Stimulation of the α7 Nicotinic Acetylcholine Receptor Protects Against Sepsis by Inhibiting Toll-like Receptor via Phosphoinositide 3-Kinase Activation

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Background. The Toll-like receptor (TLR) plays an important role in the induction of the hyperinflammatory response and tissue injury in sepsis. The cholinergic antiinflammatory pathway serves as a link between the parasympathetic and innate immune systems. We examined the antiinflammatory effect of nicotine, a potent α7 nicotinic acetylcholine receptor (α7nAChR) agonist, with regard to TLR expression and signaling during sepsis.

Methods. Polymicrobial sepsis was induced in mice by cecal ligation and puncture (CLP). The subjects received intraperitoneal nicotine (400 μg/kg) immediately after CLP for the biochemical study and 0, 24, 48, and 72 hours after CLP for the survival test. Intraperitoneal methyllycaconitine (MLA; 5 mg/kg), an α7nAChR antagonist, was administered 5 minutes prior to nicotine treatment. We evaluated the effects of nicotine using α7nAChR and phosphoinositide 3-kinase (PI3K) inhibitors in lipopolysaccharide-stimulated RAW264.7 cells.

Results. Nicotine improved sepsis-induced mortality, attenuated organ failure, and suppressed inflammatory cytokines, which were abolished by MLA. Nicotine enhanced PI3K/Akt activation and reduced PU.1 activity and TLR4 expression. MLA and PI3K inhibitors blocked this effect of nicotine.

Conclusions. Our findings suggest that stimulation of the cholinergic antiinflammatory pathway by nicotine protects against septic injury and that this may be associated with inhibition of TLR4 expression via α7nAChR/PI3K signaling.

Keywords. α7nAChR; nicotine; PI3K; sepsis; TLR; PU.1.

Severe sepsis can lead to organ failure and is associated with high mortality rates. An uncontrolled hyperinflammatory response has been proposed as the cause of multiple organ dysfunction syndrome (MODS) during sepsis. Complex Toll-like receptor (TLR) signaling and its associated downstream regulators play a crucial role in the innate immune response as the first line of defense against pathogens [1]. Furthermore, in septic patients, monocytic expression of TLR4 is significantly upregulated compared with expression in healthy individuals [2]. The proximal promoters of TLR4 genes, which show a high degree of sequence homology between human and mouse, contain features typical of myeloid-specific genes [3]. PU.1, specifically, is critical for transcriptional regulation of TLR4 in macrophages [4].

The cholinergic antiinflammatory pathway originates with signals from the vagus nerve that result in acetylcholine-dependent interactions with the α7 nicotinic acetylcholine receptor (α7nAChR) on monocytes and macrophages, which in turn reduces cytokine production [5]. Interestingly, the treatment of mice with nicotine, a potent α7nAChR agonist, significantly reduced serum high-mobility group box 1 (HMGB1) levels. HMGB1 serves as a cytokine mediator of lethality in models of systemic inflammation [6]. Recently, Tsoyi et al [7] reported that stimulation of α7nAChR by nicotine improves survival in cases of sepsis through...
heme oxygenase-1 induction via phosphoinositide 3-kinase (PI3K) signaling. However, the molecular interactions that occur between the nervous and immune systems during sepsis are not fully understood.

Therefore, in this study, we sought to elucidate the underlying mechanisms of the beneficial effects of cholinergic anti-inflammatory pathway stimulation by nicotine in cases of septic injury, with a particular focus on the modulation of TLR4 expression and signaling.

**MATERIALS AND METHODS**

**Animals**

Male BALB/c mice (20–25 g) were supplied by Daehan-Bioline. The mice were given access to water and food *ad libitum*. All animal procedures were approved by the Sungkyunkwan University Animal Care Committee and were performed in accordance with the guidelines of the National Institutes of Health.

