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*J Immunol* 2009; 183:552-559; doi: 10.4049/jimmunol.0802684
http://www.jimmunol.org/content/183/1/552

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2009/06/18/183.1.552.DC1

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The cholinergic anti-inflammatory pathway is a physiological mechanism that inhibits cytokine production and diminishes tissue injury during inflammation. Recent studies demonstrate that cholinergic signaling reduces adhesion molecule expression and chemokine production by endothelial cells and suppresses leukocyte migration during inflammation. It is unclear how vagus nerve stimulation regulates leukocyte trafficking because the vagus nerves do not innervate endothelial cells. Using mouse models of leukocyte trafficking, we show that the spleen, which is a major point of control for cholinergic modulation of cytokine production, is essential for vagus nerve-mediated regulation of neutrophil activation and migration. Administration of nicotine, a pharmacologic agonist of the cholinergic anti-inflammatory pathway, significantly reduces levels of CD11b, a β2-integrin involved in cell adhesion and leukocyte chemotaxis, on the surface of neutrophils in a dose-dependent manner and this function requires the spleen. Similarly, vagus nerve stimulation significantly attenuates neutrophil surface CD11b levels only in the presence of an intact and innervated spleen. Further mechanistic studies reveal that nicotine suppresses F-actin polymerization, the rate-limiting step for CD11b surface expression. These studies demonstrate that modulation of leukocyte trafficking via cholinergic signaling to the spleen is a specific, centralized neural pathway positioned to suppress the excessive accumulation of neutrophils at inflammatory sites. Activating this mechanism may have important therapeutic potential for preventing tissue injury during inflammation. The Journal of Immunology, 2009, 183: 552–559.

Leukocyte trafficking is critical to both innate and active immunity. Rapid and regulated leukocyte localization from the vascular compartment to sites of tissue injury and/or infection is required for successful host responses during inflammation and infection (reviewed in Ref. 1, 2). Proinflammatory mediators activate the endothelium to express cell-associated adhesion molecules that interact efficiently and selectively with circulating immune cells (reviewed in Ref. 3, 4). Selectins (e.g., E-selectin, P-selectin) facilitate the transient binding and rolling of leukocytes along the endothelium. Firm adhesion of leukocytes is mediated through endothelial expression of members of the Ig superfamily (e.g., ICAM-1 and VCAM-1) and their counterligands expressed by leukocytes (e.g., CD11b/CD18). Subsequent transmigration of circulating leukocytes through the endothelial cell barrier occurs following bidirectional signaling events. Although early leukocyte migration is critical for host responses during infection and inflammation, dysregulated or disproportionate leukocyte recruitment is associated with inflammation and tissue injury. Excessive leukocyte infiltration, which is pathogenic in acute and chronic inflammation, has been implicated in the development of autoimmune disease. The role of the endothelium in the regulation of inflammation has become a therapeutic target in pathogenic conditions associated with dysregulated immune cell trafficking as evidenced by the development of inhibitors of selectins, ICAMs, and VCAM-1, as well as the regulation of endothelium-derived NO.

The cholinergic anti-inflammatory pathway is a brain-to-immune mechanism that regulates inflammatory responses via α7-nicotinic acetylcholine receptor subunits (α7-nAChR)-dependent, vagus nerve signaling (reviewed in Ref. 5). Cholinergic stimulation (via electrical stimulation of the vagus nerve or selective cholinergic agonists) inhibits cytokine production in preclinical models of systemic inflammation, including endotoxemia, hemorrhagic shock, ischemia-reperfusion injury, and polymicrobial sepsis (6–9). Endothelial cells express the α7-nAChR, and pharmacologic stimulation of this receptor reduces both chemokine production and adhesion molecule expression by endothelium (10). During studies of vagus nerve modulation of endothelial cell activation and leukocyte migration, we were surprised to find that leukocyte recruitment to sites of localized inflammation was suppressed by cholinergic signaling (9, 10), because the endothelium and s.c. tissues are not innervated by the vagus nerve. Recent studies indicate that the spleen is an essential component of the α7-nAChR

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1This work was supported by The National Institute of General Medicine Sciences (R01 GM070727 to C.N.M. and R01 GM057226 to K.J.T.); The North Shore-Long Island Jewish Health System General Clinical Research Center (M01 RR018535, to K.J.T.); The American Heart Association (to C.N.M.), The North Shore-Long Island Jewish Health System (to C.N.M.), and the Elmezzi Graduate School of Molecular Medicine (to M.M.Y.).

