

Neuromuscular Blocking Agent Cisatracurium Attenuates Lung Injury by Inhibition of Nicotinic Acetylcholine Receptor- $\alpha 1$

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ABSTRACT

Background: Neuromuscular blocking agents (NMBAs) bind the nicotinic acetylcholine receptor $\alpha 1$ (nAChR $\alpha 1$) that also contributes to inflammatory signaling. Thus, the author hypothesized that the use of NMBA mitigates lung injury by improving ventilator synchrony and decreasing inflammatory responses.

Methods: Lung injury was induced by intratracheal instillation of hydrogen chloride in rats that were randomized to receive no NMBA with evidence of asynchronous ventilation (noNMBA/aSYNC, $n = 10$); no NMBA with synchronous ventilation (noNMBA/SYNC, $n = 10$); cisatracurium (CIS, $n = 10$); or pancuronium (PAN, $n = 10$). Mechanical ventilation was set at a tidal volume of 6 ml/kg and positive end-expiratory pressure 8 cm H₂O for 3 h. Human lung epithelial, endothelial, and CD14⁺ cells were challenged with mechanical stretch, lipopolysaccharide, lung lavage fluids (bronchoalveolar lavage fluid), or plasma obtained from patients ($n = 5$) with acute respiratory distress syndrome, in the presence or absence of CIS or small-interfering RNA and small hairpin RNA to attenuate the cell expression of nAChR $\alpha 1$.

Results: The use of CIS and PAN improved respiratory compliance (7.2 ± 0.7 in noNMBA/aSYNC, 6.6 ± 0.5 in noNMBA/SYNC, 5.9 ± 0.3 in CIS, and 5.8 ± 0.4 cm H₂O/l in PAN; $P < 0.05$), increased Pao₂ (140 ± 54 , 209 ± 46 , 269 ± 31 , and 269 ± 54 mmHg, respectively, $P < 0.05$), and decreased the plasma levels of tumor necrosis factor- α (509 ± 252 in noNMBA, 200 ± 74 in CIS, and 175 ± 84 pg/ml in PAN; $P < 0.05$) and interleukin-6 (5789 ± 79 , 1608 ± 534 , and 2290 ± 315 pg/ml, respectively; $P < 0.05$). The use of CIS and PAN or silencing the receptor nAChR $\alpha 1$ resulted in decreased cytokine release in the human cells in response to a variety of stimuli mentioned earlier.

Conclusions: The use of NMBA is lung protective through its antiinflammatory properties by blocking the nAChR $\alpha 1$. (ANESTHESIOLOGY 2016; 124:132-40)

IN patients with early, severe acute respiratory distress syndrome (ARDS), administration of the neuromuscular blocking agent (NMBA) cisatracurium (CIS) for 48 h increased 90-day survival rate and increased ventilator-free days.¹ An earlier study demonstrated attenuation of pulmonary and systemic cytokine responses after administration of CIS for 48 h in patients with ARDS.² However, it is unclear whether the decreased cytokine responses and improved outcomes were due to improved patient-ventilator synchrony and hence minimizing ventilator-induced lung injury (VILI), and/or was due to a direct antiinflammatory effect of CIS.

Nondepolarizing NMBA, such as CIS and pancuronium (PAN), bind to nicotinic acetylcholine receptor $\alpha 1$ (nAChR $\alpha 1$) at the neuromuscular junction, blocking sodium influx and muscular contraction. nAChRs are expressed on other cell types including endothelial and epithelial cells,

What We Already Know about This Topic

- Nicotinic acetylcholine receptors, which are antagonized by nondepolarizing neuromuscular blocking agents, also mediate proinflammatory effects in a variety of cell types
- The neuromuscular blocking agent cisatracurium improves outcome and reduces cytokine responses in patients with acute respiratory distress syndrome

What This Article Tells Us That Is New

- In rodent models of acute lung injury, neuromuscular blocking agents protected against ventilator-induced lung injury through their antiinflammatory properties mediated by blockade of the nicotinic acetylcholine receptor $\alpha 1$
- This protective effect was not due to improved ventilator synchrony, and the reduced cytokine release could be mimicked *ex vivo* through knockdown of nicotinic acetylcholine receptor $\alpha 1$ expression

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macrophages, mesangial cells, and fibroblasts.^{3,4} In contrast to nAChR α 7, which is a part of the cholinergic antiinflammatory reflex,^{5,6} nAChR α 1 exerts proinflammatory properties.⁷ In fact, the nAChR α 1 has been recently identified as an alternative receptor for urokinase (urokinase plasminogen activator [uPA]) promoting lung neutrophil activation after lipopolysaccharide (LPS) stimulation and release of inflammatory cytokines such as interleukin (IL)-1 α , tumor necrosis factor (TNF)- α , and macrophage inflammatory protein-2.⁸ The nAChR α 1-mediated signaling pathways of transforming growth factor- α 1 and α -smooth muscle actin have been shown to enhance renal fibrosis.^{3,9}

To elucidate the mechanisms by which NMBA protect the lung in patients with ARDS,¹ we set out to examine the inflammatory responses that may be modulated by NMBA using a rat and mouse model of lung injury, as well as in a variety of cell types including lung epithelial cells, endothelial cells, and leukocytes that are involved in innate and adaptive immunity in acute lung injury, in which nAChR α 1 was either silenced or not silenced. We hypothesized that NMBAs are protective against VILI by their antiinflammatory effects mediated by blocking the activity of nAChR α 1 on epithelial cells, endothelial cells, and leukocytes.

