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Requisite Role of the Cholinergic α7 Nicotinic Acetylcholine Receptor Pathway in Suppressing Gram-Negative Sepsis-Induced Acute Lung Inflammatory Injury

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Although activation of the α7 nicotinic acetylcholine receptor (α7 nAChR) modulates the response to sepsis, the role of this pathway in the development of sepsis-induced acute lung injury (ALI) is not known. In this study, we addressed the contribution of α7 nAChR in mediating endotoxin- and live *Escherichia coli*-induced ALI in mice. Because we found that α7 nAChR+ alveolar macrophages and neutrophils were present in bronchoalveolar lavage and injured lungs of mice, we tested whether acetylcholine released by lung vagal innervation stimulated these effector cells and thereby down-regulated proinflammatory chemokine/cytokine generation. Administration of α7 nAChR agonists reduced bronchoalveolar lavage MIP-2 production and transalveolar neutrophil migration and reduced mortality in *E. coli* pneumonia mice, whereas vagal denervation increased MIP-2 production and airway neutrophil accumulation and increased mortality. In addition, α7 nAChR−/− mice developed severe lung injury and had higher mortality compared with α7 nAChR+/+ mice. The immunomodulatory cholinergic α7 nAChR pathway of alveolar macrophages and neutrophils blocked LPS- and *E. coli*-induced ALI by reducing chemokine production and transalveolar neutrophil migration, suggesting that activation of α7 nAChR may be a promising strategy for treatment of sepsis-induced ALI. The Journal of Immunology, 2010, 184: 401–410.

The cholinergic antiinflammatory pathway (1) has been described in a series of experiments showing that vagus nerve stimulation attenuated the systemic inflammatory response to endotoxin (1–3). The α7 nicotinic acetylcholine receptor (α7 nAChR) expressed in macrophages regulates this pathway during inflammation (4) such that cholinergic activation of these cells dampens the inflammatory response. The studies showed that activation of α7 nAChR by agonists (e.g., nicotine) downregulated expression of HMGB1 protein (a late mediator of sepsis) and improved survival (5, 6). Stimulation of the cholinergic antiinflammatory pathway also appears to play a protective role in other inflammatory models, peritonitis (7, 8), and renal ischemia and reperfusion injury (9).

Activation of α7 nAChR in macrophages and monocytes may downregulate production of proinflammatory cytokines and attenuate the inflammatory response by several possible but poorly understood mechanisms: 1) suppression of NF-κB translocation and IκB phosphorylation (5, 10), 2) activation of Jak2-STAT3 signaling (11), and 3) inhibition of expression of LPS receptors and binding proteins CD14 and TLR4 (12). Some studies have also indicated a possible role of specific α7 nAChR agonists GTS-21 (13) and choline (14) or cholinesterase inhibitors (15) for improving outcome in experimental sepsis.

Sepsis-induced acute lung injury (ALI) causes acute respiratory failure in critically ill patients and has a mortality rate of 40% (16, 17). The most common causes of ALI are pneumonia and sepsis (17, 18). Although activation of the α7 nAChR modulates the response to sepsis, the role of this pathway in the development of sepsis-induced ALI is not known. Our objectives here are to determine the role of activation of alveolar macrophages and neutrophils expressing α7 nAChR on the production of proinflammatory cytokines and chemokines, whether α7 nAChR affects the ALI response induced by endotoxin and *Escherichia coli* pneumonia, and whether antagonism or deficiency of α7 nAChR activity and vagus denervation has the opposite effects of lung inflammation and injury and survival in mouse models.

Materials and Methods

Reagents

(-)-nicotine, acetylcholine (ACh), methyllycaconitine (MLA), and LPS were purchased from Sigma-Aldrich (St. Louis, MO). Dimethylaminobenzaldehyde (DMAB), PNU 282987 (PNU), and PHA 568487 (specific agonists of α7 nAChR) were purchased from Tocris Bioscience (Ellisville, MO) and dissolved in 0.9% saline before each experiment. H-302, an anti-α7 nAChR Ab used to detect α7 nAChR on the production of mouse and human origin, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PE antimouse CD11, Ly-6G (Gr-1, Gr1), and corresponding isotype Abs were purchased from eBioscience (San Diego, CA).

