MAJOR ARTICLE

The Cholinergic Anti-Inflammatory Pathway Regulates the Host Response during Septic Peritonitis

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Background. The nervous system, through the vagus nerve, can down-regulate inflammation in vivo by decreasing the release of tumor necrosis factor–α by endotoxin-stimulated macrophages. This anti-inflammatory effect is mediated by an interaction between acetylcholine, the principal neurotransmitter of the vagus nerve, and cholinergic nicotinic acetylcholine receptors on macrophages.

Methods. We determined the role of this "cholinergic anti-inflammatory pathway" during septic peritonitis induced in mice by intraperitoneal injection of live *Escherichia coli.* Septic peritonitis was preceded by inhibition of the cholinergic anti-inflammatory pathway by unilateral cervical vagotomy, by stimulation of this pathway by pretreatment of mice with nicotine, or by a combination of both interventions.

Results. Initial cytokine release during septic peritonitis was enhanced after previous vagotomy and was decreased after nicotine pretreatment, independently of the integrity of the vagus nerve. Further study established that vagotomy before septic peritonitis resulted in an enhanced influx of neutrophils and a marked increase in proinflammatory cytokine levels and liver damage. Conversely, nicotine pretreatment strongly decreased cell influx, proinflammatory cytokine levels, and liver damage, whereas bacterial clearance and survival were impaired.

Discussion. These data provide the first evidence, to our knowledge, of an important role of the vagus nerve in regulating the innate immune response to a severe bacterial infection.

Innate immunity is the first line of defense against invading pathogens [1]. The innate immune system is tightly regulated and consists of a plethora of cell-associated receptors, cytokines, chemokines, and other mediators that orchestrate the early response to infection [2–4]. At the first encounter with pathogens, the host seeks to ensure an adequate inflammatory reaction to combat infection but at the same time tries to prevent collateral damage to tissues due to excessive immune activation. Failure to control inflammation during infection may result in the clinical syndrome of sepsis, characterized by a damaging systemic inflammatory response and distant organ injury. As such, limiting the acute inflammatory response to an infection is an important task of the immune system, and several counterregulatory mechanisms exist to accomplish this, including the release of anti-inflammatory cytokines, soluble cytokine inhibitors, and stress hormones [2–4].

Recently, the cholinergic nervous system was identified as a pathway that reflexively monitors and modifies the inflammatory response [5, 6]. The most compelling evidence for a role of the cholinergic nervous system in the regulation of inflammation is derived from studies of rodents challenged with endotoxin (lipopolysaccharide [LPS]), the proinflammatory component of the outer membrane of gram-negative bacteria [7, 8]. In studies of experimental endotoxemia in rats, surgical dissection of the vagus nerve led to enhanced systemic tumor necrosis factor (TNF)–α production and accelerated the development of shock; in turn, electrical stimulation of the vagus nerve down-

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Table 1. Enhancement of early cytokine release, as a result of vagotomy, and inhibition of early cytokine release (independent of the integrity of the vagus nerve), as a result of nicotine pretreatment.

	Control plus		Nicotine pretreatment plus	
Measure	Sham surgery	Vagotomy	Sham surgery	Vagotomy
Cell count, 10^5 cells/mL				
Total	6.7 ± 0.6	8.9 ± 0.5	7.0 ± 0.8	6.3 ± 0.9
Granulocytes	4.8 ± 0.4	6.9 ± 1.1	4.7 ± 0.6	4.1 ± 0.6
Macrophages	1.7 ± 0.2	1.7 ± 0.3	2.1 ± 0.3	2.2 ± 0.3
Cytokine/chemokine level, pg/mL				
TNF-α	677 ± 61	1131 ± 132[a]	334 ± 52[a]	456 ± 32[a]
IL-1β	278 ± 41	412 ± 21[a]	105 ± 21[a]	142 ± 19[a]
IL-6	2478 ± 478	4878 ± 512[a]	1228 ± 216[a]	1218 ± 412[a]
KC	568 ± 32	705 ± 102	532 ± 62	612 ± 58

NOTE. Data are means ± SEs of 8 mice/group at 6 h after infection. Cell counts in the table have been divided by 1×10^5. Septic peritonitis was induced by intraperitoneal injection of 1×10^4 cfu of *Escherichia coli*. Four days before infection, mice were subjected to sham surgery, left-sided cervical vagotomy, pretreatment with drinking water supplemented with nicotine (100 μg/mL), or a combination of vagotomy and nicotine pretreatment. Differences between groups were calculated by 1-way analysis of variance. IL, interleukin; KC, cytokine-induced neutrophil chemoattractant; TNF, tumor necrosis factor.

[a] $P < .05$ vs. sham-operated mice with septic peritonitis not treated with nicotine.

regulated TNF-α production and protected the animals from hypotension [7]. Vagus nerve stimulation also inhibited the acute inflammatory response to acute hypovolemic hemorrhagic shock [9]. The vagus nerve exerts anti-inflammatory effects through its major neurotransmitter acetylcholine, which interacts with nicotinic acetylcholine receptors on macrophages, resulting in inhibition of LPS-induced release of TNF-α and other proinflammatory cytokines [7, 8]; the acetylcholine receptor α7 subunit is required for this effect [8]. Hence, this "cholinergic anti-inflammatory pathway" provides the host with a powerful mechanism for "sensing" inflammation via sensory pathways that relay information to the brain, as well as for counteracting excessive inflammation in a very fast, discrete, and localized way through acetylcholine released by the efferent vagus nerve.

Knowledge of the role of the anti-inflammatory cholinergic pathway during infection is not available. Therefore, in the present study, we sought to determine whether this anti-inflammatory pathway regulates host responses during experimental abdominal sepsis induced by intraperitoneal injection with live *Escherichia coli*. We studied the host response to infection in mice in which this pathway was disrupted by cervical vagotomy and in mice in which the peripheral part of this pathway—nicotinic acetylcholine receptors on macrophages—was stimulated by pretreatment with nicotine.

METHODS

Mice. Female C57BL/6 mice (Harlan; Horst), 8–10 weeks old, were used in all experiments. The protocol was approved by the Institutional Animal Care and Use Committee of the Academic Medical Center, University of Amsterdam.