Materials and Methods

Animal Model and Experimental Groups

All experiments were conducted in compliance with the Animal Care and Use Committee of St. Michael's Hospital (Toronto, Ontario, Canada). Male Sprague-Dawley rats (300 to 400 g; Charles River Labs, Canada) were anesthetized, and the right carotid artery was cannulated (Angiocath intravenous (IV) Catheter 24-gauge; Becton Dickinson, Canada) to monitor mean arterial pressure and for blood gas sampling (Ciba-Corning Model 248 blood gas analyzer; Corning Medical, USA). A catheter was placed in the right internal jugular vein to administer normal saline at 1.5 ml/h.

A tracheostomy was performed, and a 14-gauge cannula (Angiocath IV Catheter, 2.1 \times 48 mm) was inserted into the trachea. Animals were ventilated with the pediatric module of Servo 300 ventilator (Maquet, Germany) using a fraction of inspired oxygen (FIO₂) of 1.0, a tidal volume (Vt) of 6 ml/kg, positive end-expiratory pressure (PEEP) of 8 cm H₂O, and respiratory rate adjusted to maintain PaCO₂ between 35 and 45 mmHg.

Lung Injury Model

Lung injury was induced by intratracheal instillation of 2.5 ml/kg of lactated Ringer's solution, titrated to pH 1.0 with hydrogen chloride. This dose was chosen based on the results of our pilot study, which demonstrated a significant decrease in the ratio of PaO₂:FIO₂ in the absence of hemodynamic impairment. Rats were included in the study if the PaO₂ fell below 250 mm Hg on FIO₂ of 1.0, 30 min after hydrogen chloride instillation. Rats were mechanically ventilated for 3 h with Vt 6 ml/kg, PEEP 8 cm H₂O, and respiratory rate

adjusted to maintain PaCO₂ between 35 and 45 mmHg. A PEEP of 8 cm H₂O was associated with the lowest respiratory system elastance in our preliminary studies.

Randomization

A statistical power calculation was performed based on our pilot study. In five animals that received the anesthesia plan to prevent asynchrony in the absence of NMBA (noNMBA/SYNC), we observed a PaO₂:FIO₂ ratio of 209 \pm 46 at 3 h. Therefore, to detect a 25% increase in PaO₂:FIO₂ ratio in animals treated with CIS with a power of 80%, α = 0.05, a minimum of 10 rats was required per group.

After stabilization for 30 min after surgical preparation, the rats were randomized into four groups: group 1 (noNMBA/asynchronous ventilation [aSYNC], n = 10) received no NMBA and had evidence of asynchrony as reflected by active inspiratory efforts after an end-inspiratory occlusion, assessed hourly. Animals received midazolam (5 mg kg⁻¹ h⁻¹) and fentanyl (10 μ g kg⁻¹ h⁻¹) to maintain an anesthesia level in which the rats had no response to toe pinch; group 2 (noNMBA-SYNC, n = 10) received no NMBA and asynchrony was prevented by increasing the dose of anesthesia with midazolam (15 mg kg⁻¹ h⁻¹) and fentanyl (20 μ g kg⁻¹ h⁻¹); group 3 (CIS, n = 10) received CIS (bolus 0.2 mg/kg IV, followed by 10 μ g kg⁻¹ min⁻¹, IV)¹; and group 4 (PAN, n = 10) received PAN (bolus 0.5 mg/kg IV, followed by 5 μ g kg⁻¹ min⁻¹, IV).^{10,11} The CIS and PAN groups received the same dose of anesthesia as group 2 (noNMBA/SYNC). The efficiency of NMBA was monitored, and paralysis ensured, by measurement of the response to train-of-four stimulation (2 to 4 mA with a frequency of 2 Hz for 2 s) in the tibialis anterior muscle.

Lung Mechanics

Peak airway pressure was continuously monitored, and plateau airway pressure (P_{plat}) was measured hourly with a 5-s end-inspiratory hold. Total PEEP (PEEP_{TOT}) was measured hourly with a 5-s double occlusion at end expiration. Quasi-static respiratory system elastance (E_{RS}) was calculated as (P_{plat} - PEEP_{TOT})/Vt.