**Cecal Ligation and Puncture**

Polymicrobial sepsis was induced by cecal ligation and puncture (CLP) according to the method described by Chaudry et al [8]. Sham-operated animals were subjected to laparotomy and intestinal manipulation; however, the cecum was neither ligated nor punctured. Mice received intraperitoneal nicotine (400 μg/kg) or vehicle (saline) 0 hour after CLP (immediately after CLP) in the biochemical study and 0, 24, 48, and 72 hours after CLP in the survival test. Methyllycaconitine (MLA, 5 mg/kg; Tocris Bioscience), a selective α7nAChR antagonist, was dissolved in saline and intraperitoneally administered 5 minutes prior to nicotine treatment. Wortmannin (1 mg/kg; Calbiochem), a PI3K inhibitor, was dissolved in 1% dimethyl sulfoxide (DMSO) and administered 1 hour before CLP. The dose and timing of MLA and wortmannin treatment were selected based on previous reports [9, 10] and our preliminary studies.

**Endotoxemia**

Endotoxemia was induced by intraperitoneal injection of lipopolysaccharide (LPS; 10 mg/kg). Mice were intraperitoneally treated with nicotine (400 μg/kg) 30 minutes before and 6, 30, and 54 hours after LPS injection. MLA (5 mg/kg) was intraperitoneally administered 5 minutes prior to nicotine treatment.

**Assessment of Organ Damages**

Under anesthesia, the blood sample was obtained 1, 3, 6, 12, 24, and 48 hours after CLP. Alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine, and lactate dehydrogenase (LDH) levels were determined using a Hitachi 7600 automatic analyzer (Hitachi).

**Histology**

Twenty-four hours after CLP, lung and liver tissues were removed and fixed in 10% neutral-buffered formalin. Samples were then embedded in paraffin, sliced into 5-μm sections, and stained with hematoxylin-eosin in preparation for blinded histologic assessment. Histologic changes were evaluated in random, nonconsecutive fields magnified at ×200 (Olympus BX51/ Olympus DP71; Olympus).

**Cytokines**

Serum levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 were measured 1, 3, 6, 12, and 24 hours after CLP and interferon (IFN)-γ was measured 6 hours after CLP using commercially available enzyme-linked immunosorbent assay kits (BD Biosciences) according to the manufacturer’s instructions.

**HMGB1 Preparation**

The serum was obtained 30 hours after CLP and concentrated using YM-100 and YM-10 Centricrons (Millipore) with centrifugation (7500 g for 15 minutes). The concentrated samples were then subjected to Western blot analysis.

**Cell Culture and Treatment**

RAW264.7 cells were obtained from the American Type Culture Collection. The cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco/Invitrogen) and maintained at 37°C in an atmosphere of 5% CO2. MLA (30 μM), wortmannin (20 nM; Calbiochem), or vehicle (DMSO) were applied 30 minutes before nicotine (30 μM; Sigma Chemical Co.) treatment. One hour after nicotine administration, cells were treated with LPS (1 μg/mL; *Escherichia coli* serotype O111: B4; Sigma Chemical Co.) for the indicated time periods, and the culture media and cells were harvested for further analysis.

**siRNA Transfection**

RAW264.7 cells were transfected with either control siRNA (60 pmol) or α7nAChR siRNA (60 pmol) using siRNA transfection reagent (Santa Cruz Biotechnology). The cells were collected 48 hours after transfection, washed, and then pretreated with or without nicotine (30 μM) for 1 hour following LPS (1 μg/mL) treatment. After incubation with LPS for the indicated time periods, the culture media were harvested for further analysis. The level of α7nAChR protein expression was measured 48 hours after transfection to monitor expression of α7nAChR.

**Electromobility Shift Assay**

Nuclear protein (2 μg) was preincubated with electromobility shift assay (EMSA) binding buffer (Panomics) and 1 μg poly d [I-C] at room temperature for 5 minutes. Next, 10 ng of biotinylated PU.1 element probe (Panomics; 5'-GGCTCTCTAATT CTCCTTTTCT-3'), with or without an excess of unlabeled competitor DNA, was added and incubated for 30 minutes at 15°C. Specific binding of the PU.1 to labeled PU.1 was assessed by introducing unlabeled PU.1 in a 66-fold molar excess. After...
incubation, reaction products were separated on a 6% nondenaturing polyacrylamide gel in 1 × Tris-borate EDTA buffer. The gel was run and transferred to a Biodyne B nylon membrane (Pierce Biotechnology). Chemiluminescent detection of biotinylated DNA was performed using a Panomics EMSA kit according to the manufacturer’s instructions.

