring of this neuro-immune circuit involves splenic nerve-derived NE, which synapses on acetylcholine-producing splenic T cells (7). To interrogate this recently identified circuit in our infection model, peritoneal monocyte recruitment was measured 4 h postinfection in T cell–deficient nude mice after treatment with 6-OHDA or vehicle control. Despite the absence of T cells, mice in which noradrenergic neurons were ablated recruited more monocytes to the infected peritoneum than controls (Fig. 5E). These results suggest that T cells are not essential for the egress of splenic monocytes during infection. These experiments did not test the importance of splenic B cells, which are another target of splenic nerve-derived NE and may play a role in both humoral and cellular defense against intra-abdominal infection.

**Effector Ly-6C\(^{+}\) and Ly-6C\(^{-}\) monocytes migrate from the spleen to the peritoneum during infection in noradrenergically ablated mice**

Recent studies suggest the spleen houses a reservoir of monocytes that are deployed during inflammatory conditions such as myocardial infarction and stroke (21, 22). These cells reside in the SRP of the spleen and can be identified by expression of CD11b in this anatomic location (22). To measure the migration of monocytes out of the spleen during infection in noradrenergically ablated or control mice, spleens were harvested 4 h postinfection and stained for CD11b. Representative spleen sections show a decrease in CD11b\(^{+}\) cells in the SRP of 6-OHDA–treated mice (Fig. 6A, 6B). When this visual difference was quantified, there was a significant decrease in CD11b\(^{+}\) cells in the SRP of noradrenergically lesioned mice, which was not observed in control mice (Fig. 6C). To further define the subtype of monocytes recruited to the peritoneum during infection,
in mice lacking noradrenergic neurons, mice in which enhanced GFP (eGFP) has been knocked into the CX3CR1 locus were used (23). Heterozygous mice express one functional copy of CX3CR1 and express variable levels of eGFP in phagocytic cells. Monocytes in CX3CR1/eGFP mice retain the ability to chemotax to the peritoneum during inflammatory responses (23), and both Ly-6C<sup>+</sup> and Ly-6C<sup>−</sup> monocytes express eGFP. The recruitment of Ly-6C<sup>+</sup> and Ly-6C<sup>−</sup> monocytes to the peritoneum was measured by multicolor flow cytometry. GFP<sup>+</sup>CD11b<sup>+</sup> (CD11c<sup>−</sup>/F4/80<sup>−</sup>/B220<sup>−</sup>/CD3<sup>−</sup>/NK1.1<sup>−</sup>) monocytes were considered Ly-6C<sup>+</sup> monocytes, whereas GFP<sup>+</sup>CD11b<sup>+</sup> (CD11c<sup>−</sup>/F4/80<sup>−</sup>/B220<sup>−</sup>/CD3<sup>−</sup>/NK1.1<sup>−</sup>) monocytes were considered Ly-6C<sup>−</sup> monocytes. Importantly, there was a significant increase in both Ly-6C<sup>+</sup> and Ly-6C<sup>−</sup> monocytes recruited to the peritoneum 4 h postinfection in mice lacking noradrenergic neurons when compared with baseline monocyte levels or monocyte levels 4 h postinfection in control mice (Fig. 6D). These results suggest Ly-6C<sup>+</sup> and Ly-6C<sup>−</sup> monocytes exit the spleen and migrate to the peritoneum during infection and that NE from the splenic nerve may serve as a retention signal for these monocytes.

Discussion

To our knowledge, these experiments illustrate for the first time that modulation of noradrenergic neurons can improve survival during Gram-negative infection in mice. In addition, these data suggest that noradrenergic neurons regulate the host response through two distinct pathways. First, NE directly suppresses MCP-1 production by peritoneal macrophages after stimulation with bacterial products. Second, splenic nerve-derived NE acts as a retention signal for SRP monocytes. In our experiments, removal of these two immunosuppressive functions of noradrenergic neurons results in a more effective inflammatory response to infection, as evidenced by increased serum IL-6, increased peritoneal MCP-1, and improved monocyte recruitment to the infected peritoneum. This enhanced monocyte recruitment leads to more effective bacterial clearance and improved survival in our model of Gram-negative bacterial peritonitis.