Experimental groups. In a first study (study 1) we evaluated the role of the vagus nerve and nicotinic receptors in the initial host response during septic peritonitis. Mice were subjected to sham surgery, unilateral cervical vagotomy, nicotine pretreatment, or a combination of vagotomy and nicotine pretreatment. To inhibit the cholinergic anti-inflammatory pathway, we subjected mice to unilateral (left-sided) cervical vagotomy or sham surgery 4 days before induction of septic peritonitis, as described elsewhere [7]. For this procedure, mice were anesthetized by intraperitoneal injection of 0.07 mL/g FFM mixture (0.315 mg/mL fentanyl [Janssen], 10 mg/mL fluanisone, [Janssen], and 5 mg/mL midazolam [Roche]). A ventral cervical midline incision was used to expose the left cervical vagus trunk, which was ligated with 4-0 silk sutures and divided. Subsequently, the skin was closed with 3 sutures. In sham-operated mice, the left vagus nerve was exposed and isolated from surrounding tissue but was not transected. A unilateral vagotomy was chosen because early experiments showed that bilateral cervical vagotomy is lethal in mice (data not shown). In initial experiments, we compared the effect of left-sided versus right-sided vagotomy and found no major differences (see Results). The peripheral part of the cholinergic anti-inflammatory pathway (nicotinic acetylcholine receptors on macrophages) was stimulated by pretreatment of mice with nicotine (Sigma) added to the drinking water (100 μg/mL), starting 4 days before induction of septic peritonitis [10, 11]; control mice received normal drinking water. All mice were killed 6 h after infection. Hence, 4 groups of mice were studied ($n = 8$ mice/group): (1) normal drinking water plus sham surgery, (2) normal drinking water plus vagotomy, (3) nicotine pretreatment plus sham surgery, and (4) nicotine pretreatment plus vagotomy.

In a separate study (study 2), the effects of vagotomy on host

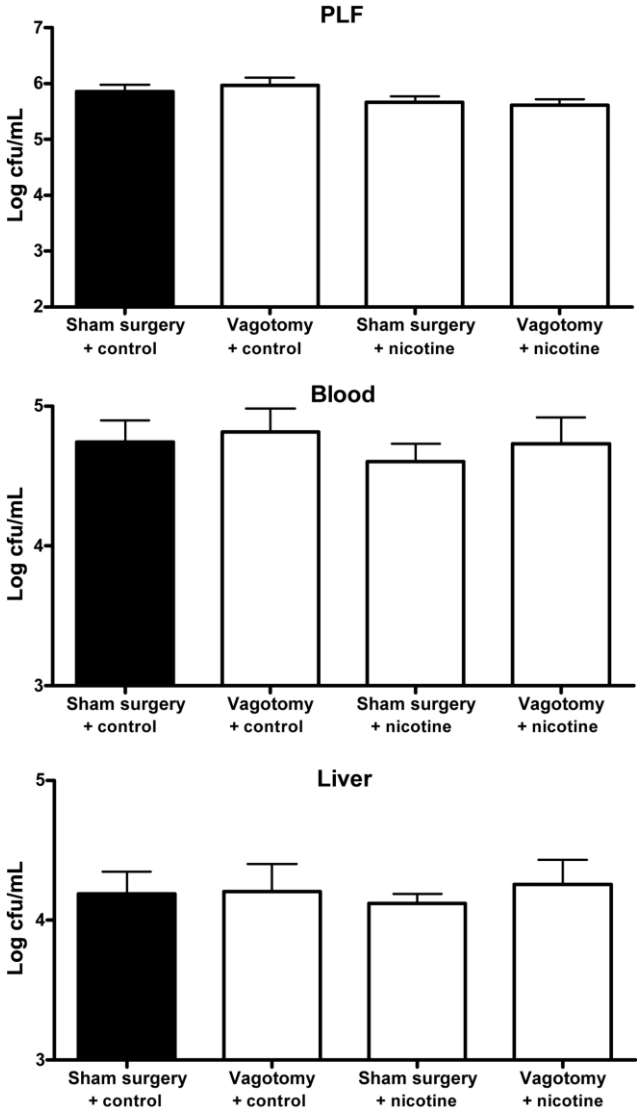

Figure 1. Lack of influence of interference with the cholinergic anti-inflammatory pathway on early bacterial outgrowth. Septic peritonitis was induced by intraperitoneal injection of *Escherichia coli*. Four days before infection, mice were subjected to left-sided cervical vagotomy, nicotine pretreatment (100 μg/mL added to drinking water), or a combination of vagotomy and nicotine pretreatment. Data are means ± SEs of 8 mice/group at 6 h after infection. Differences between groups were calculated by 1-way analysis of variance and were not found to be significant. PLF, peritoneal lavage fluid.

defense and organ damage were evaluated during more-established sepsis. In this study, mice ($n = 8$ mice/group) were subjected to sham surgery or vagotomy as described above and were killed 24 h after infection. In addition, in separate groups of mice ($n = 12$ mice/group), survival was monitored for 3 days.

In another separate study (study 3), the effects of nicotine pretreatment on host defense and organ damage were evaluated during more-established sepsis. In this study, mice ($n = 8$ mice/group) were subjected to control or nicotine pretreatment as described above and were killed 24 h after infection. In addition, in separate groups of mice ($n = 12$ mice/group), survival was monitored for 3 days.

Induction of septic peritonitis. Septic peritonitis was induced as described elsewhere [12–14]. In brief, *E. coli* O18:K1 was cultured in Luria-Bertani medium (Difco) at 37°C, harvested at mid–log phase, and washed twice before inoculation. Mice were injected intraperitoneally with ~1×10^4–5×10^4 cfu of *E. coli* in 200 μL of sterile saline. The inoculum was plated on blood agar plates to determine the exact number of viable bacteria (in retrospect, 1×10^4 cfu in studies 1 and 2 and 5×10^4 cfu in study 3). Some mice were not injected with *E. coli*, for determination of plasma ALT and AST levels in nonseptic animals. Mice were killed 6 or 24 h after infection; preceding this procedure, mice were anesthetized by inhalation of isoflurane, and peritoneal lavage was performed with 5 mL of sterile isotonic saline by use of an 18-gauge needle. Peritoneal lavage fluid was collected in sterile tubes and put on ice. After collection of peritoneal lavage fluid, deeper anesthesia was induced by intraperitoneal injection of 0.07 mL/g FFM (as described above). After opening of the abdomen, blood was drawn from the vena cava inferior, collected in sterile tubes containing heparin, and immediately placed on ice. Livers were subsequently harvested for histological analysis.

Cell counts and differentials. Cell counts were determined in each peritoneal lavage sample by use of a hemocytometer (Türck counting chamber). The cells were then diluted to a final concentration of 1×10^5 cells/mL, and differential cell counts were performed on cytospin preparations stained with Giemsa.

Assays. Cytokines and chemokines (TNF-α, interleukin

Table 2. Increases in cell influx and cytokine and chemokine levels in peritoneal lavage fluid (determined 24 h after infection), as a result of vagotomy.