**Western Blot**

Protein samples were prepared using PRO-PREP protein extraction solution (iNtRON Biotechnology Inc.) for total fractions and NE-PER (Pierce Biotechnology) for nuclear and cytosolic fractions according to the manufacturer’s instructions. Protein samples were loaded on 7.5%–15% polyacrylamide gels, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked for 1 hour, incubated overnight at 4°C with primary antibodies, and then incubated with the appropriate secondary antibodies for 1 hour. The intensity of the immunoreactive bands was determined using TotalLab TL120 software (Nonlinear Dynamics Ltd.). Primary antibodies against mouse α7nAChR, TLR4, HMBG1 (Abcam), PI3K, Akt, phospho-PI3K, phospho-Akt, and PU.1 (Cell Signaling Technology) were used, and the signals were standardized against that of β-actin (Sigma Chemical Co.) for whole and cytosolic lysates and lamin B1 (Abcam) for the nuclear fractions.

**Real-Time Reverse-Transcription Polymerase Chain Reaction**

Total RNA was prepared from tissues and cells using RNAsino (Takara Bio Inc.) according to the manufacturer’s protocol. Reverse transcription of total RNA was performed for synthesis of cDNA using a Takara RNA polymerase chain reaction (PCR) kit. Real-time (RT)-PCR was performed using the LightCycler Nano instrument (Roche). The gene-specific primers used are listed in Table 1. The PCR amplification cycling conditions were as follows: holding 94°C for 30 seconds, 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds, total of 30 cycles for TLR4; holding 94°C for 30 seconds, 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, total of 25 cycles for β-actin.

**Statistical Analysis**

Survival data were analyzed using a Kaplan-Meier curve and a log-rank test. All other data were analyzed by 1- or 2-way analysis of variance, and the Bonferroni test was used for post-hoc comparisons. Differences between the groups were considered statistically significant at $P < .05$. Results are expressed as the mean ± standard error of the mean.

**RESULTS**

**Stimulation of α7nAChR by Nicotine Reduces CLP- and LPS-Induced Mortality, Organ Injury, and Inflammatory Cytokines**

In the survival test, nicotine 50, 100, and 200 μg/kg at 0, 24, 48, and 72 hours after CLP, reduced mortality (log-rank test, $n = 10$, control 20% vs 30% $P = .724$, 40% $P = .554$, and 60% $P = .104$, respectively; data not shown). Mice that were administered 400 μg/kg of nicotine showed improved survival in polymicrobial sepsis (log-rank test, $n = 10$, 20% vs 80%, $P = .006$) and endotoxemia (log-rank test, $n = 10$, 10% vs 70%, $P = .004$) compared with control animals. This protective effect of nicotine likely occurs via α7nAChR, given that it was abolished by administration of MLA (Figure 1A and 1B). Nicotine (400 μg/kg) and MLA alone did not affect mortality (data not shown). On the basis of this result, a dose of 400 μg/kg of nicotine was chosen as the optimal dose for biochemical studies. Serum levels of ALT, BUN, creatinine, and LDH significantly increased during sepsis. Nicotine attenuated these increases, and MLA abolished this effect (see Supplementary Figure 1A–D). Twenty-four hours after CLP, marked histopathologic changes occurred in both liver (inflammatory cell infiltration, portal inflammation, and hepatocellular necrosis) and lung tissues (edema, interstitial inflammation, and inflammatory cell infiltration). These pathologic changes in liver and lung were attenuated by nicotine, and the beneficial effects were again abolished by MLA (Figure 1C and 1D). Sepsis is characterized by a dysregulated production of cytokines. In the present study, nicotine attenuated the production of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (see Supplementary Figure 2A–C). Nicotine also attenuated the production of HMBG1, a late lethal mediator of sepsis and ligand for TLR4, at 30 hours after CLP (see Supplementary Figure 2D). MLA abolished the effects of nicotine on serum cytokine and HMBG1 levels.

