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Noradrenergic neurons regulate monocyte trafficking and mortality during gram-negative peritonitis in mice[1]

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Abstract

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 to 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-OHDA mediated noradrenergic nerve ablation, we show that elimination of noradrenergic neurons improves survival during *K. pneumoniae* peritonitis (67% vs. 23%, $p<0.005$) in mice. The survival benefit results from enhanced monocyte chemotactic protein (MCP-1) dependent monocyte recruitment and a subsequent decrease in bacterial loads. Splenectomy eliminated both the survival benefit of 6-OHDA 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 (NE) directly restrains MCP-1 production by peritoneal macrophages during infection. Second, NE 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.

Keywords

Sepsis; neuro-immune interactions; sympathetic nervous system; spleen; monocytes; catecholamines; autonomic nervous system

Introduction

Sepsis is a major medical problem that is associated with a high mortality and escalating hospital costs (1). In the US, there are more than 700,000 cases of sepsis each year, leading to more than 200,000 deaths, and the incidence of sepsis 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

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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 central nervous system, where they are integrated and then descend via the parasympathetic vagus nerve to the periphery where neurotransmitters act through neuro-immune 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$^{-/-}$, C57BL/6 IL-6$^{-/-}$ and C57BL/6 CX_3CR1^{eGFP} mice were either purchased from The Jackson Laboratory or gifts from other laboratories. C57BL/6 nude/nude were purchased from the NCI. 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 of 6-Hydroxydopamine (6-OHDA) dissolved in PBS. 6-OHDA solutions were prepared freshly from 6-OHDA powder for each experiment. Mice were used for experimental studies 4 days after injection. For blocking studies, desipramine (10 mg/kg) was dissolved in PBS and delivered i.p. 30 minutes before 6-OHDA injection. Sterile PBS without 6-OHDA was used as a vehicle control.

Induction of K. pneumoniae peritonitis

K. pneumoniae (strain 43816, serotype 2; American Type Culture Collection) was resuspended in 5 ml of Nutrient Broth (Difco) and cultured overnight at 37°C. Forty microliters of this suspension was added to 20 ml of Nutrient Broth and grown for 3-4 h to log phase when CFUs were determined by OD_{600} readings and confirmed by culture. *K. pneumoniae* septic peritonitis was induced by injecting 150 CFU of *K. pneumoniae* bacteria suspended in 200 microliters of sterile PBS into the peritoneum of mice. Mice were monitored for morbidity or death three times daily. Moribund mice were euthanized by CO_2 inhalation and cervical dislocation.

Induction of LPS mediated Shock

For survival experiments, LPS (*E. Coli* 0111:B4) was solubilized in PBS and sonicated for 30 minutes before injection. Mice were injected intraperitoneally with 30 mg/kg of LPS and monitored for morbidity or death three times daily. For sub-lethal time-point experiments, mice were injected with 6 mg/kg of LPS and mice were sacrificed 90 or 240 minutes 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 reclaimed. 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, re-centrifuged, 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: MCP-1 (R&D Systems), IL-6 (R&D Systems), IFN-γ(R&D Systems) and IL-1β (R&D Systems), 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. Red blood cells were lysed and after washing, cells were incubated with anti-mouse FcRII/III (2.4G2) for 20 minutes at 4 degrees C in FACS buffer (BD) and then stained with anti-mouse antibodies specific for the anti-CD11b (M1/70, Alex 647 or Biotin, UCSF hybridoma core), Ly-6C (AL-21 PE-Cy7,BD) anti-CD3 (145-2C11 APC-eFluor780, eBiosciences), anti-NK1.1 (PK136 APC-eFluor780, eBiosciences), anti-B220 (RA3-6B2 APC-eFluor780, eBiosciences), anti-CD11c (N418, eFluor450, eBiosciences), anti-F4/80 (C1:A3-1 eFluor450, eBiosciences). Cells were analyzed on a 9 color BD LSRII and data were analyzed using Flowjo 8.7 software. Monocytes were identified as $GFP^{+}CD11b^{+}$(NK1.1 CD3 CD220 CD11c f4/80)$^{-}$. These monocytes were then divided into Ly-6C high (+) or low (−).

Catecholamine Analysis

NE levels were measured in the serum of mice after 6-OHDA treatment and during infection using an ELISA kit (Rocky Mountain Diagnostics) according to the manufacturers protocol.

Splenectomy

Mice were anesthetized with ketamine/xylezine. Local anesthesia was provided with bupivacaine injection and analgesia with buprenorphrine. Ongoing anesthesia was provided with continuous isoflourane delivered via inhalational mask. Using sterile forceps and scissors, a midline incision was made and the spleen 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 2 layers

and betadine was applied to the incision site. Mice were subjected to intraperitoneal *K. pneumoniae* peritonitis 2 weeks later.

Immunofluorescence of Spleen Sections

Spleens were harvested as above and immediately deposited in OTC and then flash frozen in a dry ice and isopropanol slurry. Tissues were stored at −80 °C prior to use. 5 micron sections of spleen were fixed in acetone at −20 °C for 10 minutes then stained overnight at 4 °C with rat anti-mouse CD11b antibody (M1/70, UCSF hybridoma core). Sections were washed and then stained with a fluorophore conjugated secondary antibody for 1 hr at room temperature. Images of spleen sections immunostained with anti-CD11b were obtained using a Nikon Eclipse TE300 microscope. $CD11b^+$ cells in the subcapsular red pulp were counted and the number per unit area quantified using ImageJ software.

NE suppression assays

Primary peritoneal macrophages (obtained as above) were plated at 100,000 cells per 200 microliters in RPMI supplemented with penicillin/streptomycin and 5% fetal calf serum. 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 hours of heat-killed *K. pneumoniae* stimulation, supernatants were harvested and levels of MCP-1 quantified by ELISA.