Measure	Sham surgery plus septic peritonitis	Vagotomy plus septic peritonitis
Cell count, 10^6 cells/mL		
Total	5.5 ± 0.4	27.2 ± 7.3[a]
Granulocytes	4.1 ± 0.3	22.7 ± 1.9[a]
Macrophages	1.3 ± 0.2	4.0 ± 0.9[a]
Cytokine/chemokine level, pg/mL		
TNF-α	200 ± 109	685 ± 111[a]
IL-1β	309 ± 41	488 ± 43[a]
IL-6	4070 ± 1015	7875 ± 1207[a]
KC	684 ± 92	1057 ± 122[a]

NOTE. Data are means ± SEs of 8 mice/group at 24 h after infection. Cell counts in the table have been divided by 1×10^6. Septic peritonitis was induced by intraperitoneal injection of 1×10^4 cfu of *Escherichia coli*. Four days before infection, mice were subjected to left-sided cervical vagotomy or sham surgery. Differences between groups were calculated by Mann-Whitney *U* test. IL, interleukin; KC, cytokine-induced neutrophil chemoattractant; TNF, tumor necrosis factor.

[a] $P < .05$ vs. sham-operated mice with septic peritonitis.

Table 3. Reduction of cell influx and cytokine and chemokine levels in peritoneal lavage fluid (determined 24 h after infection), as a result of nicotine pretreatment.

Measure	Control plus septic peritonitis	Nicotine pretreatment plus septic peritonitis
Cell count, 10^6 cells/mL		
Total	6.4 ± 0.8	2.9 ± 0.4[a]
Granulocytes	5.1 ± 0.6	1.9 ± 0.3[a]
Macrophages	1.2 ± 0.2	0.9 ± 0.2
Cytokine/chemokine level, pg/mL		
TNF-α	687 ± 270	238 ± 128[a]
IL-1β	652 ± 132	230 ± 35[a]
IL-6	10,719 ± 5413	6643 ± 937[a]
KC	1487 ± 356	953 ± 156[a]

NOTE. Data are means ± SEs of 8 mice/group at 24 h after infection. Cell counts in the table have been divided by 1×10^6. Septic peritonitis was induced by intraperitoneal injection of 5×10^4 cfu of *Escherichia coli*. From 4 days before infection, mice received either normal drinking water of drinking water supplemented with nicotine (100 μg/mL). Differences between groups were calculated by Mann-Whitney *U* test. IL, interleukin; KC, cytokine-induced neutrophil chemoattractant; TNF, tumor necrosis factor.

[a] $P < .05$ vs. control mice with septic peritonitis.

[IL]–1β, IL-6, and cytokine-induced neutrophil chemoattractant [KC]) were measured using specific ELISAs (R&D Systems) in accordance with the manufacturer's instructions. The detection limits were 31 pg/mL for TNF-α, 16 pg/mL for IL-1β, 16 pg/mL for IL-6, and 12 pg/mL for KC. Concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined with commercially available kits (Sigma) by use of a Hitachi analyzer (Boehringer Mannheim) in accordance with the manufacturer's instructions.

Histological assessment. Livers for histological assessment were harvested 24 h after infection, fixed in 10% formalin, and embedded in paraffin. Four-micrometer sections were stained with hematoxylin-eosin (HE) and analyzed by a pathologist who was blinded to the groups. To score liver injury, the following parameters were analyzed: formation of thrombi, hepatocellular necrosis, and portal inflammation. Each parameter was graded on a scale of 0–4, with 0 meaning "absent," 1 meaning "occasional," 2 meaning "mild," 3 meaning "moderate," and 4 meaning "severe." The total injury score was expressed as the sum of the scores for all parameters, the maximum being 12. Granulocyte staining was performed as described elsewhere [15, 16]. In brief, slides were deparaffinized, and endogenous peroxydase activity was quenched by a solution of methanol/0.03% H_2O_2 (Merck). After digestion with a 0.25% solution of pepsin (Sigma) in 0.01 mol/L HCl, the sections were incubated in 10% normal goat serum (Dako) and then exposed to fluorescein isothiocyanate (FITC)–labeled anti–mouse Ly-6 monoclonal antibody (Pharmingen). Slides were then incubated with a rabbit anti-FITC antibody (Dako) and then were further incubated with a biotinylated swine anti-rabbit antibody (Dako), rinsed again, incubated in a streptavidin-ABC solution (Dako), and developed using 1% H_2O_2 and 3.3′-diaminobenzidin-tetra-hydrochloride (Sigma) in Tris-HCl. After light counterstaining with methyl-green, the sections were mounted in glycerin gelatin and analyzed. Anti–active caspase 3 staining was used to detect apoptotic bodies, as described elsewhere [15]. In brief, deparaffinized slides were boiled for 5 min 2 times in citrate buffer (pH 6.0). Nonspecific binding and endogenous peroxydase activity were blocked, incubated with a rabbit anti-human active caspase 3 polyclonal antibody (Cell Signaling), and then incubated with a biotinylated swine anti-rabbit antibody (Dako). The slides were further developed as described above for the Ly-6 protocol. All antibodies were used in concentrations recommended by the manufacturers. The intensities of the granulocyte and anti–active caspase 3 staining were scored on a semiquantitative scale (0, absent; 1, few positive cells; 2, moderate staining; 3, frequent staining; 4, abundant staining).

Enumeration of bacteria and monitoring of survival. Liver lobes were harvested and homogenized at 4°C in 4 volumes of sterile saline, by use of a tissue homogenizer (Biospec Products). Colony-forming units were determined from serial dilutions of peritoneal lavage fluid, liver, and blood; were plated on blood agar plates; and were incubated for 16 h at 37°C before colonies were counted. All culture plates revealed pure cultures of *E. coli* O18:K1 only. Of note, nicotine did not influence the growth of *E. coli* in vitro. In survival studies, mortality was assessed every 2 h for 72 h.

Statistical analysis. Differences between groups were calculated by Mann-Whitney *U* test or by 1-way analysis of variance followed by Tukey's posttest when >2 groups were compared. For survival analysis, Kaplan-Meier analysis followed by a log-rank test was performed. Values are expressed as means ± SEs unless indicated otherwise. $P < .05$ was considered to be statistically significant.