Measurements

On completion of the study, blood was collected through cardiac puncture; plasma was snap frozen at -80°C for measurement of cytokines (TNF- α and IL-6) by enzyme-linked immunosorbent assay (ELISA; Invitrogen, USA). Lungs were excised through a midline sternotomy; the left lung was used for histology and the right upper lobe for measurement of wet:dry weight ratio. Bronchoalveolar lavage (BAL) was performed (5 ml cold saline \times 3) in the middle and lower lobes of the right lung. The diaphragm was excised and snap frozen at -80°C for RNA extraction.

Ex Vivo Lung Injury Model

Male 8- to 10-week-old wild-type C57BL/6 mice were anesthetized with isoflurane inhalation, and 1 mg/kg of LPS

(Sigma-Aldrich, USA; $n = 16$) or phosphate-buffered saline ($n = 16$) was instilled intratracheally under direct visualization of vocal cords. Animals were allowed to fully recover. After 12 h, mice were anesthetized by intraperitoneal injection of ketamine–xylazine (100 and 5 mg/kg, respectively), and lungs were isolated, perfused, and ventilated as described previously.¹² After ascertaining pulmonary perfusion, the mode of ventilation was changed to negative pressure to minimize hydrostatic edema formation.¹² Lungs were ventilated with end-inspiratory pressure of -10 cm H_2O and end-expiratory pressure of -3 cm H_2O , and after stabilization (30 min), lungs were randomized to be perfused with or without CIS (bolus 0.2 mg/kg, IV, followed by $10 \mu\text{g kg}^{-1} \text{min}^{-1}$) for 4 h. Lungs were perfused, in a nonrecirculating fashion, through the pulmonary artery at a constant flow of 1 ml/min. Roswell Park Memorial Institute medium containing 4% bovine serum albumin lacking phenol red (37°C, pH 7.4) was used to perfuse the pulmonary vasculature. Ventilation and perfusion were stopped after 240 min. BAL was performed by instilling 1 ml of saline into the lungs through the tracheal cannula. BAL was centrifuged at 12,000g for 10 min (4°C), and supernatant was stored at -80°C for further analysis. Supernatants were assayed in a blinded fashion using specific commercial ELISA kits specific for mice (DuoSet ELISA Development Systems, R&D Systems, USA) to determine the concentrations of TNF- α and IL-6.

Lung Epithelial Cell Stretching

Primary human small airway epithelial cells (SAECs; Lonza, USA) were cultured on collagen-coated silicon elastic plates (Flexcell plate 1, Flexcell International Corporation, USA) in the designated medium (SAGM BulletKit, USA). SAECs were transfected for 48 h with a nAChR $\alpha 1$ siRNA at 50 nM or a scrambled siRNA (ON-TARGETplus SMARTpool siRNA; Thermo Scientific, Dharmacon Division, Canada), by using DharmaFECT at 1.5 $\mu\text{l/ml}$ (Thermo Scientific, USA). The cells were subjected to cyclic stretch for 6 or 24 h at 30% elongation and 30 cycles/min (FX-4000; Flexcell International Corporation, USA) in the presence or absence of CIS at concentration of 0.023 μM (based on a dose-dependent response curve in a pilot study) that was administered 30 min before cell stretch. The same volume of phosphate-buffered saline was used as a vehicle control.

Stable Transfection of Human Endothelial ECV304 Cells with nAChR $\alpha 1$ shRNA

For stable knockdown of the nAChR $\alpha 1$ gene, a shRNA specific for nAChR $\alpha 1$ was used (Thermo Scientific Open Biosystems, by M-Medical, Italy). A scrambled shRNA served as a control. Briefly, human endothelial cells ECV304 (CRL-1998, ATCC, USA) were cultured in Dulbecco modified Eagle medium supplemented with 10% of fetal bovine serum (FBS). ECV304 cells were infected with lentiviral particles plus polybrene (8 $\mu\text{g/well}$) in antibiotic-free medium supplemented with 10% of FBS in six-well plates, centrifuged for 2 h at 300g, and incubated for a total of 24 h

at 37°C. Medium was replaced with fresh medium supplemented with puromycin (1 $\mu\text{g/ml}$) for cell selection.

Human Endothelial and CD14 $^+$ Cell Cytokine Profiles in Response to Stimuli

The human endothelial cells (ECV304) transfected with nAChR $\alpha 1$ shRNA were stimulated with LPS (1 $\mu\text{g/ml}$, *Escherichia coli* 055:B5, Sigma, Italy) or plasma or BAL fluid (BALF; both diluted in a ratio of 1:1 in culture medium) in Roswell Park Memorial Institute medium with 10% FBS, obtained from five patients with ARDS enrolled in previous studies.¹³ In separate experiments, ECV304 cells and human CD14 $^+$ cells purified from peripheral blood mononuclear cells (Miltenyi Biotech, Italy)¹⁴ were challenged with LPS for 4 h, and the cell culture medium was replaced with fresh medium containing CIS or PAN at specified concentrations and incubated for additional 24 h. Vehicle solution served as a control group. Supernatants were analyzed in a blinded fashion for the presence of IL-6, IL-8, and TNF- α (ELISA, R&D System by SPACE, Italy) and lactate dehydrogenase (Promega by DBA, Italy).