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5 Abbreviations used in this paper: α7-nAChR, α7-nicotinic acetylcholine receptor; VNS, vagus nerve stimulation; CLP, cecal ligation and puncture; MFI, mean fluorescence intensity; HSP, heat shock protein.

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dependent pathway because neural-to-spleen signals regulate cytokine production during endotoxemia (11, 12). In addition, innervation of the splenic nerve is critical for mediating the anti-inflammatory effects of vagus nerve stimulation (VNS) on LPS-induced cytokine production (13). In this study, we report that neural signals to the spleen via the cholinergic anti-inflammatory pathway regulate leukocyte trafficking to peripheral inflammatory sites by controlling neutrophil surface CD11b levels.

Materials and Methods

Abs and reagents

**Lambda carrageenan** (type IV), LPS (LPS from Escherichia coli 0111: B4), fMLP, and nicotine were purchased from Sigma-Aldrich.

**Animal studies**

All experimental procedures using animals were approved by the Institutional Animal Care and Use Committee (IACUC) of The Feinstein Institute for Medical Research. Animals were housed in a pathogen-free room under standard 12 h light/dark cycles with free access to food and water. All experiments were performed at least twice.

**Carrageenan air pouch model**

The carrageenan air pouch model was performed as previously described (10). In brief, dorsal air pouches were generated in female Swiss Webster mice (weighing 26–33 g, Taconic Farms) by injecting 6 ml of sterile air s.c. on days 0 and 3. On day 6, mice were given nicotine (1 mg/kg, i.p.) or vehicle and then challenged with an intrapouch injection of 1% carrageenan (1 ml) 15 min later. Animals were euthanized by CO2 asphyxiation and cellular infiltrates were collected following the injection of 3 ml of PBS containing 2 mM EDTA. RBC-free cellular infiltrates were counted using a hemocytometer. In some experiments, both pouch cells and heparinized whole blood were collected.

**Vagus nerve stimulation**

Stimulation of the left cervical vagus nerve was performed on mice 15 min before carrageenan challenge, as previously described (10, 11).

**Splenectomy**

Approximately 1 wk before further experimentation, spleens were removed from anesthetized (inhaled isoflurane) mice and sham animals underwent laparotomy without splenectomy, as previously described (11). For air pouch studies, mice underwent surgery (sham or splenectomy) on day 0 and received an injection of air on days 0 and 3. Sham-surgery and splenectomized mice were treated with nicotine or vehicle and challenged with carrageenan, as described for the pouch model on day 6. For analyses of blood neutrophils, all mice were rested days 6 before euthanasia and collection of blood.

**Cecal ligation and puncture (CLP)-induced sepsis**

Sepsis induced by CLP in BALB/c mice (Charles River Laboratories) was performed as previously described (11). Mice received antibiotic, Primaxin (0.5 mg/kg, s.c.), and 0.9% saline solution (20 ml/kg, s.c.) immediately following surgery. Mice were given nicotine (400 µg/kg, i.p.) 6 and 18 h post CLP surgery and were euthanized 24 h post CLP surgery. Peritoneal cavities were lavaged with PBS (10 ml) containing 2 mM EDTA to collect neutrophils for enumeration.

**Splenic neurectomy procedures**

Surgical ablation of the splenic nerve was performed as previously described (13). In brief, female Swiss Webster mice were anesthetized using isoflurane. After opening the abdominal cavity through a lateral incision on the left side, the splenic nerve was isolated and incised repeatedly at multiple points using forceps (avoiding manipulation of the celiac ganglion). Sham surgeries were performed similarly, except the splenic nerve was not incised. Mice recovered for 7 to 12 days before VNS or sham surgery (as described above). After euthanasia by CO2 asphyxiation, peripheral blood was collected for assessment of neutrophil surface CD11b levels (described below) and spleens were collected, frozen in OCT, sectioned, and stained for nerve fibers using the glyoxylic acid method (as previously described; Ref. 13, 14). Data from mice whose spleens were depleted of ≥75% nerve fibers were used in the final data analyses.