The role of catecholamines in shaping host defense in vitro and in vivo has been controversial and may depend on whether catecholamines are derived from nerves or phagocytes and on the inflammatory environment into which catecholamines are released (15, 24–27). In addition, noradrenergic regulation of immunity may be pathogen dependent (28). In models of E. coli and Pseudomonas aeruginosa peritonitis, 6-OHDA–treated mice had lower bacterial loads 7 h postinfection (28). In contrast, treatment with 6-OHDA prior to infection with the Gram-positive organism Staphylococcus aureus resulted in higher peritoneal bacterial loads (28). In a mouse model of Influenza pneumonia, 6-OHDA treatment improved survival (29). Other recent studies suggest that physiologic stressors that increase endogenous NE or the exogenous administration of NE can be directly immunosuppressive (10). For example, in a mouse model of stroke, heighted endogenous NE directly impaired innate immune defenses and predisposed mice to poststroke bacterial pneumonia (10), and, in humans, epinephrine infusion prior to i.v. LPS administration led to a decreased circulating TNF (14). In vitro studies support an anti-inflammatory role for catecholamines during LPS stimulation as well (20, 30). Collectively, our findings are largely in agreement with prior studies, and suggest that neurally derived NE is immunosuppressive and acts to restrain inflammatory cytokine secretion. In addition, our study supports a new role for NE in modulating chemokine expression and suggests a critical role for NE in regulating splenic leukocyte trafficking. Future studies, utilizing receptor-blocking agents or alternative methods of noradrenergic neuron modulation, will lend additional insights to the neural modulation of immunity.

A central tenant of autonomic physiology is that the sympathetic and parasympathetic branches of the autonomic nervous system act in opposition to maintain physiologic homeostasis. However, our study, as well as other recent studies (7, 18), suggests that this tenant does not apply to the autonomic regulation of immunity. A series of neurons originating in the dorsal motor nucleus, traveling via the efferent vagal nerve, eventually give rise to the splenic nerve. The postganglionic splenic nerve is exclusively noradrenergic (6, 18) and is destroyed by 6-OHDA treatment (8). Thus, parasympathetic outflow directly modulates splenic NE content (6). This neural circuitry underlies the commonalities between studies of leukocyte trafficking that alter cholinergic signals and others, such as ours, that attenuate noradrenergic signals to the spleen. For example, vagotomy, which decreases splenic NE, leads to a 5-fold increase in the peritoneal recruitment of macrophages and neutrophils after E. coli peritonitis (31), and mice lacking the a7 acetylcholine receptor, which transduces signals from the vagus to splenic nerve, show more effective granulocyte recruitment to the peritoneum in an E. coli peritonitis model (32). Similarly, vagal nerve stimulation, which decreases splenic NE content, reduces the recruitment of leukocytes to sites of inflammation in a carrageenan air pouch model of inflammatory cell trafficking (33). Our observation that 6-OHDA–treated mice have enhanced recruitment of Ly-6C<sup>+</sup> and Ly-6C<sup>−</sup> monocytes to the peritoneum following K. pneumoniae infection is consistent with these findings. Collectively, our experiments, as well as others (6, 7), support biochemical and functional immunologic overlap between cholinergic and noradrenergic alterations in splenic neural signaling and suggest that, in addition to regulating circulating cytokine levels (34) and B cell function (8), the splenic nerve also regulates monocyte trafficking.

It is notable that vagal nerve stimulation or administration of cholinergic agonists, which increase splenic NE content, can at-
tenuate splenocyte-derived TNF and improve survival during LPS-induced inflammation or antibiotic-treated cecal ligation and puncture (CLP) (34–36). We attribute this apparent contradiction, that increasing splenic NE content increases survival during CLP or LPS, with our findings that decreasing splenic NE improves survival during bacterial infection, to important differences in the experimental model of inflammation or infection used. Immuno-
logic alterations that enhance host antibacterial defense do not necessarily improve survival during LPS-induced inflammation or antibiotic-treated CLP. In fact, immunologic alterations that en-
hance either the early cytokine response or leukocyte recruitment in LPS or antibiotic-treated CLP may worsen shock and lead to increased organ injury and increased mortality. In contrast, in models that require effective antibacterial immunity, such as our model of K. pneumoniae peritonitis, interventions that enhance early host defense mechanisms, such as ablation of noradrenergic neurons, result in improved bacterial clearance and improved survival (31, 32, 37).