RT-PCR for Adrenergic Receptor Expression

Expression of all 9 adrenergic receptors was detected by RT-PCR. The following primers were used (5′→3′): α1aAR (fwd) CCAGTGTCTTCGCAGAAGG, (rev) TGGTTATGGTCTGTAGTCTCGG α1bAR (fwd) CGGACGCCAACCAACTACTT, (rev) GCCAGGACGATAACCGACAT α1dAR (fwd) AGTGGGTGTCTTCCTAGCC, (rev) CCACGAGTAGGCCCATACC α2aAR (fwd) GTGACACTGACGCTGGTTTG, (rev) ACTTGCCCGAAGTACCAGTAG α2bAR (fwd) TCTTCACCATTTTCGGCAATGC, (rev) AGAGTAGCCACTAGGATGTCG α2cAR (fwd) CTGTGGTGGGTTTCCTCATCG, (rev) CCAGTAACCCATAACCTCGTTG β1 AR (fwd) GAACCCTGCAACCTGTCGTC, (rev) GCCTAGAACCTCCATAGTGGC β2 AR (fwd) GGGAACGACAGCGACTTCTT, (rev) AACACAGGACATCAACCGTCTG β3 AR (fwd) AGAAACGGCTCTCTGGCTTTG, (rev) CAGVAGCAGACCTGCAAAAA b-actin (fwd) TGGAATCCTGTGGCATCCATGAAAC, (rev) TAAAACGCAGCTCAGTAACAGTCCG 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's t test. Non-normally distributed data were compared using the Mann-Whitney test. All statistical tests were performed using GraphPad Prism 5.0 software (GraphPad, La Jolla, CA, USA). 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-hydroxydopamine (6-OHDA). This compound, which is structurally similar to dopamine, is taken up through the

norepinephrine 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 days after injection (13). Thus, 4 days after 6-OHDA administration, mice were infected by intraperitoneal (i.p.) injection with 150 CFU of *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 pre-treatment with desipramine (DES), a compound that blocks the uptake of 6-OHDA into noradrenergic nerves (Fig. 1B). In contrast, noradrenergic nerve ablation, performed 4 days prior to LPS administration, did not reduce LPS induced mortality (Fig. 1C). These results show that 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 noradrenergically ablated mice compared to controls (Fig. 2 A-C). However, 4 hours after intraperitoneal injection of *K. pneumoniae*, noradrenergically ablated mice had greater numbers of peritoneal mononuclear cells, which persisted until 10 hours after infection (Fig. 2B). The peritoneal bacterial loads in control and noradrenergically ablated mice (Fig. 2D) were equivalent 4 and 10 hours after infection. However, by 24 hours after infection, noradrenergically ablated mice had significantly lower intraperitoneal bacterial loads than control mice (Fig. 2D). Serum bacterial loads were equivalent 10 and 24 hours after infection, 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 after infection or 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). In order to assess how the absence of NE affects cytokine production in response to infection, IL-6, TNF, IL-1β and INF-γ were measured in the serum and peritoneal fluid 4 and 24 hours after *K. pneumoniae* peritonitis. There were no statistical differences between TNF, IL-1b and INF-g levels in 6-OHDA treated mice compared to controls (Supplemental Fig. 2). However, IL-6 levels were elevated in the serum of 6-OHDA treated mice 4 hours after intraperitoneal infection with *K. pneumoniae* (Fig. 3B). By 24 hours following intraperitoneal infection, the intraperitoneal levels of IL-6 were lower in 6-OHDA treated mice, likely because 6-OHDA treated mice had lower bacterial loads 24 hours after infection (Fig. 2D-E).

In order to avoid the confounding effects of asymmetric bacterial growth on inflammatory cytokine production at latter time points in our infection model (Fig. 2D-E), we measured IL-6 levels after administration of sub-lethal LPS. Serum and peritoneal IL-6 levels were higher after LPS administration in mice lacking noradrenergic neurons (Fig. 3C-D). 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 intraperitoneal 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 $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice. The ablation of noradrenergic neurons in $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ 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, monocyte chemotactic protein-1 (MCP-1) levels in peritoneal fluid were determined after infection or LPS administration. MCP-1 levels were higher 4 hours after *K. pneumoniae* peritonitis and 1.5 hours after LPS in 6-OHDA treated mice (Fig. 4A and 4B). These results suggested that NE inhibits the production of MCP-1 during infectious or inflammatory stimuli.

In order 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_{1A}, α_{2A}, β1, β2, and β3 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-$1^{-/-}$ mice. Ablation of noradrenergic 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 after infection 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 (CMG), and then travel to the spleen via the splenic nerve where acetylcholine producing T cells act as intermediaries between splenic