Animals

Most experiments were done with CD1 mice (purchased from Charles River Laboratories, Wilmington, MA). α7 nAChR-deficient mice (C57BL/6 background, B6.129S7-ChRNA7tm1Bay, number 003232) and wild type (WT) littermates (C57BL/6J, 8 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME) (4). Anesthesia was induced with an i.p. injection of a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg). The Committee on Animal Research of the University of Illinois at Chicago and the University of California, San Francisco approved the protocols.
Isolation and culture of alveolar macrophages

Alveolar macrophages were isolated by bronchoalveolar lavage (BAL) (19, 20). Greater than 90% alveolar macrophage purity was confirmed with a cytospin preparation (Cytospin 3, Thermo Electron, Milford, MA) and Hema 3 staining (Fisher Scientific, Kalama zoo, MI). Alveolar macrophages were cultured in RPMI 1640 medium (2.5 × 10^5/ml), α7 nAChR agonists or an antagonist (MLA) were added 20 min before LPS stimulation (3 × 10^5 CFU) for early infection compared with the WT mice. E. coli (107 CFU) was instilled into the air spaces of lungs. Immediately before exposure to agonists or an antagonist (MLA) were added 20 min before LPS stimulation (3 μM). PBS was used as a negative control. The media was collected after 12 h incubation for TNF-α and MIP-2, and after 20 h for HMGB1 measurements.

Neutrophil isolation and culture

As described (20), mice were euthanized by cervical dislocation, and the bone marrow from the femurs and tibias was flushed with PBS using a 25-gauge needle. The whole bone marrow was centrifuged and washed in PBS, and red blood cells were hypotonically lysed with 0.2% NaCl. This solution was resuspended in PBS and then centrifuged after 70-μm nylon cell strainer (BD Discovery Labware, Bedford, MA). The solution was centrifuged and resuspended in PBS and then delicately applied over a 62% Percoll gradient. The Percoll solution was centrifuged for 30 min at 1500 × g. The neutrophil pellet was then isolated, washed, and centrifuged twice, and counted with a Coulter counter (Z1 series; Beckman Coulter, Fullerton, CA). Greater than 90% neutrophil purity was confirmed with the cytospin preparation and Hema 3 staining. Neutrophils were suspended in RPMI 1640 medium (10^6/ml). α7 nAChR agonists or an antagonist (MLA) were added 20 min before LPS stimulation (3 μM). PBS was used as a negative control. The media was collected after 12 h incubation for measuring MIP-2 or TNF-α concentration.

LPS-induced ALI mouse model

As described, mice were intratracheally (IT) instilled with PBS (5 mg/kg) by a direct visualized instillation method (21). Mice were monitored for 24 and 48 h, and killed to perform BAL or measure extravascular lung water (ELW).

Acute E. coli pneumonia mouse model and survival study

Live E. coli were obtained from American Type Culture Collection, Manassas, VA (ATCC 25992) (22). E. coli (10^7 CFU) was instilled into the air spaces of lungs. Immediately before exposure to E. coli, mice received 0.05 μCi [125I]-albumin via the right jugular vein. Mice were monitored for 4 h and killed to measure ELW and lung endothelial permeability to protein or to perform BAL.

We found a correlation between the degree of lung injury and mortality and the dose of IT E. coli. Therefore, different doses were used depending on the experimental objectives: 1) E. coli (10^7 CFU) for early experiments at 4 h to ensure that the lungs were substantially injured; 2) E. coli (2.5 × 10^6 CFU) for longer experiments (24 h) to ensure that there was no death in both control and treated groups; 3) E. coli (5 × 10^5 CFU) for the survival study, to be certain that some mice died of substantial lung injury within 24 h, which facilitated observing the difference between the control and treated groups; and 4) E. coli (4 × 10^6 CFU) for the survival study with vagotomized or α7 nAChR −/− mice, because they were more susceptible to the E. coli infection compared with the WT mice.