RESULTS

Left-sided versus right-sided cervical vagotomy. All mice tolerated unilateral cervical vagotomy well; other than a transient weight loss (maximum, 10%–15%) during the first 3 days after the procedure, no sickness behavior or mortality occurred in any mouse up to several weeks thereafter. Sham-operated mice also demonstrated a transient weight loss (5%–10%) during the first 2 days after the procedure. At the time that septic peritonitis was induced, all mice had regained their original body weight. Left-sided and right-sided unilateral cervical vagotomy influenced host responses during septic peritonitis (described below) in a similar way. To simplify the figures and data presentation, only the results obtained after left-sided unilateral vagotomy are given.

Regulation of initial cytokine release during septic perito-

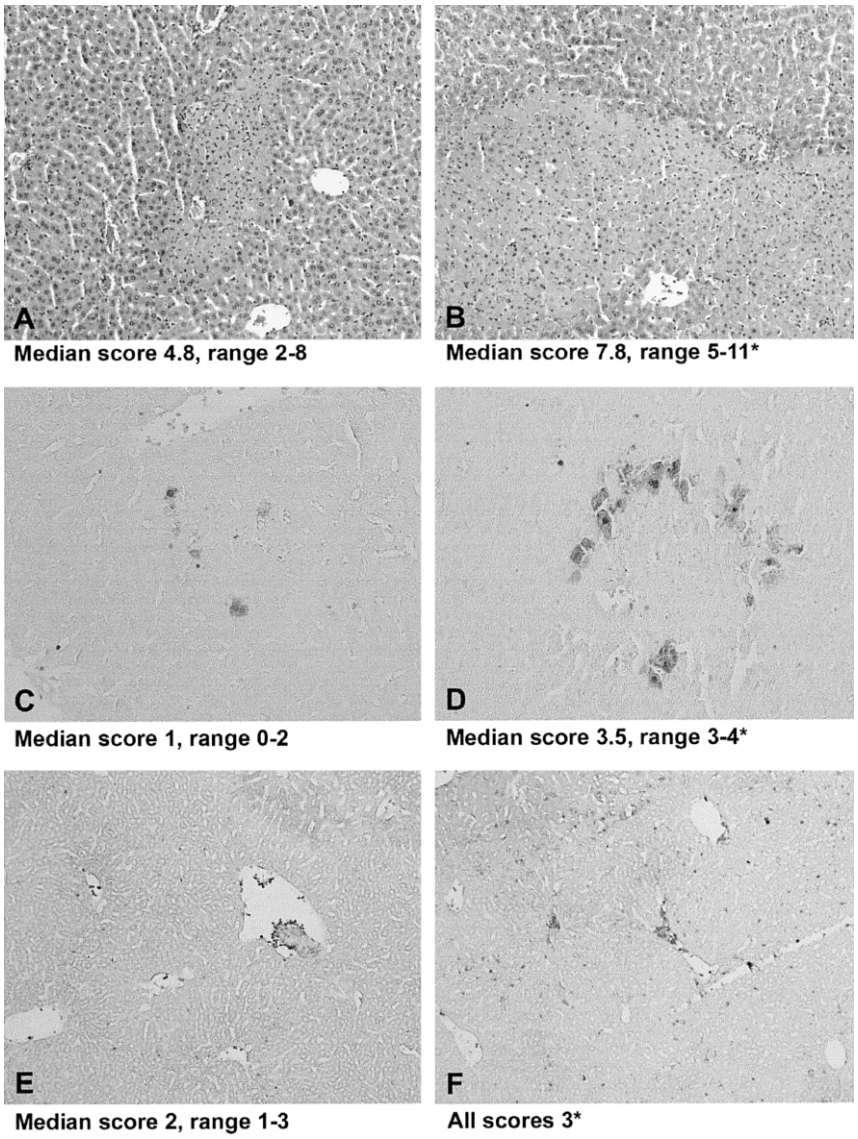

Figure 2. Increase in histopathological damage to the liver during septic peritonitis, as a result of vagotomy. Septic peritonitis was induced by intraperitoneal injection of *Escherichia coli.* Four days before infection, mice were subjected to left-sided cervical vagotomy (*B, D,* and *F*) or sham surgery (*A, C,* and *E*). Representative liver sections, obtained 24 h after infection, are shown, with medians and ranges derived from semiquantitative analyses (see Methods) of 8 mice/group indicated below each panel. *A* and *B,* Hematoxylin-eosin staining (×10). *C* and *D,* Anti–active caspase 3 staining (×20). *E* and *F,* Anti–Ly-6 (granulocyte) staining (×10). Differences between groups were calculated by Mann-Whitney *U* test. *$P < .05$ vs. sham-operated mice.

nitis by nicotinic receptors and the vagus nerve. Intraperitoneal injection of *E. coli* results in a strong and rapid inflammatory response within the abdominal cavity, characterized by the release of inflammatory mediators and the recruitment of leukocytes into the peritoneal lavage fluid [12, 14]. First, we evaluated whether the initial inflammatory response during septic peritonitis is mediated by the vagus nerve and nicotinic receptors. Six hours after infection, mice subjected to vagotomy showed increased levels of TNF-α, IL-6, and IL-1β in peritoneal lavage fluid ($P < .05$) (table 1) and plasma (not shown), compared with those in sham-operated mice. Conversely, mice pretreated with nicotine displayed lower levels of these mediators in peritoneal lavage fluid ($P < .05$) (table 1) and plasma (not shown). Interestingly, the proinflammatory effects of vagotomy were reversed in mice subjected to vagotomy that were concurrently pretreated with nicotine ($P < .05$) (table 1). Of note, at this early time point, the number of neutrophils (table 1) and colony-forming units recovered from the peritoneal lavage fluid, blood, and liver (figure 1) were comparable in all groups.

Vagotomy exaggerates, whereas nicotine attenuates, the inflammatory response to established septic peritonitis. Unilateral cervical vagotomy before induction of septic peritonitis was associated with a significantly enhanced influx of leukocytes into the peritoneal fluid 24 h after infection ($P < .05$ vs.