nerve derived NE and α7 AChR 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). In order to isolate the role of splenic nerve derived catecholamines, splenectomies were performed on mice prior to administration of 6-OHDA. Survival after infection was then assessed in 4 groups; 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 hours after infection when compared to 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. In order to functionally characterize why splenectomized mice did not show improved survival after noradrenergic nerve ablation, peritoneal cell counts and differentials were performed 4 hours after infection in each of the four groups of mice (Fig. 5C-D). Mice treated with 6-OHDA after splenectomy failed to recruit monocytes to the peritoneum 4 hours after infection (Fig. 5 C-D), 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 hours after infection 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 norepinephrine 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 MI and stroke (21, 22). These cells reside in the subcapsular red pulp (SRP) of the spleen and can be identified by expression of CD11b in this anatomic location (22). In order to measure the migration of monocytes out of the spleen during infection in noradrenergically ablated or control mice, spleens were harvested 4 hours after infection and stained for CD11b. Representative spleen sections show a decrease in CD11b+ cells in the SRP of 6-OHDA treated mice (Fig. 6A-B). 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. 6 C). To further define the sub-type of monocytes recruited to the peritoneum in mice lacking noradrenergic neurons, mice in which eGFP has been knocked into the CX_3CR1 locus were utilized (23). Heterozygous mice express one functional copy of CX_3CR1 and express variable levels of eGFP in phagocytic cells. Monocytes in $CX_3CR1^{+/eGFP}$ mice retain the ability to home to the peritoneum during inflammatory responses (23) and both Ly-6C$^+$ and Ly-6C$^-$ monocytes express eGFP. The recruitment of Ly-6C$^+$ and Ly-6C$^-$ monocytes to the peritoneum was measured by multicolor flow cytometry. GFP$^+$CD11b$^+$ (CD11c F4/80 B220 CD3 NK1.1)$^-$Ly-6C$^+$ were considered Ly-6C$^+$ monocytes while GFP$^+$CD11b$^+$ (CD11c F4/80 B220 CD3 NK1.1)$^-$Ly-6C$^-$ were considered Ly-6C$^-$ monocytes. Importantly, there was a significant increase in both Ly-6C$^+$ and Ly-6C$^-$ monocytes recruited to the

peritoneum 4 hours after infection in mice lacking noradrenergic neurons when compared to baseline monocyte levels or monocyte levels 4 hours after infection in control mice (Fig. 6D). These results suggest ly-6C^{+} and Ly-6C^{-} 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

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 2 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 *P. aeruginosa* peritonitis, 6-OHDA treated mice had lower bacterial loads 7 hours after infection (28). In contrast, treatment with 6-OHDA prior to infection with the gram-positive organism *S. 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, heightened endogenous NE directly impaired innate immune defenses and predisposed mice to post stroke bacterial pneumonia (10) and in humans, epinephrine infusion prior to intravenous 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 the splenic nerve. The post-ganglionic splenic nerve is exclusively noradrenergic (6, 18) and is destroyed by 6-OHDA treatment (8). Thus, parasympathetic outflow directly modulates splenic norepinephrine 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 α7 acetylcholine receptor (AChR), 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 increases 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$^+$ and Ly-6C$^-$ monocytes to the peritoneum following *K. pneumoniae* infection is consistent with these findings. Collectively, our experiments, as well 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 cells 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 attenuate splenocyte derived TNF and improve survival during LPS induced inflammation or antibiotic treated cecal ligation and puncture (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 utilized. Immunologic alterations that enhance host antibacterial defense do not necessarily improve survival during LPS induced inflammation or antibiotic treated cecal ligation and puncture (CLP). In fact, immunologic alterations that enhance 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 inflammation during MI and stoke (22). Based on the findings that splenectomy attenuates the recruitment of monocytes to the peritoneum and 6-OHDA treated mice have fewer CD11b$^+$ cells in the spleen 4 hours after infection, 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 four-fold increase in peritoneal monocytes just 4 hours after the injection of 150 CFU of *K. pneumoniae*. Our data also indicate that the deployment of splenic monocytes may be an important component of normal host defense, since 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 further 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 immunity *in vivo* (18, 19, 38). Neural modulation of immunity appears to be evolutionarily conserved from nematodes to mammals (4, 14, 38-41), suggesting an important evolutionary benefit to effective neuro-immune communication. Our results are potentially 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 immunosuppression by inhibiting inflammatory cytokine secretion and reducing effective monocyte trafficking. In addition, administration of catecholamine vasopressors to patients with septic shock may predispose them to subsequent infections by suppressing monocyte recruitment to regions of infection. Finally, adrenergically active drugs, including beta-blockers, may have important unrecognized immunomodulatory effects. In the future, modulating noradrenergic neurons may provide a new therapeutic option for patients predisposed to or suffering from infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

NE	norepinephrine
LPS	lipopolysaccharide
MCP-1	monocyte chemotactic protein-1
6-OHDA	6 hydroxydopamine
IL-6	Interleukin-6

References

1. Martin GS, Mannino DM, Eaton S, Moss M. The epidemiology of sepsis in the United States from 1979 through 2000. N Engl J Med. 2003; 348:1546–1554. [PubMed: 12700374]
2. Bone RC, Fisher CJ Jr. Clemmer TP, Slotman GJ, Metz CA, Balk RA. A controlled clinical trial of high-dose methylprednisolone in the treatment of severe sepsis and septic shock. N Engl J Med. 1987; 317:653–658. [PubMed: 3306374]
3. Ziegler EJ, Fisher CJ Jr. Sprung CL, Straube RC, Sadoff JC, Foulke GE, Wortel CH, Fink MP, Dellinger RP, Teng NN, et al. Treatment of gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin. A randomized, double-blind, placebo-controlled trial. The HA-1A Sepsis Study Group. N Engl J Med. 1991; 324:429–436. [PubMed: 1988827]
4. Andersson U, Tracey KJ. Neural reflexes in inflammation and immunity. J Exp Med. 2012; 209:1057–1068. [PubMed: 22665702]
5. Tracey KJ. Reflex control of immunity. Nat Rev Immunol. 2009; 9:418–428. [PubMed: 19461672]
6. Vida G, Pena G, Deitch EA, Ulloa L. alpha7-cholinergic receptor mediates vagal induction of splenic norepinephrine. J Immunol. 2011; 186:4340–4346. [PubMed: 21339364]
7. Rosas-Ballina M, Olofsson PS, Ochani M, Valdes-Ferrer SI, Levine YA, Reardon C, Tusche MW, Pavlov VA, Andersson U, Chavan S, Mak TW, Tracey KJ. Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. Science. 2011; 334:98–101. [PubMed: 21921156]
8. Mina-Osorio P, Rosas-Ballina M, Valdes-Ferrer SI, Al-Abed Y, Tracey KJ, Diamond B. Neural signaling in the spleen controls B cell responses to blood-borne antigen. Mol Med. 2012
9. Grebe KM. Editorial: regulation of the regulator: sympathetic nervous system control of regulatory T cells. J Leukoc Biol. 2009; 86:1269–1270. [PubMed: 19948519]
10. Wong CH, Jenne CN, Lee WY, Leger C, Kubes P. Functional innervation of hepatic iNKT cells is immunosuppressive following stroke. Science. 2011; 334:101–105. [PubMed: 21921158]
11. Kostrzewa RM, Jacobowitz DM. Pharmacological actions of 6-hydroxydopamine. Pharmacol Rev. 1974; 26:199–288. [PubMed: 4376244]