**Stimulation of α7nAChR by Nicotine Suppresses TLR4 Protein and mRNA Expression as Well as DNA Binding of PU.1**

In our study, increases in TLR4 protein and mRNA expression in the liver at 6 hours after CLP were reduced by nicotine, and this effect was abolished by MLA (Figure 2A and 2B). The level of nuclear PU.1 protein expression in the liver at 6 hours after CLP was significantly increased. This increase was reduced by nicotine treatment, and MLA reversed the effect of nicotine (Figure 2C). To further verify the involvement of α7nAChR in the downregulation of TLR4 by nicotine, α7nAChR siRNA transfection was performed in RAW264.7 cells. Nicotine did not show cellular toxicity in RAW264.7 cells up to 100 μM (Figure 3A). We confirmed that α7nAChR siRNA diminished comparisons. Differences between the groups were considered statistically significant at $P < .05$. Results are expressed as the mean ± standard error of the mean.

<table>
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<th>Gene</th>
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<td>TLR4</td>
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<tr>
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<td>(NM_007393)</td>
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α7nAChR protein expression 48 hours after siRNA transfection (Figure 3B). The inhibitory effect of nicotine on TLR4 protein expression was abolished in α7nAChR siRNA-transfected cells (Figure 3C). Furthermore, LPS markedly increased nuclear translocation of PU.1, and these increases were inhibited by nicotine (Figure 3D and 3E). DNA binding of PU.1 was markedly increased by LPS treatment. Nicotine inhibited the binding of PU.1 to its element, and MLA abolished the effects of nicotine (Figure 3F). Nicotine (400 μg/kg) did not show cytotoxicity in RAW264.7 cells (data not shown).

Stimulation of α7nAChR by Nicotine Suppresses TLR4 Expression Through PI3K/Akt Activation in LPS-Treated RAW264.7 Cells

In the present study, PI3K/Akt activation, which was further augmented by nicotine, was induced at 1 hour after LPS treatment. This effect of nicotine was abolished by MLA (Figure 4A and 4C). To better characterize the signaling cascade responsible for nicotine-induced PI3K/Akt activation, we used the PI3K inhibitors, wortmannin and LY294002. At 1 hour after LPS treatment, nicotine-induced PI3K/Akt activation in

Figure 1. Stimulation of α7 nicotinic acetylcholine receptor by nicotine reduces cecal ligation and puncture (CLP)- and lipopolysaccharide (LPS)-induced mortality and organ injury. Sham-operated animals were subjected to laparotomy and intestinal manipulation; however, the cecum was neither ligated nor punctured. A and B, Survival rate. Mice were given intraperitoneal nicotine (400 μg/mg) or vehicle (saline) 0, 24, 48, and 72 hours after CLP (A, n = 10 per group) and 6, 30, and 54 hours after LPS treatment (B, n = 10 per group). Mice were treated with intraperitoneal methyllycaconitine (MLA; 5 mg/kg) 5 minutes prior to nicotine treatment. All animals were monitored for 10 days after CLP surgery and LPS administration. *P < .01 (log-rank test; significant difference from CLP or LPS group). **P < .05 (log-rank test; significant difference from CLP + nicotine or LPS + nicotine group). Histological micrographs of liver (C) and lung (D). Mice were given intraperitoneal nicotine (400 μg/mg) or vehicle 0 hour after CLP. Mice were treated with intraperitoneal MLA (5 mg/kg) 5 minutes prior to nicotine treatment. Twenty-four hours after CLP, liver and lung tissues were obtained. Histological micrographs of liver and lung tissues stained with hematoxylin and eosin are shown (magnification ×200; scale bar = 100 μm). Representative images were chosen from each of the experimental groups.
LPS-stimulated cells was markedly inhibited by pretreatment of cells with wortmannin and LY294002 (Figure 4B and 4D). The inhibitory effect of nicotine on the binding of PU.1 to its element (Figure 3F) as well as TLR4 protein and mRNA expression were also abolished by wortmannin (Figure 4E and 4F).