Gene Expression of Atrogin-1 and MuRF1 in Rat Diaphragm

Rat diaphragm muscle was lysed, and total RNA was extracted using the TRIzol[®] RNA Isolation Reagent (Life Technologies Inc., USA). RNA quality was assessed with a bioanalyzer (Model 2100, Agilent Technologies, USA) and quantified by absorption spectrophotometry at 260 and 280 nm. For removal of any contaminating genomic DNA, the total RNA samples were treated with DNA-free treatment (Ambion, USA). The cDNA synthesis kit (Fermentas First Strand cDNA Synthesis Kit; Thermo Scientific) was used to reverse transcribe 0.4 mg of total RNA from each sample with a random hexamer primer. For each gene, real-time polymerase chain reaction was performed in triplicate wells.

Relative quantification of Atrogin-1 and muscle-specific ring finger protein 1 (MuRF1) was determined using the cycle threshold ($\Delta\Delta\text{CT}$) method according to instructions (Applied Biosystems, Canada). *Hypoxanthine phosphoribosyltransferase*, a gene we previously identified to be stably expressed in the diaphragms of animals from each experimental group, was used as a housekeeping gene. Primer amplification efficiencies were equal for all genes tested and the housekeeping gene.

Protein Expression of nAChR $\alpha 1$

The isolated CD14 $^+$ cells, SAEC transfected with nAChR $\alpha 1$ siRNA, and ECV304 cells transfected with nAChR $\alpha 1$ -shRNA (sh#1 and sh#2) were lysed in RIPA buffer. Briefly, the protein lysate (50 μg) was run in reducing conditions with 2-ethanesulfonic acid buffer using 4 to 12% NuPAGE Bolt gels (Life Technologies, Italy). Gels were blotted on nitrocellulose membranes, blocked with 5% nonfat dry milk

in Tris-buffered saline, and then incubated in the presence of the primary antibody specific for nAChR α 1 (Santa Cruz by DBA, Italy; 1:200 dilution) and anti- β -actin (1:2000; Sigma) in Tris-buffered saline containing 1% of Tween with 5% BSA overnight at 4°C. Membranes were subsequently exposed to a goat anti-rat-horseradish peroxidase and donkey anti-rabbit-horseradish peroxidase (GE Healthcare, Canada) antibodies (both 1:2,000 dilution in 5% nonfat dry milk in a mixture of Tris-buffered saline and Tween20) (Santa Cruz) for 1 h. The protein was detected by ECL 2 Substrate (Thermo Fisher by VWR, Italy). Images were recorded with the ProXpress scanner equipped with the ProSCAN 4.0 and ProFinder 2 software (PerkinElmer, Italy).

Statistics

All data are presented as mean \pm SD or otherwise indicated. Comparison among groups was performed by either one-way ANOVA or Kruskal-Wallis test for normally and nonnormally distributed data, respectively, followed by the appropriate Dunn *post hoc* test for multiple comparisons or two-way ANOVA for repeated measures with Bonferroni *post hoc* analysis (SPSS Inc., USA, and GraphPad Software, Inc., USA). A *P* value < 0.05 was considered statistically significant.

Results

Rat Model of ARDS

Hemodynamics. Mean arterial pressures were similar at baseline and decreased significantly after hydrogen chloride instillation (fig. 1A).

Arterial Blood Gases. Mean Pao₂ was similar throughout the study in the animals that received no hydrogen chloride instillation and were ventilated with synchrony in the presence of CIS or PAN (data not shown). Pao₂ values were similar at baseline and decreased after hydrogen chloride instillation. There was a further decrease at 3 h during mechanical ventilation in the noNMBA/aSYNC group compared with CIS and PAN (fig. 1B). After increasing from baseline secondary to hydrogen chloride instillation, Paco₂ was similar in all groups during the mechanical ventilation period (fig. 1C).

Respiratory Mechanics. Respiratory elastance (E_{RS}) increased similarly in all groups after hydrogen chloride instillation and remained similar for the initial 2-h mechanical ventilation period. Thereafter, E_{RS} increased in the noNMBA/aSYNC group compared with CIS and PAN (both *P* < 0.05 ; fig. 1D).

Lung Injury Indexes. Lung wet:dry weight ratio was 6.7 ± 1.1 in the noNMBA/aSYNC and 6.1 ± 0.7 in the noNMBA/SYNC groups. The wet:dry ratio was significantly lower in the CIS (3.8 ± 0.6) and PAN (3.9 ± 0.6) groups (both *P* < 0.05 vs. noNMBA/aSYNC and noNMBA/SYNC groups; fig. 1E). Similarly, BALF protein concentrations were significantly higher in noNMBA/aSYNC and noNMBA/SYNC groups than in CIS and PAN (fig. 1F).