The bone marrow has been considered the primary site of monocyte generation and deployment during infection. However, recent studies suggest that the spleen also contains a population of Ly-6C+ and Ly-6C− monocytes that are deployed to sites of in-
flammation during myocardial infarction and stroke (22). Based on the findings that splenectomy attenuates the recruitment of mono-
cytes to the peritoneum and 6-OHDA-treated mice have fewer CD11b+ cells in the spleen 4 h postinfection, we suggest that Ly-
6C+ and Ly-6C− monocytes from the splenic SRP are deployed within the initial hours of infection in noradrenergic nerve-ablated mice. Deployment appears to be sensitive and rapid because there is a 4-fold increase in peritoneal monocytes just 4 h after the in-
jection of 150 CFU K. pneumoniae. Our data also indicate that the deployment of splenic monocytes may be an important component of normal host defense, because monocytes eventually arrive in the peritoneum in control mice, but this normal process is accelerated by noradrenergic nerve ablation. The absence of neutrophils in 6-OHDA–treated mice is also notable. We suggest that the rapid recruitment of splenic monocytes in 6-OHDA–treated mice, with subsequent decreases in bacterial loads, obviates the need for fur-
ther neutrophil recruitment. Thus, it appears that neural signals mediate monocyte retention in the spleen and that ablating these neurons leads to more rapid monocyte deployment and improved survival.

This study further advances accumulating evidence that nerves, through the secretion of neurotransmitters, directly regulate im-
munity in vivo (18, 19, 38). Neural modulation of immunity appears to be evolutionarily conserved from nematodes to mam-
mals (4, 14, 38–41), suggesting an important evolutionary benefit to effective neuroimmune communication. Our results are poten-
tially relevant to human health and disease for several reasons. First, conditions leading to catecholamine excess (e.g., severe anxiety or acute illness) may lead to catecholamine-induced immuno-suppression by inhibiting inflammatory cytokine secretion and reducing effective monocyte trafficking. In addition, admin-
istration of catecholamine vasopressors to patients with septic shock may predispose them to subsequent infections by sup-
pressing monocyte recruitment to regions of infection. Finally, adrenergically active drugs, including β-blockers, may have im-
portant unrecognized immunomodulatory effects. In the future, modulating noradrenergic neurons may provide a new therapeutic option for patients predisposed to or suffering from infection.

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References

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Supplemental Figure Legends

**Supplemental Figure 1.** (A) Mice were treated with 6-OHDA and then sacrificed a specific timepoints after treatment and Serum NE content was analyzed by ELISA. (B) Mice were treated with 6-OHDA or vehicle control and then infected 4 days later with 150 *K. pneumonia* i.p.. Serum NE content was assessed at multiple time points in 6-OHDA or vehicle control treated mice. (n=3 mice per timepoint). (C) Serum NE levels 4 hours after infection in control and splenectomized (SPLX) mice (n=8-10 mice per group). *P < 0.05.

**Supplemental Figure 2.** Serum and peritoneal cytokine levels during infection in control and 6-OHDA treated mice. IL-6, INFγ, IL-1β and TNF levels were determined by ELISA in the serum and peritoneal fluid of control of 6-OHDA treated mice 4 and 24 hours after infection. (n=8-15 mice per time point, repeated 1-2 times) *P < 0.05

**Supplemental Figure 3.** The survival benefit of 6-OHDA is not mast cell dependent. Mast cell deficient Kit^W-sh/Kit^W-sh mice were treated with 6-OHDA or vehicle control and infected with *K. pneumoniae* i.p. 4 days later. (n=8-15 mice, repeated twice) *P < 0.05.

**Supplemental Figure 4.** Primary peritoneal macrophages express α and β adrenergic receptors. Primary peritoneal macrophages were harvested by lavage and PCR was performed for mRNA from the α and β adrenergic receptors. A homogenate of brain, liver and kidney was used as a positive (pos) control. Peritoneal macrophages express the α1A, α2A and β1, β2 and β3 adrenergic receptors.
Supplemental Figure 1.

A

B

C

Supplemental Figure 1.
Supplemental Figure 2

Serum and peritoneal cytokine levels during infection in control and 6-OHDA treated mice. IL-6, INFγ, IL-1β and TNF levels were determined by ELISA in the serum and peritoneal fluid of control and 6-OHDA treated mice 4 and 24 hours after infection. (n=8-15 mice per time point, repeated 1-2 times) *P < 0.05
Supp Figure 3
Supp Figure 4