Complete blood count and differentials

Following CO2 asphyxiation, mouse blood was collected via cardiac puncture into EDTA-microtubes and analyzed for complete blood count and differential by AnimLytics.

**Flow cytometry staining for neutrophil integrins**

Cell surface integrin expression by neutrophils was determined using a whole blood staining FACScalibur assay, as previously described (15–17). In brief, whole blood was collected in heparinized syringes via cardiac puncture (post CO2 euthanasia) from control, sham, or splenectomized mice (1 wk post surgery) either after sham or VNS procedures (as described above) or after vehicle or nicotine (1 mg/kg) administration for up to 1 h before euthanasia. Whole blood was mixed 1:1 with RPMI 1640 and then either untreated or stimulated in vivo with LPS (5 mg/ml) or (fMLP 10−7 M) for 15 min at 37°C. For some experiments, whole blood was incubated with nicotine ex vivo for 30–60 min at 37°C before stimulation. Neutrophils were then stained with fluorochrome-conjugated Abs specific for mouse Ly-6G, CD11a, CD11b, CD62L, or isotype control Ab at 4°C according to the manufacturer’s recommendations (BD Biosciences) and Refs. 16, 17. Following RBC lysis, cells were fixed and analyzed by flow cytometry, as described above. Neutrophils isolated from nicotine-challenged air pouches and whole blood cells were stained for Ly-6G, CD11b, CD11a, CD62L, or isotype control Ab. The neutrophils (determined by Ly-6Gbright staining and specific SSC/FCS “neutrophil” gating, as previously described by Ref. 17) were analyzed by flow cytometry using the FACScalibur with CellQuest software. For intracellular CD11b staining, neutrophils were first stained with Ly6-G and then permeabilized using Cytofix/Cytoperm (BD Biosciences) before staining with fluorochrome-labeled anti-CD11b (or isotype control) and analyzed as described above. All staining studies were performed (in duplicate) at least three times (for each experimental condition).

**Neutrophil F-actin staining**

Mice were treated with vehicle or nicotine (1 mg/kg, i.p.) 1 h before euthanasia. Peripheral blood neutrophils were isolated from heparinized mouse blood collected by cardiac puncture after CO2 asphyxiation according to Ref. 18. Neutrophil purity was >95% according to the M20/79/79 staining and specific SSC/FCS neutrophil gating, as previously described by Ref. 17. Neutrophil F-actin staining was performed using FITC-phalloidin (Sigma-Aldrich) according to previously published methods (19). In brief, neutrophils were resuspended in HBSS at 2 × 106 cells/ml (in 50 µl) and prewarmed to 37°C for 2 min before no stimulation or stimulation with 10 µM fMLP for 30 s. Following fMLP stimulation, neutrophils were fixed with 3.7% formaldehyde and permeabilized with 0.05% Triton X-100 (PBS), neutrophil F-actin staining was determined using FITC-phalloidin (50 µM) and flow cytometry methods according to (19). Neutrophil F-actin content was expressed as the ratio of mean fluorescence intensity (MFI) of fMLP-stimulated neutrophils to MFI of unstimulated neutrophils (or relative F-actin).

**Neutrophil CD11b mRNA expression**

Quantitative expression of CD11b mRNA was performed using the RT2 Profiler PCR array (mouse inflammatory cytokines and receptors) purchased from SuperArray Biosciences. In brief, RNA was isolated from pouch-derived neutrophils obtained from vehicle-treated and nicotine-treated (1 mg/kg, i.p.) Swiss Webster mice obtained 3.5 h post carrageenan challenge. Quantitative RT-PCR was performed according to the manufacturer’s protocols (this method reliably detects 2-fold changes in mRNA expression). PCR data were analyzed using the RT2 Profiler template according to the manufacturer’s recommendation and data were presented as fold up- or down-regulation (test/control sample).