Pulmonary and Systemic Cytokine Responses. The levels of TNF- α in BALF and plasma and IL-6 in plasma were significantly lower in CIS and PAN compared with noNMBA/aSYNC (both *P* < 0.05 ; fig. 2, A–D).

Diaphragm Mediators of Muscle Atrophy. The levels of Atrogin-1 mRNA and MuRF1 mRNA in the rat diaphragm muscle were not statistically different across the groups (fig. 2, E and F).

CIS-attenuated Cytokine Responses in In Vitro Single-cell Models

Cytokine Responses Were Attenuated by CIS in CD14⁺ Cells Stimulated with Samples from ARDS Patients. Stimulation of CD14⁺ cells with plasma from patients with ARDS resulted in increased IL-6 and IL-8, which was attenuated by CIS (fig. 3, A and B). A similar but markedly attenuated expression of the cytokines was observed when CD14⁺ cells were stimulated with BALF from patients with ARDS, which might be due to the dilution factor in the BALF (fig. 3, C and D).

Cytokine Responses Were Attenuated by Treatment with CIS or nAChR α 1 siRNA in Human Lung Epithelial Cells Subjected to Mechanical Stretch

The protein expression of nAChR α 1 was detectable in the three cell types tested (fig. 4A). Mechanical stretch of human SAEC induced a time-dependent increase in granulocyte-macrophage colony-stimulating factor, IL-6, and IL-8 24 h after mechanical stretch, which was attenuated in the presence of CIS (fig. 4, B–E). TNF- α increased at 6 h and remained high at 24 h during mechanical stretch; CIS at the dose used transiently decreased TNF- α at 6 h (fig. 4E).

To further examine the specific effects of nAChR α 1 in mediating cytokine responses, the SAECs were treated with nAChR α 1 siRNA or scrambled siRNA before exposure to mechanical stretch (fig. 4, F–I). Compared with control, cells treated with scrambled nAChR α 1 siRNA had a significantly attenuated release of granulocyte-macrophage colony-stimulating factor, IL-6, and IL-8 after 24 h of mechanical stretch, which might be due to nonspecific effects. This attenuation was more pronounced in the nAChR α 1 siRNA group (fig. 4, F–H). TNF- α levels were not affected (fig. 4I).

Cytokine Responses Were Attenuated by NMBA in LPS-stimulated Human CD14⁺ and Endothelial Cells. The increased production of TNF- α and IL-6 after LPS stimulation of human CD14⁺ cells for 4 h was attenuated by CIS (fig. 5, A and B) or PAN (fig. 5, C and D) in a dose-dependent manner. Similarly, treatment with CIS and PAN inhibited IL-6 production in human endothelial cells (ECV304) 4 h after LPS stimulation (fig. 5, E and F). TNF- α was not detected in culture supernatants of the human endothelial cells. In the perfused mouse lung model, the administration of CIS attenuated the production of TNF- α and IL-6 in the lung lavage fluids after LPS challenge (fig. 5, G and H).