12. Madden KS, Thyagarajan S, Felten DL. Alterations in sympathetic noradrenergic innervation in lymphoid organs with age. Ann N Y Acad Sci. 1998; 840:262–268. [PubMed: 9629254]
13. Tang Y, Shankar R, Gamboa M, Desai S, Gamelli RL, Jones SB. Norepinephrine modulates myelopoiesis after experimental thermal injury with sepsis. Ann Surg. 2001; 233:266–275. [PubMed: 11176134]
14. van der Poll T, Coyle SM, Barbosa K, Braxton CC, Lowry SF. Epinephrine inhibits tumor necrosis factor-alpha and potentiates interleukin 10 production during human endotoxemia. J Clin Invest. 1996; 97:713–719. [PubMed: 8609227]
15. Flierl MA, Rittirsch D, Nadeau BA, Sarma JV, Day DE, Lentsch AB, Huber-Lang MS, Ward PA. Upregulation of phagocyte-derived catecholamines augments the acute inflammatory response. PLoS One. 2009; 4:e4414. [PubMed: 19212441]
16. Sutherland RE, Olsen JS, McKinstry A, Villalta SA, Wolters PJ. Mast cell IL-6 improves survival from Klebsiella pneumonia and sepsis by enhancing neutrophil killing. J Immunol. 2008; 181:5598–5605. [PubMed: 18832718]
17. Wang XS, Lau HY. Beta-adrenoceptor-mediated inhibition of mediator release from human peripheral blood-derived mast cells. Clin Exp Pharmacol Physiol. 2006; 33:746–750. [PubMed: 16895550]
18. Rosas-Ballina M, Ochani M, Parrish WR, Ochani K, Harris YT, Huston JM, Chavan S, Tracey KJ. Splenic nerve is required for cholinergic antiinflammatory pathway control of TNF in endotoxemia. Proc Natl Acad Sci U S A. 2008; 105:11008–11013. [PubMed: 18669662]
19. Huston JM, Ochani M, Rosas-Ballina M, Liao H, Ochani K, Pavlov VA, Gallowitsch-Puerta M, Ashok M, Czura CJ, Foxwell B, Tracey KJ, Ulloa L. Splenectomy inactivates the cholinergic antiinflammatory pathway during lethal endotoxemia and polymicrobial sepsis. J Exp Med. 2006; 203:1623–1628. [PubMed: 16785311]
20. Vida G, Pena G, Kanashiro A, Thompson-Bonilla Mdel R, Palange D, Deitch EA, Ulloa L. beta2-Adrenoreceptors of regulatory lymphocytes are essential for vagal neuromodulation of the innate immune system. FASEB J. 2011; 25:4476–4485. [PubMed: 21840939]
21. Leuschner F, Rauch PJ, Ueno T, Gorbatov R, Marinelli B, Lee WW, Dutta P, Wei Y, Robbins C, Iwamoto Y, Sena B, Chudnovskiy A, Panizzi P, Keliher E, Higgins JM, Libby P, Moskowitz MA, Pittet MJ, Swirski FK, Weissleder R, Nahrendorf M. Rapid monocyte kinetics in acute myocardial infarction are sustained by extramedullary monocytopoiesis. J Exp Med. 2012; 209:123–137. [PubMed: 22213805]
22. Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, Figueiredo JL, Kohler RH, Chudnovskiy A, Waterman P, Aikawa E, Mempel TR, Libby P, Weissleder R, Pittet MJ. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. Science. 2009; 325:612–616. [PubMed: 19644120]
23. Jung S, Aliberti J, Graemmel P, Sunshine MJ, Kreutzberg GW, Sher A, Littman DR. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. Mol Cell Biol. 2000; 20:4106–4114. [PubMed: 10805752]
24. Flierl MA, Rittirsch D, Nadeau BA, Chen AJ, Sarma JV, Zetoune FS, McGuire SR, List RP, Day DE, Hoesel LM, Gao H, Van Rooijen N, Huber-Lang MS, Neubig RR, Ward PA. Phagocyte-derived catecholamines enhance acute inflammatory injury. Nature. 2007; 449:721–725. [PubMed: 17914358]
25. Straub RH, Rauch L, Rauh L, Pongratz G. Sympathetic inhibition of IL-6, IFN-gamma, and KC/CXCL1 and sympathetic stimulation of TGF-beta in spleen of early arthritic mice. Brain Behav Immun. 2011; 25:1708–1715. [PubMed: 21767631]
26. Harle P, Mobius D, Carr DJ, Scholmerich J, Straub RH. An opposing time-dependent immune-modulating effect of the sympathetic nervous system conferred by altering the cytokine profile in the local lymph nodes and spleen of mice with type II collagen-induced arthritis. Arthritis Rheum. 2005; 52:1305–1313. [PubMed: 15818682]
27. Spengler RN, Chensue SW, Giacherio DA, Blenk N, Kunkel SL. Endogenous norepinephrine regulates tumor necrosis factor-alpha production from macrophages in vitro. J Immunol. 1994; 152:3024–3031. [PubMed: 8144901]