**Statistical analyses**

All experiments were repeated at least twice. All data in figures are expressed as mean ± SEM or SD, as indicated. One-way ANOVA using the Bonferroni correction was used to compare the mean values between the groups. p values <0.05 were considered significant.

**Results**

Splenectomy reverses the inhibitory effect of cholinergic agonist on leukocyte migration

In agreement with previous studies (10), administration of the cholinergic agonist nicotine significantly reduced leukocyte accumulation in the carrageenan-filled air pouches as compared with vehicle-treated controls (Fig. 1A). Using this model of acute
inflammation, >90% of the cells in the pouch at 5 h post challenge are neutrophils (10), indicating that the anti-inflammatory cholinergic agonist significantly inhibited neutrophil trafficking. To investigate the role of the spleen in this anti-inflammatory pathway, mice were subjected to splenectomy or sham surgeries 1 wk before carrageenan challenge. Spleenectomy reversed the inhibitory effect of nicotine on leukocyte migration in vivo as compared with sham-surgery conditions (Fig. 1B). Likewise, administration of nicotine to mice following polymicrobial sepsis induced by CLP significantly reduced neutrophil recruitment to the peritoneal cavity (Fig. 1C) and this inhibitory effect was blunted in the absence of a spleen (Fig. 1D).

**Cholinergic agonist administration reduces the appearance of CD11b on the surface of neutrophils**

Based on the critical role of integrins on neutrophil trafficking in vivo, we explored the effect of cholinergic agonists on neutrophil integrin expression using the air pouch model. Similar to other models of acute inflammation, the appearance of CD11b, the β2 integrin critical for neutrophil adhesion to the endothelium and subsequent trafficking to sites of inflammation, is significantly increased on the surface of neutrophils found in the blood and in the air pouches of animals following carrageenan challenge (supplemental Table I). By contrast, neutrophil surface CD11a levels were unchanged and neutrophil CD62L surface levels declined following carrageenan challenge (supplemental Table I). To determine the effect of cholinergic stimulation on the levels of CD11b on the surface of neutrophils, circulating neutrophil CD11b expression was assessed using whole blood assay methods following treatment with vehicle or nicotine (1 mg/kg i.p.). Nicotine administration significantly reduced basal CD11b levels on circulating neutrophils obtained from mice in a dose-dependent manner (Fig. 2A). Likewise, nicotine administration in vivo reduced both LPS- and fMLP-induced CD11b surface expression by blood neutrophils (Fig. 2, B and C, respectively). By contrast, using whole blood cell assays we found that nicotine treatment ex vivo had no effect on neutrophil surface CD11b levels (supplemental Table II). In addition, nicotine administration had no effect on either CD11a or CD62L levels on the surface of circulating neutrophils (data not shown). Next, we investigated the effect of nicotine administration on CD11b levels on circulating neutrophils obtained from sham vs splenectomized mice. We found that while nicotine administration suppressed CD11b levels present on peripheral blood neutrophils by >50% in sham mice, nicotine only weakly reversed CD11b levels (<20%) on circulating neutrophils obtained from mice without spleens (Fig. 3).

**VNS decreases leukocyte trafficking and neutrophil surface CD11b levels in animals with intact spleens**

Because previous studies show that electrical stimulation of the vagus nerve dampens inflammatory responses including neutrophil trafficking (9, 10), we examined the effect of vagus nerve stimulation on neutrophil trafficking to the carrageenan-filled air pouch.
As surgery (trophil surface CD11b levels is dependent on the spleen. Mice underwent sham surgery (solid) or splineectomy (SPLX, gray) surgery 1 wk before administration of nicotine (1 mg/kg, i.p.). One hour later, blood was collected to determine the levels of CD11b on surface of neutrophils. Data from three experiments (three to five mice per group) were combined and data are shown as percentage of change in neutrophil surface CD11b levels in sham vs splenectomized mice (induced by nicotine treatment) \( \pm \) SEM. *, \( p < 0.05 \) comparing the percentage of change in neutrophil CD11b levels in sham vs splenectomized mice.

and CD11b levels on the surface of neutrophils. Similar to our observations with nicotine administration, VNS significantly reduced leukocyte accumulation in the carrageenan air pouch model (Fig. 4A). Likewise, splenectomy significantly blocked the inhibitory effect of VNS on leukocyte migration in vivo as compared with vehicle treatment (Fig. 4B).