ELW and lung extravascular plasma equivalent

As described (19), homogenate and supernatant of lung, and blood were weighed and then desiccated in an oven (60°C for 24 h). ELW was calculated by standard formula:

\[
ELW = \left(\frac{QW_{exp}\times Qd_{exp}}{QW_{control}\times Qd_{control}}\right)\times 1000 \mu l
\]

where QW exp equals water volume of the lung in the experimental group; Qd exp equals dry weight of lung in the experimental group. The control was the normal mice with the same age as the experimental group. Lung extravascular plasma equivalents (EPEs: index of lung vascular permeability to protein) were calculated as the counts of [125I]-album in the blood free lung tissue divided by the counts of [125I]-album in the plasma.
Flow cytometric analysis

Monoclonal Abs PE anti-mouse CD11b (M1/70), Gr1 (RB6-8C5), and IgG isotype controls were obtained from ebioscience. Rabbit anti-mouse α7 nAChR and control Abs were obtained from Santa Cruz Biotechnology. All samples were pretreated with Fc receptor blocking reagent to prevent nonspecific binding. After BAL, red blood cells were lysed. The BAL cell pellets were washed with PBS (2.5% FCS), fixed in 2% paraformaldehyde, permeabilized with 0.2% Triton, and then labeled with corresponding Abs. Fluorescent cells were analyzed after exclusion of debris and aggregates with CyAn ADP or MoFlo (DakoCytomation, Carpinteria, CA). Data were analyzed by Summit 4.3 software (DakoCytomation).

α7 nAChR Western blotting of BAL samples

As described (19), denatured proteins (20 μg) from BAL cells were loaded and run on a 4–12% gradient Bis-Tris gel (Invitrogen, Carlsbad, CA). The proteins were then transferred to a nitrocellulose membrane and incubated with anti-α7 nAChR Ab (H-302). Membranes were exposed to HRP-labeled Ab and developed with an ECL kit (Amersham, Piscataway, NJ).

Statistical analysis

Statistical analysis was done with SPSS software (SPSS, Chicago, IL). An unpaired t test was used unless there were multiple comparisons, in which case we used ANOVA with post hoc Bonferroni test (significance level was set at p ≤ 0.05). The log-rank test was used for comparing survival data by GraphPad Prism software (GraphPad, San Diego, CA). The results are shown as means ± SD.

Results

Alveolar macrophages express α7 nAChR

In normal BAL cells, alveolar macrophages are dormant or resting cells (Fig. 1A). α7 nAChR immunoreactivity was found in the cell membrane and cytoplasm (Fig. 1B, 1C). Western blotting demonstrated that normal BAL cells (mainly alveolar macrophages) expressed α7 nAChR with a band at 55 kDa, using PC12 cells and rat brain extract as positive controls (Fig. 1D). To study alveolar macrophage cell surface expression of α7 nAChR, normal BAL cell pellets were double stained with anti-mouse CD11b (Mac-1, a surface marker of macrophages) and α7 nAChR Abs to perform flow cytometry. We observed that 14.7 ± 8.7% of normal BAL cells coexpressed CD11b and α7 nAChR (Fig. 1E, 1F). To investigate coexpression of α7 nAChR and CD11b in E. coli–injured lungs, lung sections were obtained from pneumonia mice at 12 h after IT challenge of E. coli for immunostaining. Coexpression of α7 nAChR and CD11b was found in injured lung sections (Fig. 1G–J).

Effects of nicotine and MLA on TNF-α and MIP-2 production in alveolar macrophages

To test whether activation of α7 nAChR by nicotine in alveolar macrophages alters proinflammatory cytokine production and whether MLA (a specific α7 nAChR antagonist) reverses the effects of nicotine, alveolar macrophages were pretreated separately with PBS, 10^{-6} M nicotine, or 10^{-5} M nicotine and MIP-2 in the media. Untreated cells with PBS challenge were used as controls. LPS stimulation increased MIP-2 (545 ± 24 versus 31 ± 18 pg/ml in PBS group) and TNF-α (704 ± 6 versus 16 ± 7 pg/ml in PBS group) production in alveolar macrophages. Nicotine reduced LPS-induced MIP-2 and TNF-α production. MLA counteracted this effect of nicotine on TNF-α and MIP-2 production (Fig. 1K, 1L).