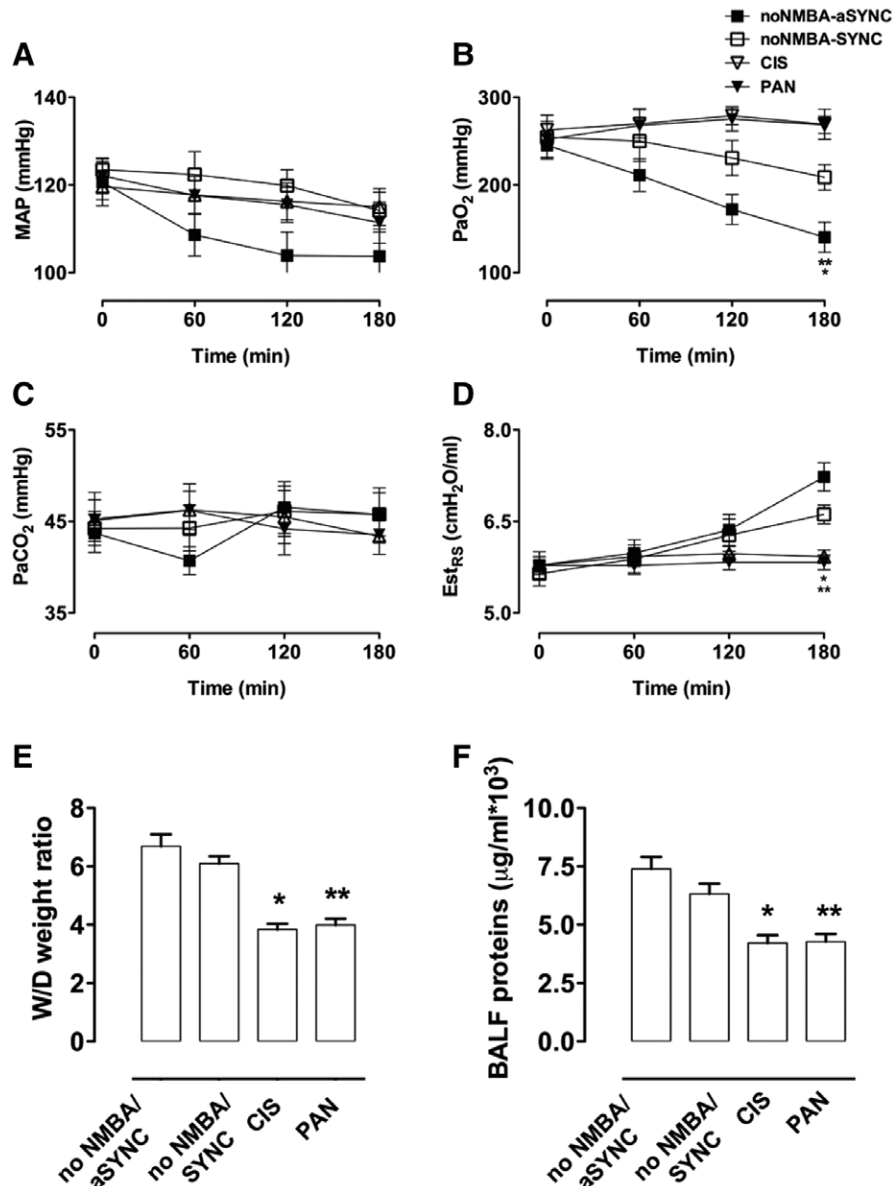


Fig. 1. Hemodynamic, gas exchange, and lung injury indexes. Time course (A–D) of MAP, P_{aCO_2} , elastance of respiratory system, and P_{aO_2} during 3-h period after initial lung injury. * $P < 0.05$ noNMBA/aSYNC versus CIS; ** $P < 0.05$ noNMBA/aSYNC versus PAN. Lung wet to dry ratio (W/D) and BALF protein concentrations (E and F). * $P < 0.05$ noNMBA/aSYNC and noNMBA/SYNC versus CIS; ** $P < 0.05$ noNMBA/aSYNC and noNMBA/SYNC versus PAN. aSYNC = asynchronous ventilation; BALF = bronchoalveolar lavage fluid; CIS = cisatracurium; Est_{RS} = elastance of respiratory system; MAP = mean arterial pressure; NMBA = neuromuscular blocking agent; PAN = pancuronium; SYNC = synchronous ventilation.

Knockdown of nAChR α 1 Gene Prevents the Cytokine Production in Response to LPS Stimulation in Human Endothelial Cells

In human endothelial ECV304 cells transfected with a scrambled shRNA, the administration of CIS (at both doses of 0.06 and 0.16) resulted in a significantly dose-dependent decrease in IL-6 after stimulation with LPS and plasma from patients with ARDS (figs. 4A and 6). In the same cells transfected with two nAChR α 1 shRNA, where the expression of nAChR α 1 receptor was knocked down, CIS had significant less impact on attenuation of IL-6 production after LPS and

plasma stimulation, except for nAChR α 1 sh#2 at dose of CIS of 0.06 after plasma stimulation (figs. 4A and 6).

Discussion

A recent study has reported that patients with moderate to severe ARDS treated for 48 h with CIS had improved 90-day survival compared with a placebo-treated group,¹ which might be due to improved patient–ventilator synchrony that minimized VILI and/or potential direct antiinflammatory effects of the NMBA.¹⁵ In this study, we demonstrated that (1) treatment with NMBA is protective against VILI