We reasoned that vagus nerve signals, like nicotine, might suppress neutrophil trafficking by altering neutrophil surface markers associated with migration. To determine the effect of VNS on neutrophil surface CD11b levels, circulating neutrophil CD11b levels were assessed using whole blood assay methods following electrical stimulation of the vagus nerve or sham surgery. Although VNS had no effect on basal CD11b levels, we found that VNS significantly inhibited LPS-induced and fMLP-induced CD11b levels on the surface of neutrophils (Fig. 4, C and D, respectively) as compared with animals following sham surgery. These observations suggest that a reduction in neutrophil surface CD11b levels by VNS inhibits neutrophil migration to a zone of peripheral inflammation. Similar to our results with nicotine, the presence of a spleen was necessary for the suppression of CD11b levels by VNS. Together, these results indicate that both electrical and pharmacological activation of the cholinergic anti-inflammatory pathway requires the spleen to inhibit leukocyte trafficking likely via regulating the appearance of CD11b on the cell surface.

**Surgical ablation of the splenic nerve inhibits the modulation of CD11b surface levels by VNS**

Very recent studies reveal that splenic innervation is required for VNS-mediated suppression of LPS-induced TNF production in vivo (13). Therefore, we investigated whether innervation of the spleen was necessary for VNS-mediated down-regulation of neutrophil surface CD11b levels following splenic neurectomy. Although surgical ablation of the splenic nerve had no effect on LPS-induced CD11b levels following sham surgery (Fig. 5, Sham-Sham vs SNVX-Sham), surgical ablation of the splenic nerve significantly blunted the inhibitory effect of VNS on CD11b levels when compared with sham surgery (Fig. 5, sham-VNS vs SNVX-VNS).

**Cholinergic regulation of neutrophil surface CD11b levels is mediated through F-actin**

The cholinergic anti-inflammatory pathway (induced by either VNS or cholinergic agonists) is dependent on the expression of the \( \alpha 7 \) subunit of the nAChR (20). Our results showed that nicotine was more potent than VNS in regulating leukocyte trafficking in vivo and neutrophil surface CD11b levels. Therefore, we used nicotine, a pharmacologic agonist of the \( \alpha 7 \)-nAChR, to investigate the molecular basis of the cholinergic regulation of neutrophil surface CD11b levels. Although the rapid appearance of CD11b on the surface of neutrophils is believed to be regulated post-translationally, we examined the effect of nicotine administration on cellular CD11b mRNA (by quantitative RT-PCR) and protein expression (by flow cytometry methods). Nicotine administration did not significantly decrease neutrophil CD11b mRNA expression when compared with vehicle-treated animals (1.06 \( \pm \) 0.06-fold-increase vs 1.0 \( \pm \) 0.02 for vehicle). Likewise, total CD11b protein expression (determined by intracellular staining by flow cytometry methods) by neutrophils obtained from nicotine and vehicle-treated animals were similar (data not shown). CD11b is stored intracellularly and its localization to the neutrophil surface during cellular activation is closely associated with the cell’s ability to polymerize actin to F-actin (21, 22). Using FITC-phalloidin (a phallotoxin that specifically binds to F-actin) and flow cytometry methods, we observed that nicotine administration significantly reduced F-actin levels in peripheral blood neutrophils as compared...
with levels found in neutrophils obtained from vehicle-treated animals (Fig. 6). This observation is consistent with the effects of cholinergic stimulation on LPS- and fMLP-induced CD11b expression on blood neutrophils and suggests a mechanism for how cholinergic signals regulate the translocation of intracellular CD11b to the surface of neutrophils during inflammation.