Effects of nicotine and ACh on HMGB1 production in alveolar macrophages

To test whether activation of α7 nAChR agonists by nicotine and ACh affects HMGB1 production in alveolar macrophages, alveolar macrophages were pretreated separately with PBS, 10^{-7} M nicotine or ACh, 10^{-6} M nicotine or ACh, or 10^{-5} M nicotine or ACh. At 20 min, the cells from these four groups were stimulated with LPS (3 μM) for 20 h to measure HMGB1 in the media. Untreated cells with PBS challenge were used as controls. LPS stimulation increased HMGB1 production in alveolar macrophages (399 ± 204 versus 28 ± 6 pg/ml in the PBS stimulated group. Nicotine reduced HMGB1 by 25 and 32% at 10^{-6} and 10^{-5} M, respectively (Fig. 1M). ACh suppressed HMGB1 by 30% at 10^{-7} to 10^{-5} M (Fig. 1N).

Neutrophils express α7 nAChR

To determine whether neutrophils express α7 nAChR, we collected BAL cells from E. coli pneumonia (2.5 × 10^{6} CFU, IT, sacrificed at 12 h) to perform immunofluorescence and Western blotting. The smear of BAL cells by Hema 3 staining demonstrated that the number of neutrophils (Fig. 2A) was increased in BAL from E. coli pneumonia compared with normal BAL cells, which are mainly alveolar macrophages (Fig. 1A). By Western blotting, we observed that the expression of α7 nAChR in BAL cells from E. coli pneumonia (alveolar macrophages mixed with neutrophils) was markedly increased compared with normal BAL cells (Fig. 2B). Immunofluorescence demonstrated that BAL neutrophils from pneumonia expressed α7 nAChR in the cell membrane and cytoplasm (Fig. 2C, 2D, segmented neutrophils). To confirm that neutrophils expressed α7 nAChR, BAL cell pellets (from pneumonia mice at 12 h) were double stained with anti-mouse Gr1 (a surface marker of neutrophils) and α7 nAChR Abs to perform flow cytometry. We observed that 43.6 ± 1.8% of normal BAL cells coexpressed Gr1 and α7 nAChR (Fig. 2E, 2F), confirming the finding that neutrophils express α7 nAChR. To investigate coexpression of α7 nAChR and Gr1 in E. coli–injured lungs, lung sections were obtained from pneumonia mice at 12 h after IT challenge of E. coli for immunostaining. Coexpression of α7 nAChR and Gr1 was found in injured lung sections (Fig. 2G–J).

α7 nAChR activation downregulates TNF-α and MIP-2 production in neutrophils

To investigate whether activation of α7 nAChR agonists by nicotine, DMAB (a novel partial α7 nAChR agonist), or PNU (a highly specific α7 nAChR agonist, K_{i} 27 nM) affect MIP-2 production in neutrophils, neutrophils were pretreated separately with PBS; 10^{-7} M nicotine, DMAB or PNU; 10^{-6} M nicotine, DMAB or PNU; or 10^{-5} M nicotine, DMAB or PNU. At 20 min, cells from these four groups were stimulated with LPS (3 μM) for 12 h to measure MIP-2 concentration in the media. Untreated cells with PBS challenge were used as controls. The MIP-2 concentration in LPS-stimulated neutrophils was increased (1124 ± 84 pg/ml) compared with 11 ± 3 pg/ml in PBS group. Nicotine reduced MIP-2 by 23–40% at 10^{-7} to 10^{-6} M, and 50% at 10^{-5} M (Fig. 2K). DMAB inhibited MIP-2 by 51–53% at 10^{-7} to 10^{-5} M (Fig. 2L), and PNU suppressed MIP-2 by 49% at 10^{-7} to 10^{-6} M and 30% at 10^{-5} M (Fig. 2M).