28. Straub RH, Pongratz G, Weidler C, Linde HJ, Kirschning CJ, Gluck T, Scholmerich J, Falk W. Ablation of the sympathetic nervous system decreases gram-negative and increases gram-positive bacterial dissemination: key roles for tumor necrosis factor/phagocytes and interleukin-4/lymphocytes. J Infect Dis. 2005; 192:560–572. [PubMed: 16028124]
29. Grebe KM, Takeda K, Hickman HD, Bailey AL, Embry AC, Bennink JR, Yewdell JW. Cutting edge: Sympathetic nervous system increases proinflammatory cytokines and exacerbates influenza A virus pathogenesis. J Immunol. 2010; 184:540–544. [PubMed: 20018617]
30. Bosmann M, Grailer JJ, Zhu K, Matthay MA, Sarma JV, Zetoune FS, Ward PA. Anti-inflammatory effects of beta2 adrenergic receptor agonists in experimental acute lung injury. FASEB J. 2010; 26:2137–2144. [PubMed: 22318967]
31. van Westerloo DJ, Giebelen IA, Florquin S, Daalhuisen J, Bruno MJ, de Vos AF, Tracey KJ, van der Poll T. The cholinergic anti-inflammatory pathway regulates the host response during septic peritonitis. J Infect Dis. 2005; 191:2138–2148. [PubMed: 15898001]
32. Giebelen IA, Le Moine A, van den Pangaart PS, Sadis C, Goldman M, Florquin S, van der Poll T. Deficiency of alpha7 cholinergic receptors facilitates bacterial clearance in Escherichia coli peritonitis. J Infect Dis. 2008; 198:750–757. [PubMed: 18627272]
33. Saeed RW, Varma S, Peng-Nemeroff T, Sherry B, Balakhaneh D, Huston J, Tracey KJ, Al-Abed Y, Metz CN. Cholinergic stimulation blocks endothelial cell activation and leukocyte recruitment during inflammation. J Exp Med. 2005; 201:1113–1123. [PubMed: 15809354]
34. Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, Wang H, Abumrad N, Eaton JW, Tracey KJ. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature. 2000; 405:458–462. [PubMed: 10839541]
35. Wang H, Liao H, Ochani M, Justiniani M, Lin X, Yang L, Al-Abed Y, Metz C, Miller EJ, Tracey KJ, Ulloa L. Cholinergic agonists inhibit HMGB1 release and improve survival in experimental sepsis. Nat Med. 2004; 10:1216–1221. [PubMed: 15502843]
36. Pavlov VA, Ochani M, Yang LH, Gallowitsch-Puerta M, Ochani K, Lin X, Levi J, Parrish WR, Rosas-Ballina M, Czura CJ, Larosa GJ, Miller EJ, Tracey KJ, Al-Abed Y. Selective alpha7-nicotinic acetylcholine receptor agonist GTS-21 improves survival in murine endotoxemia and severe sepsis. Crit Care Med. 2007; 35:1139–1144. [PubMed: 17334244]
37. Craciun FL, Schuller ER, Remick DG. Early enhanced local neutrophil recruitment in peritonitis-induced sepsis improves bacterial clearance and survival. J Immunol. 2010; 185:6930–6938. [PubMed: 21041722]
38. Sun J, Singh V, Kajino-Sakamoto R, Aballay A. Neuronal GPCR controls innate immunity by regulating noncanonical unfolded protein response genes. Science. 2011; 332:729–732. [PubMed: 21474712]
39. Aladaileh S, Nair SV, Raftos DA. Effects of noradrenaline on immunological activity in Sydney rock oysters. Dev Comp Immunol. 2008; 32:627–636. [PubMed: 17988735]
40. Chang CC, Hung MD, Cheng W. Norepinephrine depresses the immunity and disease-resistance ability via alpha1- and beta1-adrenergic receptors of Macrobrachium rosenbergii. Dev Comp Immunol. 2011; 35:685–691. [PubMed: 21295067]
41. van der Poll T, Calvano SE, Kumar A, Coyle SM, Lowry SF. Epinephrine attenuates down-regulation of monocyte tumor necrosis factor receptors during human endotoxemia. J Leukoc Biol. 1997; 61:156–160. [PubMed: 9021920]

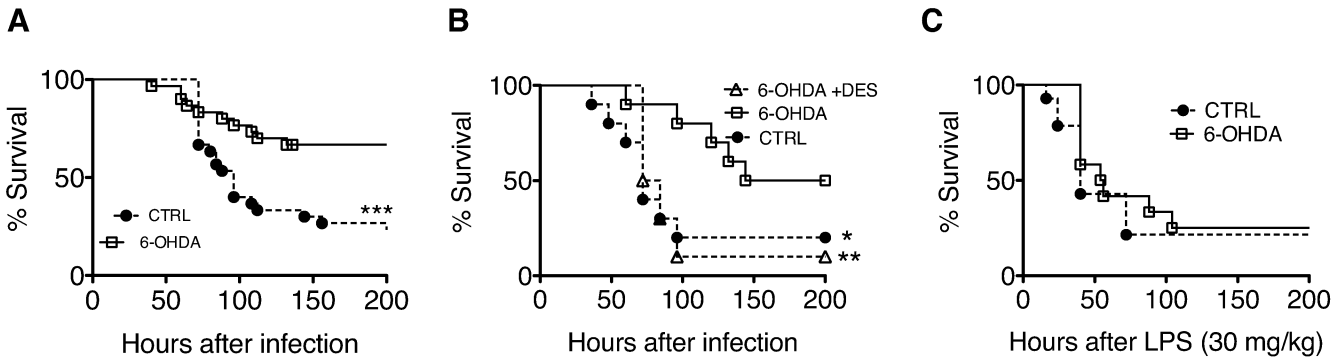

FIGURE 1.