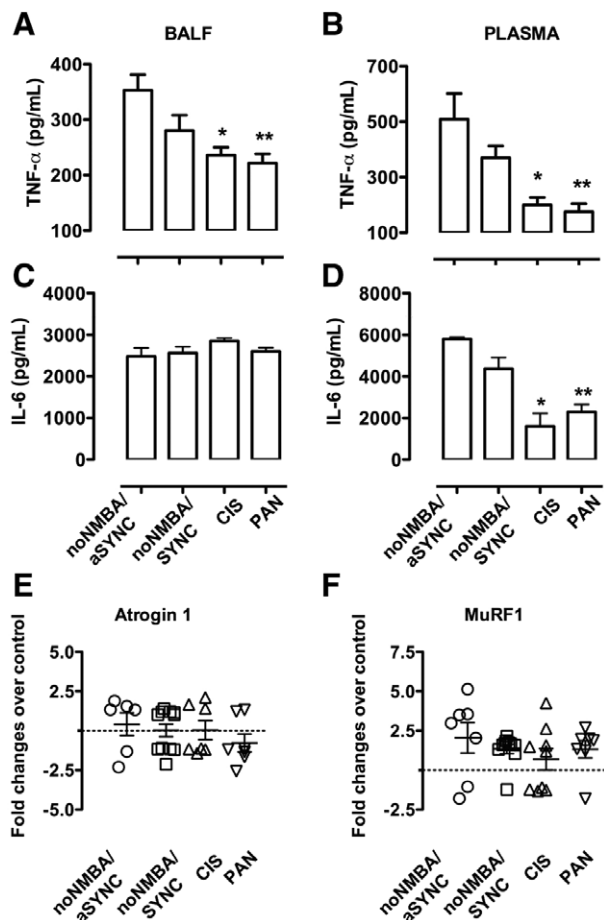


Fig. 2. Cytokine levels in BALF and plasma (A–D) and gene expression of muscle-specific ubiquitin ligases Atrogin-1 (E) and muscle-specific ring finger protein 1 (MuRF1, F) in the diaphragm determined by real-time polymerase chain reaction. Ct value HPRT = 23.42 ± 0.25 ; MuRF1 = 19.85 ± 0.90 , Atrogin-1 = 20.90 ± 0.61 . aSYNC = synchronous ventilation; BALF = bronchoalveolar lavage fluid; CIS = cisatracurium; HPRT = hypoxanthine phosphoribosyltransferase; IL-6 = interleukin-6; NMBA = neuromuscular blocking agents; PAN = pancuronium; SYNC = synchronous ventilation; TNF- α = tumor necrosis factor- α . * $P < 0.05$ CIS versus noNMBA/aSYNC; ** $P < 0.05$ PAN versus noNMBA/aSYNC.

through an antiinflammatory property; (2) this antiinflammatory property of NMBA is largely independent of the effects of synchrony; and (3) the antiinflammatory effects are largely mediated by the nAChR $\alpha 1$ expressed on epithelial, endothelial, and CD14 $^{+}$ cells.

Our study design set out to separate the effects on synchrony from that of direct attenuation of biotrauma. The nonasynchrony group managed by deep sedation showed no significant difference in all endpoints compared with the asynchrony group but had greater inflammatory responses compared with the NMBA groups. These data suggest that the beneficial effects of NMBA can largely be attributed to mechanisms other than its impact on synchrony.

Indeed, the NMBA-treated animals showed better respiratory system compliance and oxygenation associated with

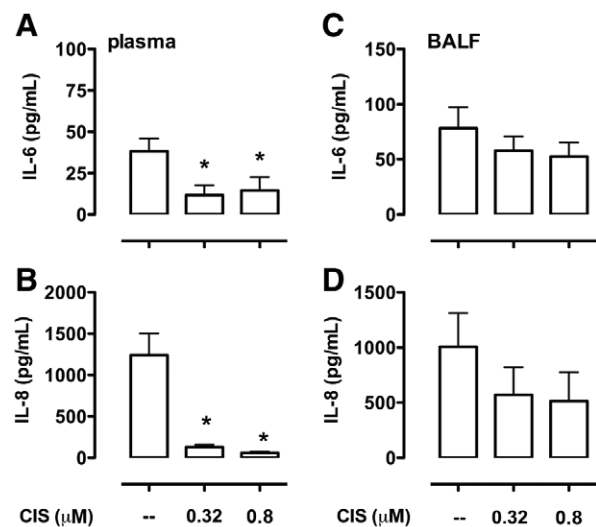


Fig. 3. The levels of IL-6 and IL-8 released from CD14 $^{+}$ purified cells after stimulation with plasma (A and B) and BALF (C and D) from patients with acute respiratory distress syndrome in the presence or absence of CIS. BALF = bronchoalveolar lavage fluid; CIS = cisatracurium; IL-6 = interleukin-6; IL-8 = interleukin-8. * $P < 0.05$ versus vehicle control (-).

attenuation of inflammatory responses seen in VILI.^{16,17} The antiinflammatory effects of NMBA were confirmed in an *ex vivo* mouse model of the LPS-induced lung injury. Moreover, we demonstrated a direct, dose-dependent antiinflammatory effect of the NMBA *in vitro* models using human epithelial, endothelial, and CD14 $^{+}$ cells challenged with LPS, mechanical stretch, or stimulated with BALF and plasma from patients with ARDS. We further identified that a mechanism of the antiinflammatory effects of NMBA acts through interaction with the nAChR $\alpha 1$ receptor.

NMBA bind to the nAChR $\alpha 1$ receptors on the muscle, which are ligand-gated ion channels known to mediate signal transduction at the neuromuscular junction.¹⁸ The nAChR $\alpha 1$ receptor is also expressed on other cell types such as endothelium, epithelium, neutrophils, macrophages, and fibroblasts and has been identified as a functional cell receptor for urokinase.^{3,8,18} Upon ligation, the nAChR $\alpha 1$ receptor changes its conformation and becomes permeable to sodium and calcium ions leading to calpain activation, nuclear factor- κ B nuclear translocation that in turn mediates inflammatory responses. In a recent study using a mouse model of hypercholesterolemia/uninephrectomy-induced nephropathy and mouse monocytes, silencing of the *nAChR $\alpha 1$* has been shown to reduce renal tissue calcium influx and calpain-1 activation, suggesting that nAChR $\alpha 1$ contributes to macrophage inflammation and tissue damage by calpain-1 activation.⁷ Activation of the nAChR $\alpha 1$ receptor has been demonstrated to play a role in the upregulation of proinflammatory cytokines and adhesion molecules,⁷ inflammatory cell migration, chemotaxis⁸ in animal models of atherosclerosis,⁹ and chronic kidney diseases.³ The increased levels of uPA, an alternative ligand for nAChR $\alpha 1$ receptor,