α7 nAChR-deficient neutrophils propagate proinflammatory cytokine production

To test whether deficiency of α7 nAChR in neutrophils facilitates proinflammatory cytokine production, the isolated bone marrow neutrophils from α7 nAChR^{−/−} and α7 nAChR^{+/−} mice were stimulated with LPS (3 μM). The media was collected at 12 h. After LPS stimulation, TNF-α and MIP-2 concentrations in the media from α7 nAChR^{−/−} neutrophils were increased compared with α7 nAChR^{+/−} neutrophils (Fig. 2N, 2O).

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α7 nAChR activation attenuates LPS-induced ALI

To study whether administration of nicotine suppresses LPS-induced ALI, mice were administered nicotine (0.4 mg/kg, i.p., every 6 h). The first dose was given 15 min before LPS (5 mg/kg, IT), and the control group received the same volume of saline (5) for 24 h. Lungs were lavaged to measure protein, and then homogenized to measure BAL neutrophil counts and TNF-α. Cells were treated with PBS, nicotine, or nicotine + MLA, then stimulated with LPS. *p < 0.05 versus LPS only; n = 3–5 in each group. Data are mean ± SD.

α7 nAChR modulates lung inflammation and edema by suppressing inflammatory mediator production in local milieu

To test whether activation of α7 nAChR reduces ELW and lung EPE in E. coli pneumonia, we divided mice into four groups: 1) saline + saline group, in which the mice were pretreated with saline and then IT saline; 2) nicotine + saline group, in which the mice were treated with nicotine (3.5 mg/kg, i.v.) (19) 5–10 min before IT saline; 3) nicotine + E. coli (107 CFU); the lungs were substantially injured at 4 h at this dose) group, in which the mice were pretreated with saline and then IT E. coli; and 4) nicotine + E. coli (107 CFU) group, in which mice were pretreated with nicotine and then IT E. coli. At 4 h, blood was withdrawn and the lungs were removed to measure ELW and lung EPE. ELW and lung EPE in the saline + E. coli group were increased eightfold to ninefold compared with the saline + saline group. ELW and lung EPE in the nicotine + E. coli group were reduced compared with the saline + E. coli group at 4 h (Fig. 4A, 4B). We also used the same experimental design to determine BAL TNF-α and MIP-2 levels and neutrophil numbers. Nicotine reduced the BAL neutrophil counts and TNF-α and MIP-2 levels compared with the saline group at 4 h (Fig. 4C–E).
To address whether activation of α7 nAChR by nicotine affects E. coli pneumonia at a 24-h time frame, mice were treated with either nicotine (0.4 mg/kg, i.p.) or saline 15 min before IT challenge with 2.5 × 10^8 CFU E. coli (at this dose, there was no death in both control and treated groups within 24 h of the experiment). The same therapy was repeated every 6 h. The mice were killed at 4, 12, and 24 h. Normal mice were used as controls. BAL was collected. BAL from normal mice was used as a control. BAL collected. BAL from normal mice was used as a control. 

AChE activity and choline concentration in BAL in E. coli pneumonia

To study AChE activity in alveolar proinflammatory cells and BAL choline concentrations in E. coli pneumonia, mice were challenged IT with E. coli (2.5 × 10^8 CFU) and sacrificed at 12 h with...
compared with nicotine- and saline-treated groups. BAL protein levels were diminished at 4, 12, and 24 h compared with nicotine- and saline-treated groups (Fig. 5D).

To test the effects of PHA 568487 (a specific agonist of α7 nAChR) on transmigration of proinflammatory cells into the airspace and lung epithelial and endothelial permeability, mice were pretreated with either PHA 568487 (0.4 mg/kg, i.p.) or saline 15 min before IT challenge with *E. coli* (2.5 × 10^6 CFU). The therapy was repeated every 6 h. The mice were killed at 4, 12, and 24 h. Normal mice were used as controls. BAL was collected to measure numbers of leukocytes, neutrophils, and monocytes, and BAL protein levels. At 4 h, BAL leukocyte (0.32 ± 0.02 versus 0.16 ± 0.04 k/μl) and neutrophil counts (0.09 ± 0.05 versus 0.01 ± 0 k/μl) were reduced (Fig. 5E) with PHA 568487 therapy. At 12 h, BAL neutrophil and monocyte counts showed a trend in reduction in the PHA 568487 group (Fig. 5F, 5G). At 24 h, BAL neutrophil count was lower in the PHA 568487-treated group (Fig. 5H).