Ablation of noradrenergic neurons improves survival during K. pneumoniae septic peritonitis. (A) Mice were injected with 250 mg/kg of 6-OHD or vehicle control 4 days prior to infection. 6-OHD treated or control mice were injected with 150 CFU of K. pneumoniae i.p. and morbidity or mortality were monitored. (B) The survival benefit of 6-OHD treatment was specific for noradrenergic nerve ablation because pretreatment with Desipramine (DES) 30 minutes before 6-OHD injection abrogated the survival benefit of 6-OHDA. (C) 6-OHD treatment prior to i.p. delivery of LPS (30 mg/kg) did not improve survival. (10-15 mice per experimental group, results are representative of 2-3 individual experiments) *P < 0.05, ** < 0.01, **P< 0.005.

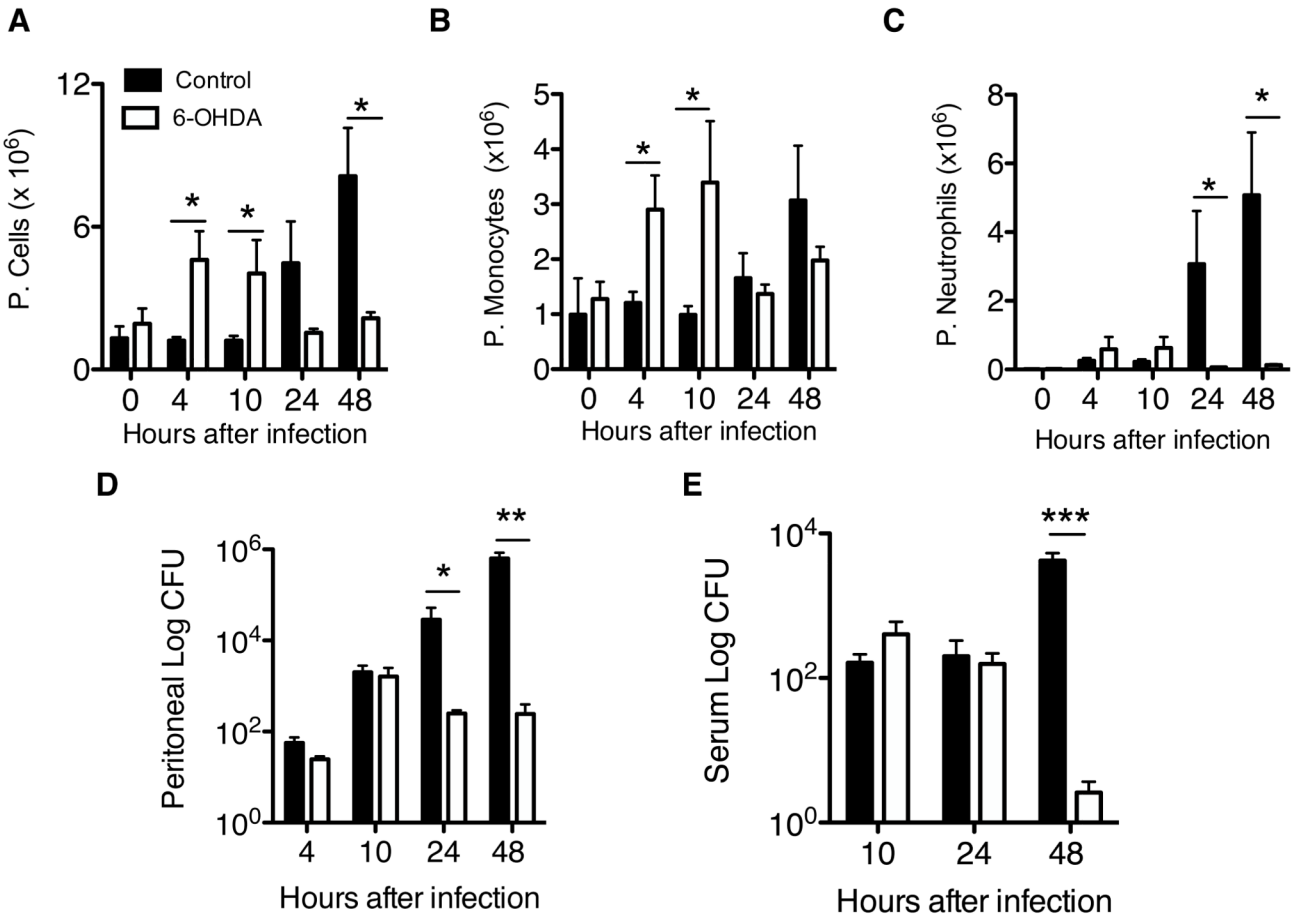

FIGURE 2.