The nervous system-spleen connection regulates leukocyte trafficking during inflammation

In agreement with clinical observations and experimental models showing that splenectomy increases peripheral blood counts (23, 24), we found a significant increase in peripheral white blood cell counts and circulating blood neutrophils in mice following splenectomy as compared with sham animals (supplemental Table III). Administration of nicotine to sham animals rapidly and significantly reduced peripheral white blood cell counts and circulating neutrophil counts, peaking at 15–30 min post administration, as compared with vehicle-treated controls (supplemental Table III). Nicotine administration failed to significantly suppress circulating white blood cell levels or neutrophil counts in the absence of a spleen (supplemental Table III). To determine the fate of circulating neutrophils following cholinergic stimulation, we counted the number of neutrophils within the spleen following nicotine administration (peaking at 30 min post administration) as compared with vehicle-treated animals (Fig. 7A). Nicotine administration did not significantly alter the localization of T lymphocytes to the spleen (Fig. 7B) indicating that signaling through the cholinergic anti-inflammatory pathway specifically mediates neutrophil trafficking to the spleen.

Discussion

Effective immune responses depend on the rapid and coordinated trafficking of circulating leukocytes to sites of tissue inflammation, injury, and/or infection. Disproportionate accumulation of inflammatory cells may lead to tissue damage, whereas insufficient recruitment of inflammatory cells to an infectious site may result in systemic infection. The cholinergic anti-inflammatory pathway modulates proinflammatory cytokine production during local and systemic inflammation through the spleen (11, 12). Recent studies show that cholinergic stimulation regulates leukocyte migration to sites of tissue inflammation (9, 10). Endothelial cells express the $\alpha_7$nAChR and down-regulate adhesion molecule expression in response to cholinergic stimulation (10). However, the endothelium is not directly innervated by the vagus nerve. Herein, we report that the spleen is a critical interface between the cholinergic anti-inflammatory pathway and systemic regulation of immune cell...
trafficking and that the cholinergic regulation of neutrophil migration is mediated, in part, through modulation of CD11b expression on the surface of neutrophils.

The anti-inflammatory effects of nicotine and VNS have been shown to be mediated through the α7-nAChR (20). Consistent with our observation revealing a significant decrease in leukocyte trafficking in carrageenan-challenged mice following cholinergic stimulation by nicotine, a pharmacologic nAChR agonist, or VNS (in the presence of a spleen) (Figs. 1 and 4), we found that nicotine administration and VNS significantly reduced both LPS- and fMLP-induced neutrophil surface CD11b expression (Figs. 2 and 4, respectively). CD11b is known to be the key β2-integrin on the surface of neutrophils regulating both neutrophil adhesion to the endothelium and neutrophil transmigration to sites of microbial infection (25). In contrast, nicotine administration did not alter basal or stimulated expression of other neutrophil adhesion molecules, including L-selectin (CD62L), or the β2-integrin, CD11a. These observations are consistent with the expression of integrins and selectins by neutrophils in our model systems. Using the carrageenan air pouch model, we measured significantly higher levels of CD11b on the surface of activated neutrophils found in the carrageenan pouch and blood during acute inflammation compared with the levels found on circulating neutrophils in naive mice (supplemental Table I). As expected, CD62L was shed from the neutrophil surface following carrageenan challenge in vivo (supplemental Table I). Whereas, neutrophil CD11a levels were unchanged following carrageenan challenge in vivo (supplemental Table I). Similar results were observed using the ex vivo whole blood assay. Although neutrophil surface CD11b expression was significantly enhanced following LPS or fMLP stimulation ex vivo (supplemental Table IV), neutrophil surface CD62L levels declined following LPS or fMLP stimulation ex vivo and there were no significant changes in neutrophil CD11a expression following stimulation (supplemental Table IV).

In previous studies, we showed that the administration of cholinergic agonists reduced cytokine and chemokine production (by 20–30%) at the site of inflammation (10). Based on the known inhibitory effect of VNS and nicotine on systemic cytokine production in vivo, VNS and nicotine-induced suppression of leukocyte trafficking might be mediated through their effects on peripheral cytokine production. We assessed circulating TNF and spleen TNF levels associated with the carrageenan air pouch model for up to 5 h post injection. We found no detectable TNF in the spleen (at 90 min, 150 min, and 5 h post injection) and almost no detectable levels of TNF in the blood (not detectable at 90 min, 36.6 ± 14.8 pg/ml TNF at 150 min post injection, and not detectable 5 h post injection). In contrast, pouch TNF levels reached 828 ± 308 pg/ml at 150 min and 1179 ± 253 pg/ml at 5 h post carrageenan injection. As shown previously, cholinergic stimulation suppresses TNF levels in the carrageenan-filled air pouch by ~20% (10). Based on our observations that there was no detectable TNF found in the spleen (which mediates the effect of cholinergic stimulation on leukocyte trafficking in vivo) and almost no detectable TNF in the blood using this model, it is unlikely that cholinergic regulation of cytokine production, particularly in the spleen, is responsible for the regulation of leukocyte trafficking in this model of localized inflammation.