Based on the above data, we hypothesized that the vagus nerve-mediated ACh-α7 nAChR pathway regulates transendothelial or epithelial migration of proinflammatory cells and lung epithelial and vascular permeability. Therefore, mice with denervation of vagus were used to test this hypothesis. Mice were first surgically prepared with a unilateral cervical vagotomy (right side) or sham operation and then instilled IT with *E. coli* (2.5 × 10^6 CFU). The mice were killed at 4, 12, and 24 h. The right lungs were lavaged. Normal mice were used as controls. BAL was collected to measure the numbers of leukocytes, neutrophils, and monocytes, and BAL protein levels. BAL leukocyte, neutrophil, and monocyte counts were increased at 12 and 24 h in the vagotomized group (Fig. 5I–K). BAL protein levels in vagotomized group were also increased compared with the sham group at 4 and 12 h (Fig. 5F).

α7 nAChR pathway negatively regulates MIP-2 production

To study the effects of nicotine (a nonselective agonist of α7 nAChR) on MIP-2 production in BAL and circulation, mice were pretreated with either nicotine (0.4 mg/kg, i.p.) or saline 15 min before IT challenge with *E. coli* (2.5 × 10^6 CFU). The therapy was repeated every 6 h. The mice were killed at 4, 12, and 24 h. Normal mice were used as controls. BAL and plasma were collected to measure MIP-2 levels by ELISA. The MIP-2 levels in the BAL were reduced at 24 h (Fig. 5A).
Reduced at 4 and 12 h in the nicotine-treated group compared with the saline group (Fig. 6A). MIP-2 levels in the plasma in the nicotine-treated group also showed a lower trend at 4 h (Fig. 6B).

To study the effects of PHA 568487 (a specific agonist of α7 nAChR) on MIP-2 production in BAL and circulation, mice were pretreated with either PHA 568487 (0.4 mg/kg, i.p.) or saline 15 min before IT challenge of E. coli 2.5 × 10^6 CFU, and then the same therapy was repeated every 6 h. The mice were killed at 4, 12, and 24 h. Normal mice were used as controls. BAL and plasma were collected to measure MIP-2 levels by ELISA. MIP-2 levels in the BAL were reduced at 4 h and showed a lower trend at 12 and 24 h in the PHA 568487-treated group (Fig. 6C). The MIP-2 levels in the plasma in the PHA 568487-treated group were also reduced at 4 h (Fig. 6D). Because vagus nerve signaling via the ACh–α7 nAChR pathway may limit MIP-2 production, we studied the effects of denervation of vagus on the response. First, unilateral cervical vagotomy (right side) was done and then mice were instilled IT with E. coli 2.5 × 10^6 CFU. The mice were killed at 4, 12, and 24 h, and the right lungs were lavaged. Normal sham-operated mice were used as controls. BAL and plasma were collected to measure MIP-2 levels by ELISA. MIP-2 levels in the BAL were increased at 4 and 12 h in the vagotomized group (Fig. 6E), and MIP-2 levels in the plasma in vagotomized group were also increased compared with the sham group at 4 and 12 h (Fig. 6F).

Nicotine activation of α7 nAChR increases survival in E. coli pneumonia

To test whether activation of α7 nAChR by nicotine affects survival in E. coli pneumonia, mice were challenged IT with E. coli (5 × 10^6 CFU) and treated with either nicotine (2.4 mg/kg, delivered by a ALZET [Cupertino, CA] osmotic pump implanted under skin) or saline. Mice were followed for 24 h, and survival was increased in the nicotine therapy group compared with saline (Fig. 7A). To address whether MLA, a specific α7 nAChR antagonist, counteracts the effect of nicotine, mice were treated with either nicotine (2.4 mg/kg, via osmotic pump) or nicotine + MLA (4 mg/kg, i.p. at 8 and 16 h). At 24 h, survival in MLA treated mice was reduced compared with the nicotine-only group (Fig. 7B).