Ablation of noradrenergic neurons enhances monocyte recruitment and bacterial clearance during septic peritonitis. Control (black bars) or 6-OHD treated mice (white bars) were sacrificed at baseline and 4, 10, 24 and 48 hours after infection with 150 CFU *K. pneumoniae*. (A) Total cell counts and differentials, including (B) total monocytes and (C) total neutrophils were measured in peritoneal lavage fluid at each time-point. (D) Peritoneal and (E) serum bacterial loads were quantified at each time point in 6-OHD treated or control mice. (10-15 mice per group, each time point performed 1-2 times). **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

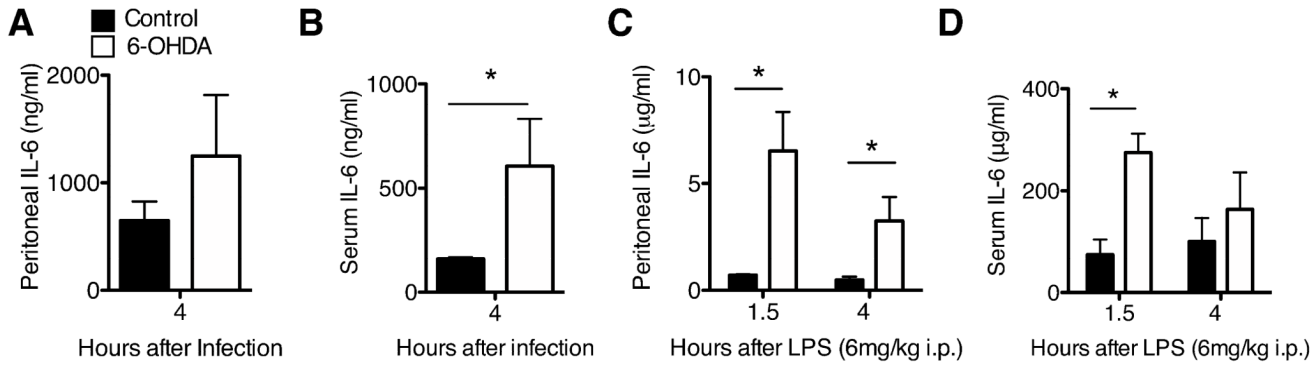

FIGURE 3.

Ablation of noradrenergic neurons leads to increased IL-6 secretion after infection or LPS mediated inflammation. (A, B) IL-6 levels were measured by ELIS in peritoneal fluid and serum harvested 4 hours after *K. pneumoniae* infection or (C, D) 1.5 and 4 hours after sub-lethal LPS administration in control (black bars) and 6-OHD treated mice (white bars). (n= 5-15 mice per group) **P*< 0.05.

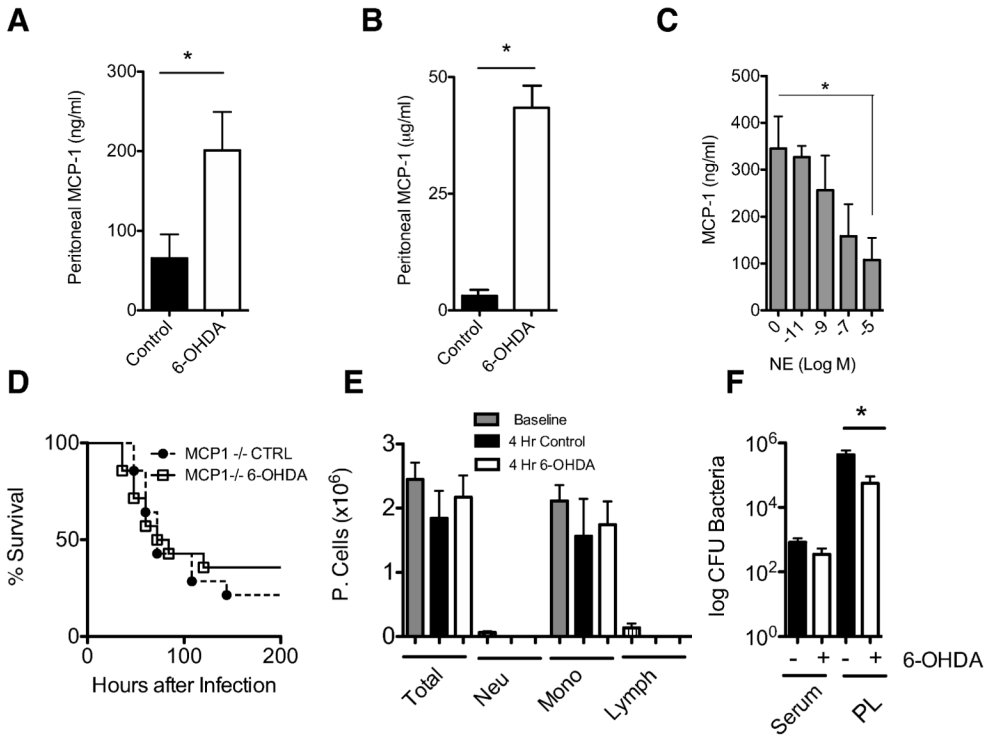

FIGURE 4.

The survival benefit of noradrenergic nerve ablation is dependent on MCP-1. (A) MCP-1 levels were measured by ELISA in the peritoneal lavage fluid of control or 6-OHD treated mice 4 hours after i.p. injection of 150 CFU *K. pneumoniae* or (B) 1.5 hours after LPS (6 mg/kg) administration. MCP-1 levels were higher in 6-OHD treated mice than in controls. (C) The addition of NE to primary peritoneal macrophages stimulated in vitro with heat killed *K. pneumoniae* suppressed the production of MCP-1 in culture supernatants. (D) Control or 6-OHDA treated MCP-1$^{-/-}$ mice were infected with 150 CFU *K. pneumoniae* and survival was monitored (n=12 mice per group). (E) Peritoneal recruitment of leukocytes was measured at baseline (gray) or 4 hours after infection in control (black) and 6-OHDA treated mice (white). (F) Serum and peritoneal lavage bacterial loads were measured 48 hours after infection in 6-OHDA treated or control mice (n=10-12 mice per group). *$P <$ 0.05.