Although neutrophil surface CD11b expression has been shown to be posttranslationally regulated, with the rapid transport of preformed intracellular CD11b to the cell surface following activation (22, 26), we explored whether cholinergic stimulation down-regulates CD11b mRNA or total protein expression. We found that nicotine had no effect on either CD11b mRNA expression or total intracellular CD11b protein levels. Another possibility is that cholinergic stimulation directly influences circulating neutrophils to down-regulate CD11b on their surface. Studies using whole blood or isolated blood neutrophils with ex vivo nicotine treatment revealed that nicotine (10−3 M to 10−5 M) had no direct effect on neutrophil CD11b expression (basal or stimulated) (supplemental Table II). This observation in conjunction with the failure of cholinergic stimulation to regulate neutrophil trafficking and neutrophil CD11b expression in vivo following splenectomy suggests that the cholinergic stimulation does not act directly on peripheral blood neutrophils.

Neutrophil surface CD11b is closely linked to the cells’ ability to polymerize actin and transport intracellular CD11b to the cell surface. The role of actin polymerization, through its involvement with cell adhesion, cell motility, exocytosis, phagocytosis, and signal transduction, on cytokine production and inflammatory responses is well described (27–31). Consistent with the inhibitory effects of VNS and nicotine administration on the appearance of neutrophil CD11b induced by LPS and fMLP ex vivo, we found that nicotine administration blocked F-actin polymerization (Fig. 6). Previous studies report nicotine-mediated suppression of F-actin levels in chromaffin cells (32). Likewise, acetylcholine, the predominant neurotransmitter released by VNS, blocked the translocation of F-actin which is necessary for leukocyte-endothelial cell adhesion (33). We observed that nicotine, at the doses used, had greater effects on both leukocyte trafficking in vivo and surface CD11b levels (Figs. 1 and 2, respectively) than VNS (Fig. 4). This difference might be due to anesthetics (used for VNS) that can impair actin assembly (34, 35), an essential step for the translocation of CD11b to the cell surface. Thus, because the assessment of VNS on actin polymerization would be hindered by the effect of anesthetics on actin polymerization, we used nicotine, a pharmacologic agonist of the α7nAChR, to explore the effects of cholinergic stimulation on actin polymerization. Previous studies have demonstrated that functional expression of CD11b is modulated by...
active rearrangement of the cytoskeleton, namely the polymeriza-
tion of F-actin (22, 26). Our data support the cholinergic regu-
lation of neutrophil CD11b expression via F-actin polymerization.
Interestingly, numerous studies suggest that in addition to altering cell
trafficking, actin serves as a structural scaffold for the assembly
of structural and signaling molecules. Therefore, cholinergic signal-
ing to the spleen might alter cell trafficking and CD11b surface
levels through effects on either signaling or structural molecules
which control actin stability, actin polymerization, and leukocyte
migration. For example, cholinergic stimulation might regulate in-
hibitors of actin polymerization including small heat shock protein
(HSP) 27 (36, 37) and HSP25, also known as inhibitor of actin
polymerization or IAP (38) to exert effects on cell trafficking. In
addition, there are reports that demonstrate the regulation of in-
flammatory mediator production, as well as cellular apoptosis by
HSP90 (39, 40). By contrast, nicotine reduced the apoptosis of
lung cancer cells (41). To our knowledge, the effect of nicotine on
HSP25 and HSP27 or HSP90 (with respect to cytokine production)
has not been explored, nor has the role of HSP90 in actin poly-
merization/stability or cell migration or the link between nicotine
and HSP90 been investigated.
Administration of nicotine rapidly reduces circulating neutro-
phil counts and increases the number of neutrophils found in the
spleen (Fig. 7). This effect is specific for neutrophils, because the
number of T lymphocytes in the spleen remains unchanged. Splen-
ectomy abolishes the ability of nicotine to reduce circulating neutro-
phil counts, which suggests that cholinergic signaling may regu-
late leukocyte trafficking by directing cells to the spleen. Under
normal conditions, the number of circulating neutrophils are
tightly regulated through the release of young neutrophils by the
bone marrow into the blood (42), and the storage and the removal
of neutrophils in the spleen, liver, and bone marrow (43–45).
In the air pouch model of acute inflammation, we found that the
number of circulating neutrophils remains the same despite the rapid
accumulation of neutrophils in the air pouch. Similar to clinical
reports and studies with splenectomized animals, we found that
splenectomy significantly increased the number of peripheral
blood leukocytes and neutrophils (supplemental Table III), with
reduced trafficking to the inflamed s.c. air pouch. Although the role
of the spleen in regulating cholinergic anti-inflammatory responses
during sepsis has been described (12), the role of the spleen in
regulating leukocyte trafficking during acute inflammation (in the
absence or presence of cholinergic stimulation) has not been pre-
viously investigated.
To determine whether the effect of VNS on neutrophil surface
CD11b levels was dependent on spleenic innervation, we surgically
ablated the spleenic nerves. We found that VNS was significantly
less effective in down-regulating surface CD11b expression fol-
lowing splenic nerveectomy (Fig. 5), suggesting that similar to its
important role in mediating the effects of VNS on LPS-stimulated
TNF production by macrophages (13), spleenic innervation is crit-
ical for the cholinergic regulation of CD11b levels on the surface
of neutrophils.
Together these findings reveal a specific, centralized neural-to-
spleen pathway for controlling peripheral leukocyte trafficking that
is positioned to respond rapidly to local tissue injury via modula-
tion of circulating neutrophil activity (Fig. 8). Based on the role of
the spleen in regulating the anti-inflammatory effects of cholin-
ergic stimulation during sepsis and endotoxemia (11, 12) and our
findings, we propose that neural signaling via the spleen “edu-
cates” or communicates with stored or circulating neutrophils to
modulate their trafficking in response to inflammation. This path-
way mediated through either cell-based or soluble splenic factors
might be manipulated through direct stimulation of the endogenous
vagus nerve pathway to the spleen (via splenic innervation) or
through administration of pharmacological cholinergic agonists
(which act through the spleen) to inhibit excessive and deleterious
neutrophil migration into inflamed or infected tissues (Fig. 8).