Cervical vagotomy and α7 nAChR deficiency reduces survival in E. coli pneumonia

To study whether vagus denervation affects survival in E. coli pneumonia, unilateral cervical vagotomy in mice (right side) or a sham operation was performed; E. coli (4 × 10^6 CFU) was instilled IT, and mice were followed for 48 h. Survival in vagotomized mice was reduced compared with the sham group (Fig. 7C). To determine whether the deficiency of α7 nAChR affects survival from E. coli pneumonia, α7 nAChR+/− and α7 nAChR−/− mice were instilled IT with E. coli (4 × 10^6 CFU) and followed for 12 h. Survival in α7 nAChR−/− mice was reduced compared with α7 nAChR+/− mice (Fig. 7D).

Discussion

The current study demonstrates for the first time that activation of α7 nAChR in alveolar macrophages and neutrophils is a critical mechanism that decreases the lung inflammatory response to either E. coli pneumonia or LPS challenge in mice. Activation of α7 nAChR in these cells reduced the production of proinflammatory cytokines and chemokines (especially MIP-2) and neutrophil...
transmigration, and thereby reduced lung injury and mortality. There was a causal relationship between α7 nAChR activation and the reduction in lung inflammation, because lung MIP-2 production, lung neutrophil infiltration, and mortality were increased in α7 nAChR-/- and vagus-denervated mice.

α7 nAChR+CD11b+ alveolar macrophages and α7 nAChR+Gr1+ neutrophils activated by α7 nAChR agonists induced a decreased production of MIP-2, TNF-α, and HMGB1 consistent with an important role of α7 nAChR activation in mediating the inactivation of proinflammatory mediator production. The question arises as to the mechanisms of vagal innervation-induced protection mediated by α7 nAChR receptors in alveolar macrophages and neutrophils. It is known that airway epithelia and perivascular tissue are vagally innervated (6), thus release of ACh in airways may activate α7 nAChR+ inflammatory cells accumulating in the airways. The ACh concentration, however, did not increase in BAL in our models consistent with rapid hydrolysis of ACh (half-life, 2 min) by AChE occurring after ACh release from nerve endings (24). This possibility was assessed by measuring AChE activity in proinflammatory cells and BAL choline concentration (50 nmol/ml); thus, it is likely that α7 nAChR+CD11b+ and α7 nAChR+Gr1+ cells are activated in the local milieu at sites of vagal innervation. Vagus denervation failed to activate α7 nAChR+CD11b+ and α7 nAChR+Gr1+, resulting in persistent MIP-2 production and neutrophil infiltration, and worsened lung inflammation and injury compared with control animals.

In LPS and E. coli pneumonia ALI mouse models, alveolar macrophages are the central effector cells in the production of proinflammatory cytokines (25, 26), which initiate and amplify neutrophil transmigration into the lungs to mediate inflammation and injury (27–29). Infiltrated neutrophils may function in a feed-forward manner to generate MIP-2 and promote further neutrophil transmigration (30, 31). Using exogenous α7 nAChR agonists to stimulate α7 nAChR+CD11b+ and α7 nAChR+Gr1+ cells in injured lungs disrupted this feed-forward inflammatory loop. In this sense, activation of α7 nAChR by endogenous ACh represents a homeostatic negative feedback mechanism that probably fine-tunes the lung–host defense system by dampening neutrophil transmigration, and thereby mitigating lung inflammation and injury.

E. coli pneumonia shares similar pathogenic mechanisms with LPS-induced ALI, including activation of alveolar macrophages, increased production of early and late proinflammatory mediators,

<FIGURE 5. Activation of α7 nAChR reduces BAL proinflammatory cells and protein in E. coli pneumonia. A–D, Effect of nicotine. A, BAL leukocytes. B, BAL neutrophils. C, BAL monocytes. D, Protein levels. *p < 0.05 for nicotine versus saline; n = 3–5 in each group. Data were pooled from three different experiments. E–H, Effect of PHA 568487. E, BAL leukocytes. F, BAL neutrophils. G, BAL monocytes. H, Protein levels. *p < 0.05 for PHA 568487 versus saline; n = 3–5 in each group. Data were pooled from three different experiments. I–L, Unilateral cervical vagotomy (right side) increases BAL proinflammatory cells and protein levels in E. coli pneumonia. I, BAL leukocytes. J, BAL neutrophils. K, BAL monocytes. L, Protein levels. *p < 0.05 for vagotomy versus sham group at 4, 12, or 24 h after IT E. coli challenge; n = 4–5 in each group. Data were pooled from three different experiments. Data are mean ± SD.