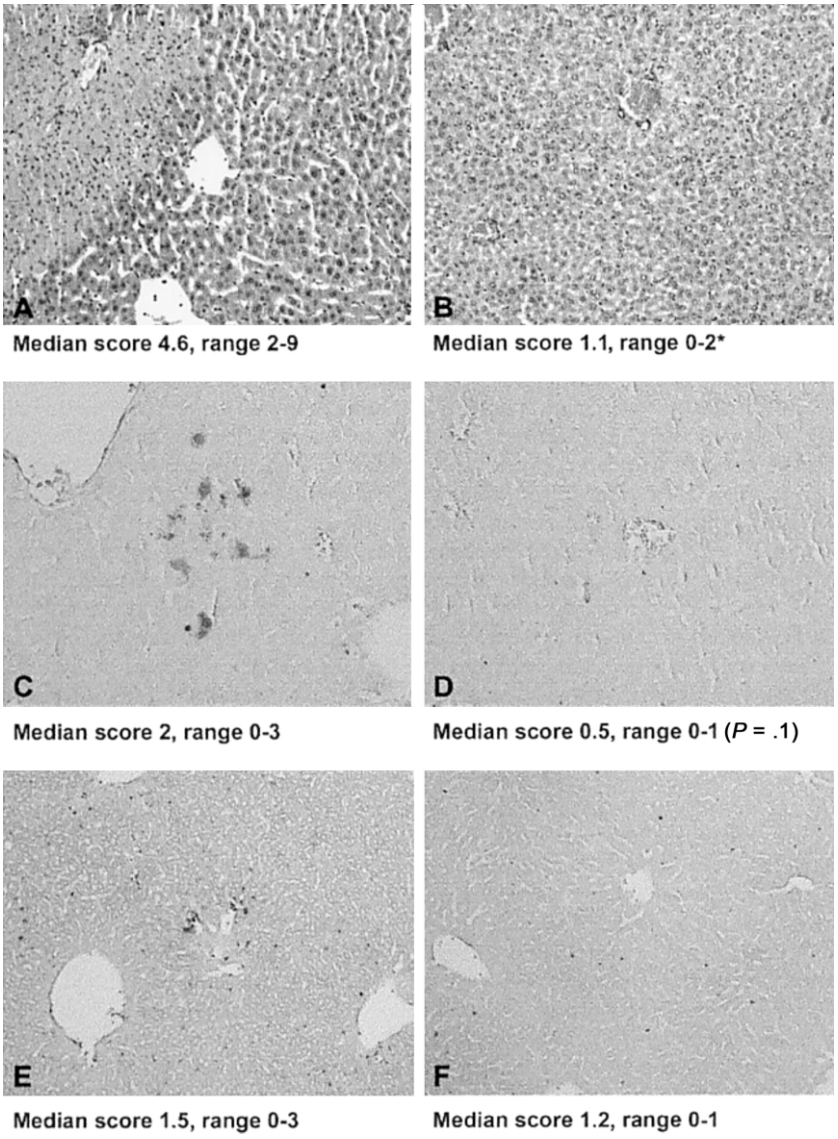

Figure 3. Attenuation of histopathological damage to the liver during septic peritonitis, as a result of pretreatment with nicotine. Septic peritonitis was induced by intraperitoneal injection of *Escherichia coli*. Starting 4 days before infection, mice received either drinking water supplemented with nicotine (100 μg/mL) (*B, D,* and *F*) or normal drinking water (*A, C,* and *E*). Representative slides, obtained 24 h after infection, are shown, with medians and ranges derived from semiquantitative analyses (see Methods) of 8 mice/group indicated below each picture. *A* and *B,* Hematoxylin-eosin staining (×10). *C* and *D,* Anti–active caspase 3 staining (×20). *E* and *F,* Anti–Ly-6 (granulocyte) staining (×10). Differences between groups were calculated by Mann-Whitney *U* test. **P* < .05 vs. control mice.

sham-operated mice), which was almost exclusively caused by an increased invasion of neutrophils (table 2). In addition, vagotomy resulted in higher local levels of TNF-α, IL-1β, IL-6, and KC during septic peritonitis, compared with those in infected sham-operated mice (all 4 cytokines/chemokines, $P <$ .05 for differences between groups) (table 2). In contrast, nicotine treatment was associated with a decreased influx of neutrophils and lower levels of cytokines and chemokines in peritoneal lavage fluid during septic peritonitis, compared with those in mice with septic peritonitis that did not receive nicotine pretreatment (all 4 cytokines/chemokines, $P < .05$ for differences between groups) (table 3). Plasma cytokine levels were influenced by vagotomy and nicotine pretreatment in a manner similar to peritoneal lavage fluid levels (data not shown).

Vagotomy increases, whereas nicotine attenuates, liver injury. Liver injury is one of the hallmarks of distant organ damage in experimental septic peritonitis and is related to the extent of systemic inflammation [12–14]. Mice not injected with *E. coli* did not display evidence of necrosis or apoptosis in their livers, irrespective of whether they were subjected to vagotomy or nicotine pretreatment (data not shown). Septic peritonitis in mice in which the cholinergic system was not manipulated by either

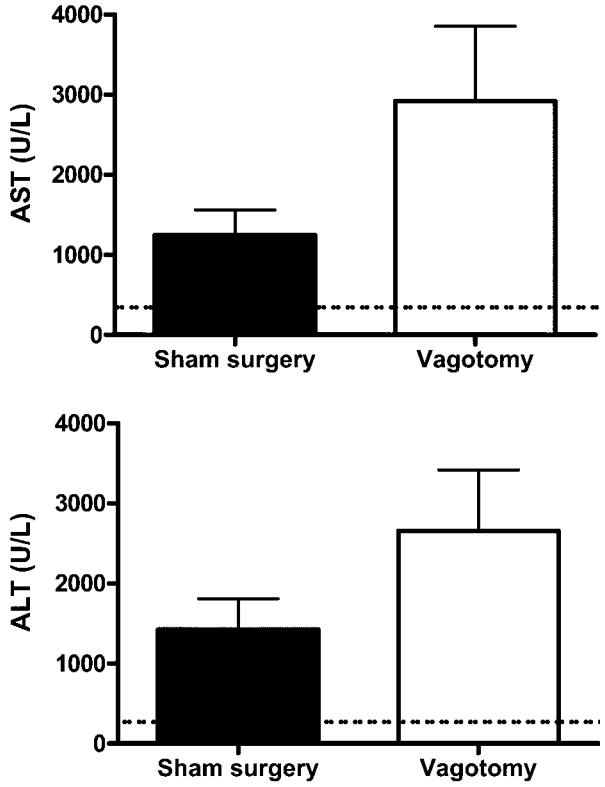

Figure 4. Effect of vagotomy on plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Septic peritonitis was induced by intraperitoneal injection of *Escherichia coli*. Four days before infection, mice were subjected to left-sided cervical vagotomy or sham surgery. Data are means ± SEs of 8 mice/group at 24 h after infection. The dotted line represents the transaminase level in uninfected mice. Differences between groups were calculated by Mann-Whitney *U* test and were not found to be significant.

vagotomy or nicotine pretreatment was accompanied by histopathological evidence of liver necrosis, inflammation, thrombosis, and apoptosis 24 h after infection (figures 2*A*, 2*C*, 3*A*, and 3*C*). In vagotomized mice, the extent of both liver damage (figure 2*B*) and apoptosis (figure 2*D*) was markedly increased during septic peritonitis ($P < .05$ vs. sham-operated mice). Conversely, nicotine pretreatment prevented liver damage after intraperitoneal infection with *E. coli* ($P < .05$ vs. control mice) (figure 3*B*). In addition, the extent of apoptosis was decreased in nicotine-pretreated mice during septic peritonitis, although the difference in comparison with that in infected control mice did not reach statistical significance in the semiquantitative analysis ($P = .10$) (figure 3*D*).