Disclosures
The authors have no financial conflict of interest.

References
2. Aird, W. C. 2003. The role of the endothelium in severe sepsis and multiple organ
7: 678–689.
4. Muller, W. A. 2003. Leukocyte-endothelial-cell interactions in leukocyte trans-
5. Tracey, K. J. 2007. Physiology and immunology of the cholinergic anti-
stimulation blunts nuclear factor-κB activation and protects against hypovolemic
8. Pavlov, V. M., M. Ochani, L. H. Yang, M. Gallowitsch-Puerta, K. Ochani, X. Lin,
acetylcholine receptor agonist GTS-21 improves survival in murine endotoxemia
2007. Stimulation of 7α cholinergic receptors inhibits lipopolysaccharide-in-
duced neutrophil recruitment by a tumor necrosis factor α-independent mecha-
10. Saeed, R. W., S. Varma, T. Peng-Nemeroff, B. Sherry, D. Balakhaneh, J. Huston,
endothelial cell activation and leukocyte recruitment during inflammation. J. Exp.
M. Gallowitsch-Puerta, A. Shigematsu, V. A. Pavlov, et al. 2006. Spleenec-
tomy inactivates the cholinergic anti-inflammatory pathway during lethal endo-
12. Huston, J. M., H. Wang, M. Ochani, K. Ochani, M. Rosas-Ballina,
Splenectomy protects against sepsis lethality and reduces serum HMGB1 levels.
2006. Distribution of peripheral

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blood cells in mice after splenectomy or autotransplantation. Microsurgery 26: 43–49.