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and neutrophil transmigration (17, 24, 29). Recently, studies have shown that nicotine (nonspecific), DMAB (partial), PNU (specific), and PHA 568487 (specific) are α7 nAChR agonists, but these chemical preparations are different in molecular structure and specificity. To compare the effects of α7 nAChR agonists on proinflammatory cytokine production and lung inflammation, we administered α7 nAChR agonists (nicotine, DMAB, PNU, or PHA 568487) to disrupt the propagation of inflammation by reducing MIP-2 production in α7 nAChRCD11b+ and α7 nAChRGr1+ cells, decreasing pulmonary edema and neutrophil infiltration in the early time frame (4 and 12 h), and thereby enhancing the survival of pneumonia in the nicotine treated mice. The role of vagal signaling was established by the vagus denervation study, which increased MIP-2 production in BAL and lung neutrophil infiltration at these early time points. These findings demonstrate that regulation of proinflammatory responses (e. g., MIP-2 production) by α7 nAChR-cholinergic antiinflammatory pathway plays an important role in the early stage of lung inflammation induced by E. coli pneumonia or LPS challenge in mice.

Studies of Streptococcus pneumoniae–induced pneumonia showed that nicotine treatment increased bacterial burden and worsened lung inflammation at 24 h (32), results that are different from the lung antiinflammatory effects of activating α7 nAChR and the previous observations that activation of α7 nAChR protected against Gram-negative sepsis (1, 5, 12–15, 33). A reason for this apparent difference in results with live bacteria may be that TLR4 recognizes the Gram-negative product LPS, whereas TLR2 recognizes Gram-positive components (34); therefore, the protective effects by α7 nAChR may occur downstream of TLR4 activation. In the sepsis models (endotoxemia and cecal ligation and puncture), the previous observations that activation of α7 nAChR by its specific antagonist MLA counteracts the protective effects of nicotine on survival in E. coli pneumonia. Mice were treated with nicotine or nicotine + MLA (2.4 mg/kg, delivered by an osmotic pump; MLA was given i.p.) and followed up for 24 h. *p < 0.05 versus nicotine (n = 9) versus nicotine + MLA (n = 5) treated mice. Data were pooled from two different experiments.

Unilateral vagotomy reduces survival in E. coli pneumonia. Sham and vagotomized mice were challenged with E. coli and followed for 48 h. *p < 0.05 for vagotomy (n = 30) versus sham (n = 30). Data were pooled from 5 different experiments.

Diagrams of α7 nAChR effects on MIP-2 in BAL from isolateral lung and plasma in experiments.

Effects of nicotine. Mice were treated with saline or nicotine (2.4 mg/kg, delivered by an osmotic pump) and followed up for 24 h. *p < 0.05 for nicotine versus saline treated mice; n = 12 in each group. Data were pooled from two different experiments.

Antagonism of α7 nAChR by its specific antagonist MLA counteracts the protective effects of nicotine on survival in E. coli pneumonia. Mice were treated with nicotine or nicotine + MLA (2.4 mg/kg, delivered by an osmotic pump; MLA was given i.p.) and followed up for 24 h. *p < 0.05 versus nicotine (n = 9) versus nicotine + MLA (n = 5) treated mice. Data were pooled from two different experiments.

Unilateral vagotomy reduces survival in E. coli pneumonia. Sham and vagotomized mice were challenged with E. coli and followed for 48 h. *p < 0.05 for vagotomy (n = 30) versus sham (n = 30). Data were pooled from 5 different experiments.

Deficiency of α7 nAChR worsens reductions in survival in E. coli pneumonia; n = 5 α7 nAChR−/− mice; n = 4 α7 nAChR+/− mice; *p < 0.05 for α7 nAChR−/− versus α7 nAChR+/− mice.

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Disclosures

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References