Granulocyte stainings of liver specimens revealed that vagotomy was also associated with an increased influx of neutrophils into the liver 24 h after induction of septic peritonitis ($P < .05$ vs. sham-operated mice) (figures 2*E* and 2*F*). Nicotine pretreatment tended to diminish hepatic neutrophil accumulation during septic peritonitis, although the effect did not reach statistical significance (figures 3*E* and 3*F*).

Clinical chemistry can be used to evaluate hepatocellular injury in a quantitative way in this model [12–14]. Thus, we measured the plasma levels of ALT and AST 24 h after infection. Septic peritonitis was associated with elevated ALT and AST levels, confirming the results of our previous studies [12–14]. Vagotomy resulted in even higher plasma ALT and AST levels, although the differences with levels in sham-operated mice were not significant, because of a relatively large amount of interindividual variation (figure 4). Nicotine-pretreated mice showed a marked reduction in plasma ALT and AST levels ($P < .05$ vs. control mice) (figure 5).

Nicotine pretreatment impairs bacterial clearance and survival during septic peritonitis. An adequate local inflammatory response is important for mounting an effective antibacterial response during septic peritonitis [12–14]. Therefore, we determined the consequences that the effect of vagotomy and nicotine

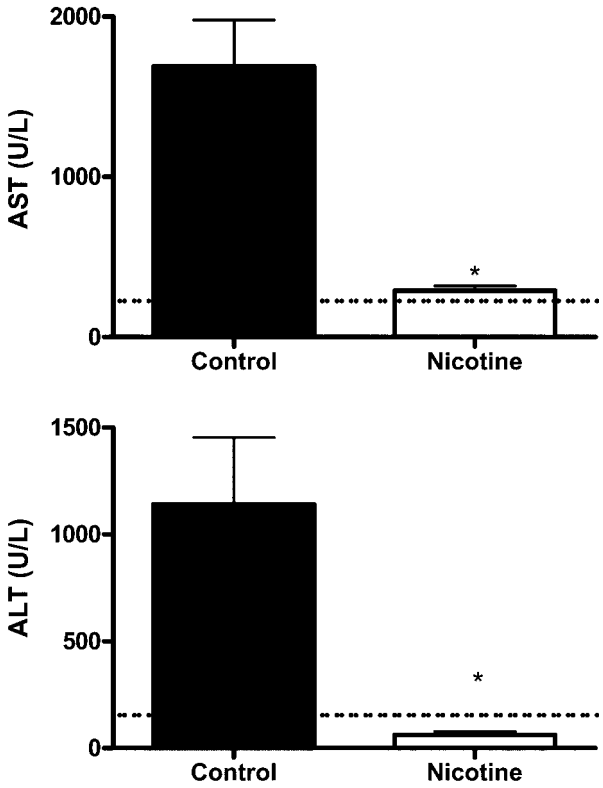

Figure 5. Reduction of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels by nicotine. Septic peritonitis was induced by intraperitoneal injection of *Escherichia coli*. Starting 4 days before infection, mice received either normal drinking water or drinking water supplemented with nicotine (100 μg/mL). Data are means ± SEs of 8 mice/group at 24 h after infection. The dotted line represents the transaminase level in uninfected mice. Differences between groups were calculated by Mann-Whitney *U* test. *$P < .05$ vs. control mice with septic peritonitis.

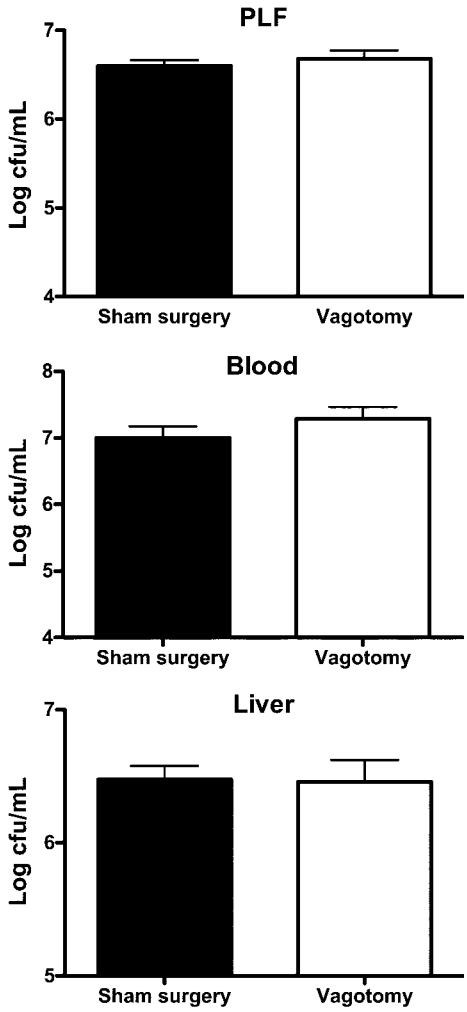

Figure 6. Lack of influence of vagotomy on bacterial clearance. Septic peritonitis was induced by intraperitoneal injection of *Escherichia coli*. Four days before infection, mice were subjected to left-sided cervical vagotomy or sham surgery. Data are means ± SEs of 8 mice/group at 24 h after infection. Differences between groups were calculated by Mann-Whitney *U* test and were not found to be significant. PLF, peritoneal lavage fluid.

pretreatment on the host inflammatory reaction to *E. coli* infection have for bacterial loads in the peritoneal lavage fluid (the site of the infection), blood (to evaluate the extent to which the infection became systemic), and liver 24 h after infection (i.e., shortly before the first deaths occurred). Vagotomy did not influence the number of *E. coli* colony-forming units recovered from these 3 body sites (figure 6). However, nicotine pretreatment facilitated the outgrowth of *E. coli* in peritoneal lavage fluid, blood, and liver (all 3 sites, $P < .05$ vs. control mice) (figure 7). Finally, we determined the effect of vagotomy and nicotine pretreatment on mortality. Consistent with their influence on the outgrowth of *E. coli* during the infection, vagotomy did not alter mortality during septic peritonitis (data not shown), whereas nicotine significantly accelerated mortality ($P < .05$ vs. control mice) (figure 8). Notably, the relatively modest adverse effect of nicotine pretreatment on survival in this fulminant model of sepsis was reproduced in 2 separate additional experiments (data not shown).