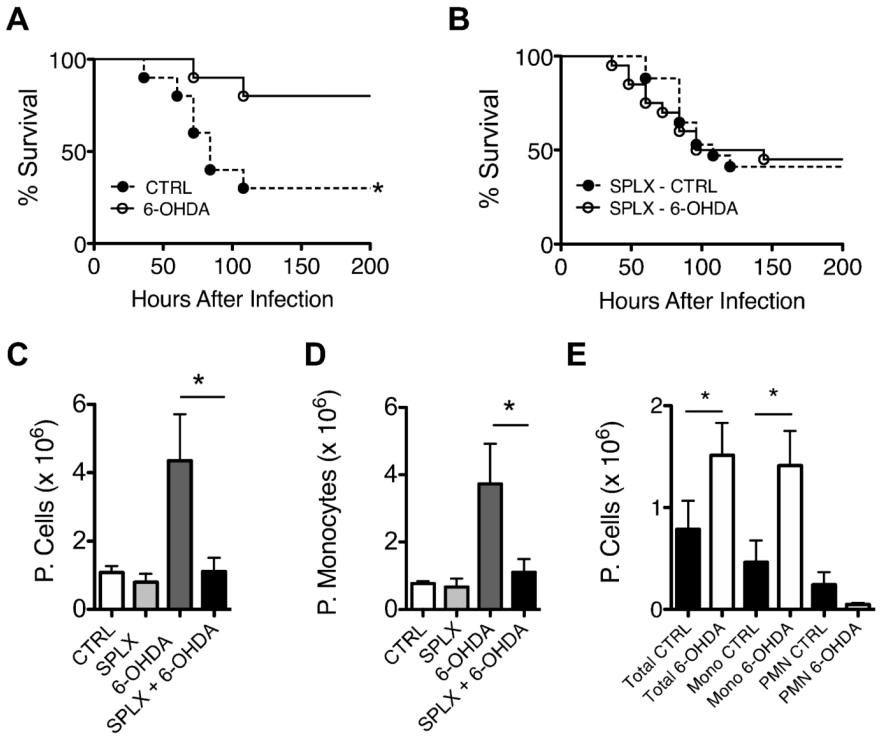

FIGURE 5.

The spleen is required for monocyte recruitment in noradrenergically ablated mice. (A-B) Splenectomies were performed and 2 weeks later mice were treated with 6-OHDA or vehicle control. 4 days after 6-OHDA, mice were infected with *K. pneumoniae* and survival was monitored. Splenectomy prior to 6-OHDA treatment eliminated the survival benefit of noradrenergic nerve ablation (experiments in panels A,B were performed at the same time). (C,D) Similar groups of mice were then sacrificed 4 hours after infection and total peritoneal cell counts and differentials were performed, showing that the spleen is required for monocyte recruitment in 6-OHDA treated mice. (E) Splenic T cells are not required for improved monocyte recruitment because 6-OHDA treatment led to enhanced leukocyte recruitment in T cell deficient (Nude) mice 4 hours after *K. pneumoniae* infection. Experiments performed 2-3 times with 5-15 mice. * $P < 0.05$.

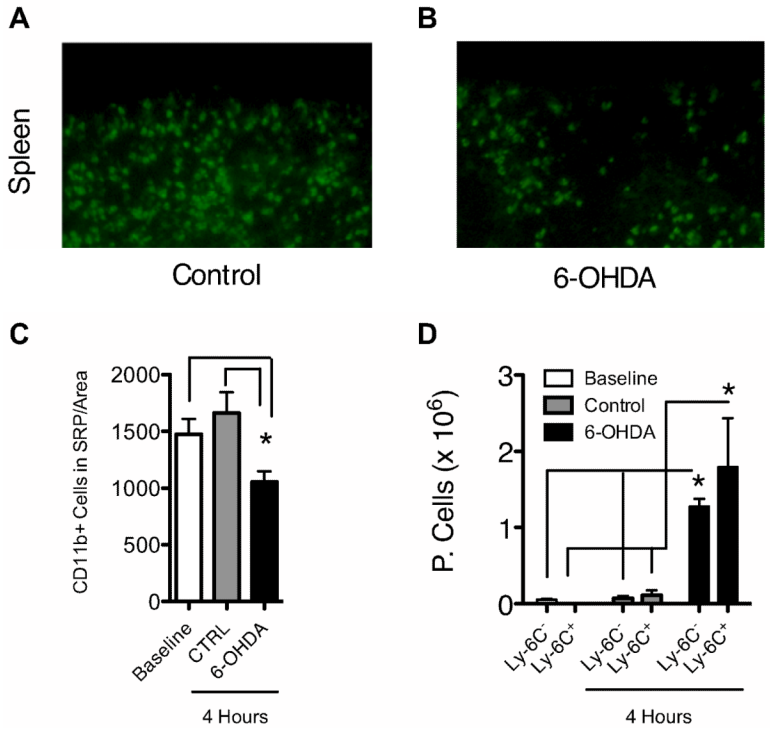

FIGURE 6.

Noradrenergic nerve ablation enhances the egress of splenic monocyte during infection. (A-B) Representative images of subcapsular red pulp (SRP) spleen sections immunostained for CD11b harvested from (A) control or (B) 6-OHDA treated mice 4 hours after infection with *K. pneumoniae*. (C) Quantification of $CD11b^{+}$ cells in the spleen shows that 4 hours after infection, 6-OHDA treated mice have fewer CD11b immunoreactive cells in the SRP than control mice. (D) Monocyte subsets recruited to the peritoneum 4 hours after infection were quantified using $Cx3Cr1^{+/eGFP}$ mice. 6-OHDA treated mice recruited both $Ly6C^{-}$ and $Ly6C^{+}$ monocytes more rapidly to the peritoneum than control mice. (n=3-6 mice per group, similar observations were made in experiments, performed 2-3 times.) $*P < 0.05$.