Nicotine Protects Against Septic Injury Through α7nAChR/PI3K/Akt Pathway Activation During Sepsis

We tested whether nicotine activates the α7nAChR/PI3K/Akt pathway in vivo during sepsis. The activation of PI3K and Akt increased 6 hours after CLP. Nicotine augmented this activation and MLA counteracted this effect of nicotine (Figure 5A).
In the CLP model, wortmannin reversed the decrease in mortality observed with nicotine treatment (log-rank test, \( n = 9-10 \), \( P = .012 \); Figure 5C). Wortmannin alone did not affect mortality (data not shown). Furthermore, wortmannin blocked the inhibitory effects of nicotine on nuclear PU.1 and TLR4 overexpression at 6 hours after CLP (Figure 5D and 5E).

Figure 3. Nicotine suppresses Toll-like receptor 4 (TLR4) protein expression and PU.1 activity through \( \alpha 7 \) nicotinic acetylcholine receptor (\( \alpha 7nAChR \)) activation in lipopolysaccharide (LPS)-treated RAW264.7 cells. A, Cytotoxicity of nicotine on RAW264.7 cells. The cells were treated with various concentrations of nicotine for 24 hours for 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide assay. B, \( \alpha 7nAChR \) protein expression. Forty-eight hours after \( \alpha 7nAChR \) siRNA transfection, \( \alpha 7nAChR \) protein expression was estimated using Western blot analysis. C, TLR4 protein expression. After siRNA transfection, the cells were treated with nicotine (30 \( \mu M \)) for 1 hour followed by LPS 1 \( \mu g/mL \) treatment for 6 hours. D and E, Translocation of PU.1 to nucleus. The cells were pre-treated with methyllycaconitine (MLA; 30 \( \mu M \)) or wortmannin (20 nM) for 30 minutes, then treated with nicotine (30 \( \mu M \)) for 1 hour, and finally LPS (1 \( \mu g/mL \)) for 1 hour. Densitometry was performed, and fold changes in protein expression are shown below the representative bands. Results are expressed as the mean ± standard error of mean of 3 independent experiments. ** \( P < .01 \) (Bonferroni test; significant difference from control siRNA or control group). †† \( P < .01 \) (Bonferroni test; significant difference from LPS group). §§ \( P < .01 \) (Bonferroni test; significant difference from LPS + nicotine group). F, DNA binding of PU.1. The cells were pretreated with MLA (30 \( \mu M \)) or wortmannin (20 nM) for 30 minutes, then treated with nicotine (30 \( \mu M \)) for 1 hour, and finally LPS (1 \( \mu g/mL \)) for 1 hour. The cold probe was in 66-fold excess of unlabeled PU.1 oligonucleotide. The autoradiogram is representative of 3 independent experiments.
Figure 4. Stimulation of α7 nicotinic acetylcholine receptor by nicotine suppresses Toll-like receptor 4 (TLR4) expression through PI3K/Akt activation in lipopolysaccharide (LPS)-treated RAW264.7 cells. A and C, PI3K and Akt activation. The cells were pretreated with methyllycaconitine (MLA; 30 μM) for 30 minutes, then treated with nicotine (30 μM) for 1 hour, and finally LPS (1 μg/mL) for 1 hour. B and D, PI3K and Akt activation. The cells were pretreated with wortmannin (20 nM) or LY294002 (20 μM) for 30 minutes, then treated with nicotine (30 μM) for 1 hour, and finally LPS (1 μg/mL) for 1 hour. TLR4 protein (E) and mRNA (F) expression. The cells were pretreated with MLA (30 μM) or wortmannin (20 nM) for 30 minutes, then treated with nicotine (30 μM), and finally LPS (1 μg/mL) for 1 hour and 6 hours prior to measuring TLR4 protein and mRNA expression, respectively. Densitometry was performed, and fold changes in protein and mRNA expression are shown below the representative bands. Results are expressed as the mean ± standard error of mean of 3 independent experiments. *P<.05, **P<.01 (Bonferroni test; significant difference from control group). \( ^{\dagger}P<.01 \) (Bonferroni test; significant difference from LPS group). \( ^{\ddagger}P<.05, ^{\ddagger\ddagger}P<.01 \) (Bonferroni test; significant difference from LPS + nicotine group).
Figure 5. Nicotine activates the α7 nicotinic acetylcholine receptor/Akt pathway during sepsis. A and B, PI3K and Akt activity. Mice were treated with methyllycaconitine (MLA; 5 mg/kg) 5 minutes prior to nicotine (400 μg/kg; 0 hour after cecal ligation and puncture [CLP]) exposure. Liver tissue was obtained 6 hours after CLP. Sham-operated animals were subjected to laparotomy and intestinal manipulation; however, the cecum was neither ligated nor punctured. The p-PI3K/PI3K (A) and p-Akt/Akt (B) ratios were determined by Western blot analysis. C, Survival rate. Mice were treated with wortmannin (1 mg/kg) 1 hour before nicotine (400 μg/kg; 0, 24, 48, and 72 hours after CLP) treatment. D and E, PU.1 and TLR4 protein expression in liver. Mice were treated with wortmannin (1 mg/kg) 1 hour before nicotine (400 μg/kg; 0 hour after CLP) treatment. Six hours after CLP, nuclear and whole extracts were prepared for Western blot analysis of PU.1 (D) and TLR4 (E), respectively. F and G, Serum tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) levels. Mice were treated with wortmannin (1 mg/kg) 1 hour before nicotine (400 μg/kg; 0 hour after CLP) treatment. Six hours after CLP, serum TNF-α (F) and IFN-γ (G) levels were measured, respectively. Densitometry was performed, and fold changes in protein and mRNA expression are shown below the representative bands. Results are expressed as the mean ± standard error of mean of 6–8 animals per group. *P < .05, **P < .01 (Bonferroni test; significant difference from sham group). †P < .05, ‡P < .01 (Bonferroni test; significant difference from CLP group). §P < .05, §§P < .01 (Bonferroni test; significant difference from CLP + nicotine group).
The inhibitory effects of nicotine on serum TNF-α and IFN-γ levels were also blocked by wortmannin (Figure 5F and 5G).