DISCUSSION

The efferent vagus nerve has been implicated as an important anti-inflammatory pathway through an interaction of its principal neurotransmitter, acetylcholine, with nicotinic cholinergic receptors—in particular, the $\alpha 7$ subunit—on resident macrophages. Although the function of this cholinergic anti-inflammatory pathway has been well established in models of sterile inflammation [7–9, 17, 18], the present study is, to our knowledge, the first to investigate its role in the innate immune response

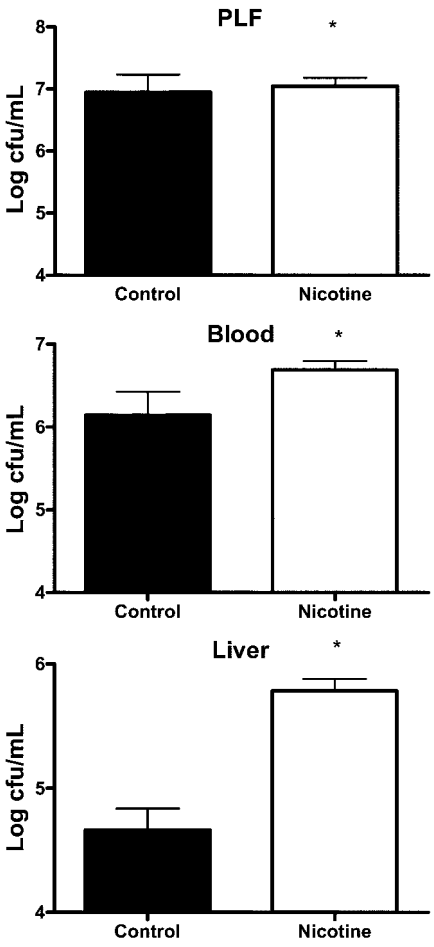

Figure 7. Nicotine impairment of bacterial clearance. Septic peritonitis was induced by intraperitoneal injection of *Escherichia coli*. Starting 4 days before infection, mice received either normal drinking water or drinking water supplemented with nicotine (100 μg/mL). Data are means ± SEs of 8 mice/group at 24 h after infection. Differences between groups were calculated by Mann-Whitney *U* test. *$P < .05$ vs. control mice with septic peritonitis. PLF, peritoneal lavage fluid.

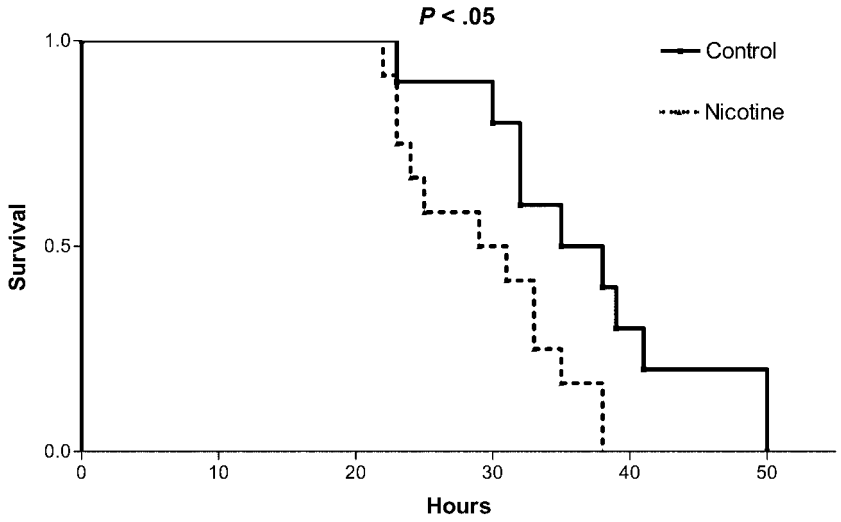

Figure 8. Acceleration of mortality during septic peritonitis, as a result of pretreatment with nicotine. Septic peritonitis was induced by intraperitoneal injection of *Escherichia coli.* Starting 4 days before infection, mice received either normal drinking water or drinking water supplemented with nicotine (100 μg/mL). $n = 12$ mice/group. The P value indicates the difference between groups, assessed by log-rank test.

to infection with live bacteria. Here, we demonstrate that inhibition of the cholinergic anti-inflammatory pathway by cervical vagotomy results in an enhanced early and late inflammatory response to septic peritonitis induced by intraperitoneal injection of *E. coli;* this response is characterized by increased cytokine release, an enhanced influx of inflammatory cells to the site of infection, and the occurrence of extensive liver damage. Conversely, activation of the peripheral component of the pathway by oral administration of nicotine attenuated early and late inflammation, as is reflected by decreased cytokine production, decreased neutrophil recruitment, and prevention of liver damage. These data suggest that the cholinergic anti-inflammatory pathway plays an essential role in the regulation of inflammatory response during septic peritonitis.

The currently reported effects of vagus nerve manipulation by vagotomy or nicotine pretreatment are in line with earlier investigations that examined the influence of vagus nerve activity on sterile inflammation. Previous studies have identified an important role for the afferent vagus nerve in the detection of inflammation by the central nervous system. Whether humoral or neural pathways are essential in relaying information to the brain is largely dependent on the magnitude of the inflammatory response. Experimental studies have shown that when the level of inflammation is low, such as when a low dose of LPS is injected intraperitoneally, vagotomy inhibits the stimulation of the hypothalamus-pituitary-adrenal axis and the induction of IL-1 in the brain [19, 20], whereas high doses of LPS induce responses by the brain independently of the vagus nerve [21, 22]. This implicates that neural pathways are essential in the relay of localized inflammation, whereas information about severe systemic inflammation reaches the brain predominantly through humoral pathways. The vagus nerve not only is essential in the detection of inflammation but also provides an important route through which the central nervous system can respond. In experimental endotoxemia, direct electrical or chemical vagus nerve stimulation reduced serum TNF-α levels and prevented shock [7, 18], whereas cervical vagotomy augmented serum TNF-α levels and sensitized animals to the lethal effects of LPS [7, 8]. In other models of systemic inflammation, induced by either ischemia reperfusion injury or hypovolemic hemorrhagic shock, stimulation of the vagus nerve decreased serum TNF-α levels and prevented the development of hypotension [9, 17]. Previous, as-yet-unpublished studies of endotoxemic mice by our group have shown that the anti-inflammatory effects of electrical vagus nerve stimulation are relatively short lived and wane 2–4 h after stimulation. On the basis of these results, given the duration of the septic peritonitis model used here, we decided not to use vagus nerve stimulation in the present study. It should be noted that nicotine was administered via the drinking water beginning from 4 days before induction of septic peritonitis. Because of this route of administration and the acute nature of the model used, we were not able to examine the effect of postponed treatment with nicotine.

Our data confirm the anti-inflammatory potential of the vagus nerve in a well-established model of abdominal sepsis. We first show that the initial inflammatory response (which has been shown to be essential for host defense in this model [13, 23]) during septic peritonitis is regulated by the vagus nerve and nicotinic receptors. Six hours after infection, mice subjected to unilateral vagotomy showed increased levels of proinflammatory cytokines, compared with those in sham-operated mice. Conversely, mice pretreated with nicotine displayed lower

levels of these mediators. Interestingly, the proinflammatory effects of vagotomy were abolished in mice subjected to vagotomy that were concurrently treated with nicotine, confirming that nicotine acts on the peripheral part of the cholinergic anti-inflammatory pathway (which is independent of the integrity of the vagus nerve). In subsequent studies, mice were killed 24 h after infection, and we investigated the effects of vagotomy and nicotine pretreatment on host defense and liver damage during more-established septic peritonitis. Interference with the function of the vagus nerve strongly influenced not only the proinflammatory cytokine response to *E. coli* septic peritonitis but also the migration of leukocytes to the site of the infection, which is one of the hallmarks of the early immune reaction to invading pathogens. Moreover, our study documented a protective role of the intact vagus nerve against liver injury accompanying experimental *E. coli* septic peritonitis. We specifically focused on hepatic injury and inflammation, since we previously documented hepatocellular damage in this infection model [12–14] and since the liver is richly innervated by the vagus nerve [24]. We used nicotine to chemically stimulate the peripheral part of the cholinergic anti-inflammatory pathway. Previous studies have shown that nicotine inhibits LPS-stimulated TNF-α release by human—as well as mouse—macrophages in vitro via a specific interaction with the α7 subunit of nicotinic acetylcholine receptors [8]. These findings were corroborated by in vivo studies using LPS-challenged α7-deficient mice, in which the anti-inflammatory effect of electrical stimulation of the vagus nerve was abolished [8]. Together with our finding that nicotine added to drinking water reduced TNF-α levels in mice challenged with live *E. coli* in vivo, we consider it likely that nicotine exerts its anti-inflammatory effects through an interaction with the α7 subunit of nicotinic acetylcholine receptors on macrophages. Of note, the same scheme and route of nicotine administration has been reported to reduce colonic damage during spontaneous colitis in IL-10 gene–deficient mice [10, 11, 25]. Unfortunately, we were not able to confirm and expand our results by use of α7-deficient mice, since these mice do not breed well (information from Jackson Laboratories), and our own prolonged efforts to breed them in our institution did not result in a colony large enough for use in in vivo experiments.

Interruption or stimulation of the vagus nerve had a profound impact on the recruitment of neutrophils to the infected peritoneal cavity. Since the local release of the neutrophil-attracting chemokine KC during septic peritonitis was enhanced by vagotomy and decreased by nicotine, it is conceivable that the alterations in neutrophil migration to the site of infection are, at least in part, mediated by KC. Alternatively, these results can also be explained by a direct effect of vagotomy or nicotine on neutrophils. Previous studies have shown that a variety of nicotinic acetylcholine receptors is present on neutrophils and that stimulation of nicotinic receptors inhibits neutrophil migration, which is, at least in part, mediated by inhibition of adhesion molecule expression on both the endothelial cell surface and neutrophils [26].

Whereas the inflammatory response to septic peritonitis was increased after vagotomy and was reciprocally decreased by nicotine pretreatment, bacterial clearance and survival were altered by nicotine pretreatment only. A possible explanation is that unilateral vagotomy induces only partial interference with the cholinergic anti-inflammatory pathway. Notably, the effect of bilateral vagotomy could not be investigated, since this procedure is lethal in mice. After nicotine pretreatment, bacterial clearance and survival were significantly reduced. Since host defense in septic peritonitis is a delicate balance between proinflammatory pathways intended to eliminate bacteria and anti-inflammatory pathways intended to prevent systemic inflammation, any imbalance in pro- or anti-inflammatory mediators might prove harmful. Indeed, our laboratory recently demonstrated that elimination of the anti-inflammatory cytokine IL-10 in septic peritonitis resulted in an uncontrolled systemic inflammatory response syndrome and lethality, in spite of the fact that IL-10 deficiency facilitated the clearance of bacteria from the peritoneal cavity [12]. In the present study, pretreatment with nicotine resulted in a reduction of local and systemic inflammation but increased lethality, because of a decrease in bacterial clearance and enhanced dissemination of bacteria. Taken together, these findings illustrate the delicacy of the balance between pro- and anti-inflammation during septic peritonitis.

Excessive activation of coagulation plays an important role in the pathogenesis of severe sepsis [27], and the model used here is associated with profound activation of the coagulation system [28]. Of note, in the present study, we did not find consistent effects of either vagotomy or nicotine pretreatment on the procoagulant response to abdominal sepsis, as measured by thrombin-antithrombin levels in peritoneal lavage fluid and fibrin staining of liver sections (data not shown).

It should be noted that variation existed in some of the end points measured in the control groups of study 2 (sham surgery) and study 3. The exact number of viable bacteria used to inoculate mice can be quantified only retrospectively; in study 2, the inoculum contained 1×10^4 cfu of *E. coli*, whereas, in study 3, 5×10^4 cfu were injected. Although an effect of sham surgery cannot be excluded, we consider it most likely that the larger bacterial challenge given in study 3 explains the somewhat higher control values in this experiment. Importantly, in both separate studies, control and intervention groups were injected with exactly the same inoculum at the same time.

Peritonitis is a common cause of sepsis [29], and *E. coli* remains one of the most frequently isolated pathogens in intraperitoneal infections [30]. Intraperitoneal administration of live *E. coli* results in a syndrome that resembles a clinical condition commonly associated with septic peritonitis [31]. By

using this model, we here demonstrate—for the first time, to our knowledge—that the cholinergic anti-inflammatory pathway is an essential regulator of the innate immune response to a severe bacterial infection. We further show that stimulation of the cholinergic anti-inflammatory pathway by nicotine impairs bacterial clearance and survival during *E. coli*–induced septic peritonitis. The cholinergic anti-inflammatory pathway may be a future target for the modulation of the host inflammatory response to sepsis.

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