**DISCUSSION**

The cholinergic antiinflammatory pathway has been implicated as a neuronal feedback system that serves to limit the inflammatory response. Stimulation of the vagus nerve prevents cytokine release in experimental models of sepsis, endotoxemia, ischemia/reperfusion injury, hemorrhagic shock, arthritis, and other inflammatory syndromes [11–13].

Cholinergic and anticholinergic drugs are widely used in intensive care units, where patients with sepsis and organ failure often have increased cytokine levels and depressed vagus nerve activity [14]. Treatment of mice with nicotine significantly reduced serum HMGB1, a cytokine mediator of lethality in clinically relevant CLP-induced sepsis models [7]. In contrast, Giebel et al [15] showed that stimulation of acetylcholine receptors impairs host defense function in patients with pneumococcal pneumonia. Nicotine pretreatment strongly decreased cell influx, proinflammatory cytokine levels, and liver damage, whereas survival was impaired. Among the various nicotinic acetylcholine receptors, α7, which has been identified in immune cells, is believed to contribute to nicotinic antiinflammatory effects [16]. In the present study, we found that nicotine had potent antiinflammatory effects that selectively attenuated the systemic release of proinflammatory cytokines and HMGB1 through α7nAChR stimulation. However, nicotine did not affect the colony forming units in peritoneal cavity and blood (data not shown).

During sepsis, excessive production of proinflammatory cytokines causes capillary leakage, tissue injury, and, ultimately, lethal organ failure. MODS is a serious clinical condition and a common cause of death in critically ill patients. Our study showed that the administration of nicotine in septic mice effectively reduced mortality. Interestingly, the time-dependent profile of markers of organ injury showed differences in the vulnerability of organs during sepsis. Treatment with nicotine resulted in decreases of serum markers of organ dysfunction, and MLA abolished this beneficial effect. These results suggest that stimulation of α7nAChR by nicotine is responsible for attenuating MODS and improving mortality.

The signaling pathways that mediate the antiinflammatory effects of nicotine have been investigated extensively; however, much less is known about its actions in immune cells. TLR plays an important role in the initiation of the inflammatory response and is a central target in sepsis therapy. Our recent report revealed that upregulated expression of TLR4 in the liver plays a role in the pathophysiology and high mortality associated with sepsis [17]. GTS-21, a potent α7nAChR agonist, modulated TLR expression after LPS stimulation and prevented cytokine release [18]. In our study, nicotine attenuated increases in TLR4 protein and gene expression, and these protective effects were abolished by MLA treatment. This suggests that nicotine suppresses TLR4 expression at the transcriptional level through stimulation of α7nAChR.

Regulation of TLR4 gene expression in macrophages is an important determinant of the intensity and/or duration of the response to endotoxins. Both human and mouse TLR4 genes contain multiple purine-rich sequence motifs that are recognized by transcription factors in the E26 transformation-specific family, including myeloid-specific factor PU.1. Several lines of evidence suggest that PU.1 is critical for both macrophage maturation and transcriptional regulation of TLR4 in macrophages [19]. Our findings demonstrate that stimulation of α7nAChR by nicotine suppresses PU.1-binding activity in macrophages, as demonstrated by EMSA. The fact that we observed suppression of PU.1 in parallel with the reduction in TLR4 mRNA 6 hours after CLP strongly suggests that transcription of TLR4 after nicotine exposure is mediated by this key transcriptional factor.

Although PU.1 was identified as a major regulator of TLR4, the molecular mechanisms that regulate TLR4 expression are largely unknown. PI3K, a signal transduction enzyme, and Akt, a downstream serine/threonine kinase, have been reported to play a role in cellular activation, the inflammatory response, chemotaxis, and apoptosis [20]. The PI3K pathway plays a critical role in immunologic defense mechanisms and acts, in part, in response to activation of intracellular proinflammatory signaling pathways [21]. Additionally, a growing body of evidence indicates that PI3K functions as a regulator of TLR signaling.

Although many studies have shown that TLR4 signaling can activate PI3K to limit production of TNF-α and that pharmacological blockade of PI3K results in enhanced activation of nuclear factor κ-B [22], the exact role of PI3K activation in TLR4 signaling remains controversial and is likely multifaceted. PI3K/Akt activation has been shown to decrease cellular apoptosis and TLR4 expression, and treatment with a specific PI3K inhibitor increased local inflammation [23]. However, it has been reported that PI3K/Akt contributes to increased expression of TLR4 in macrophages exposed to hypoxic stress via activation of hypoxia inducible factor-1α [24]. In the present study, nicotine augmented sepsis-induced PI3K/Akt activation through α7nAChR activation. PI3K inhibition blocked the beneficial effects of nicotine, as evidenced by increased mortality and augmented inflammatory responses. Moreover, nicotine-induced attenuation of TLR4 protein expression and production of early and late proinflammatory cytokines were blocked by PI3K inhibition. The negative role of PI3K in TLR4 signaling was also observed in LPS-treated RAW264.7 cells. Pretreatment of cells with PI3K inhibitors markedly decreased nicotine-induced inhibition of proinflammatory cytokines. Moreover, in LPS-stimulated RAW264.7 cells, nicotine-activated α7nAChR/PI3K inhibited nuclear translocation of PU.1, and this was further confirmed using an EMSA assay in which DNA binding of PU.1 was increased by a PI3K inhibitor.
In conclusion, stimulation of α7nAChR by nicotine improves mortality rates and MODS during sepsis. This protective effect of nicotine can be associated with the inhibition of TLR4 overexpression through the PI3K/Akt signaling pathway. Although the therapeutic potential of nicotine is still limited by its nonspecific effects, this study may provide an impetus for further development of therapeutic strategies for modifying the cholinergic anti-inflammatory pathway in the treatment of various inflammatory diseases.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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Potential conflict of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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