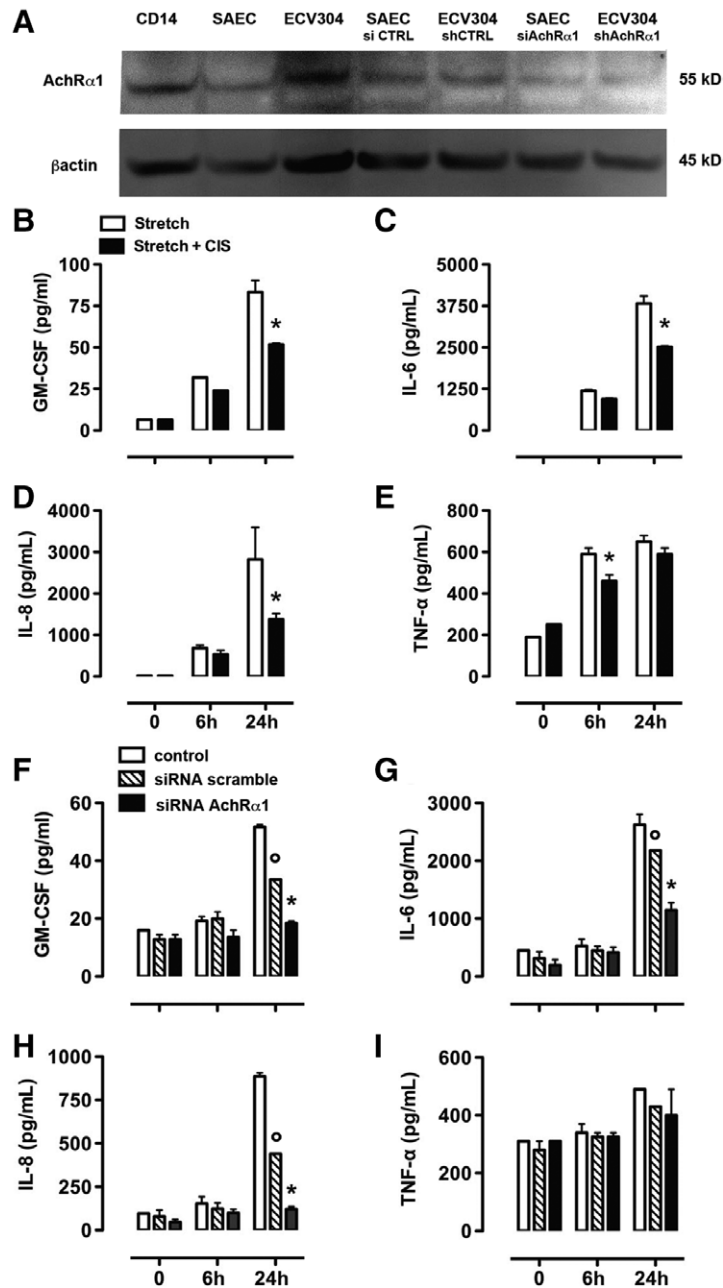


Fig. 4. Protein expression in primary human CD14⁺ cells and knockdown of nAChR α 1 in human cell lines (A) and time course of cytokine response in SAEC after mechanical stretch for 48 h (B–I). CIS = cisatracurium; ECV304 = human endothelial cells; GM-CSF = granulocyte-macrophage colony-stimulating factor; IL-6 = interleukin-6; IL-8 = interleukin-8; nAChR α 1 = nicotinic acetylcholine receptor α 1; SAEC = primary human small airway epithelial cells; shAChR α 1 = nAChR α 1 small hairpin RNA; shCtrl = scrambled small hairpin RNA; siAChR α 1 = nAChR α 1 small-interfering RNA; siCtrl = scrambled small-interfering RNA; TNF- α = tumor necrosis factor- α . * P < 0.05 versus Stretch in (B–E). ° P < 0.05 scrambled siRNA versus vehicle control; * P < 0.05 nAChR α 1 siRNA versus scrambled siRNA and control in (F–I).

have been demonstrated in plasma of septic patients, suggesting that neutrophils activated by uPA and LPS migrate from the systemic circulation into the lungs and contribute to the development of acute lung injury.^{8,19}

In the nAChR α 1 receptor knockdown studies, the administration of CIS slightly attenuated IL-6 after stimulation with LPS or plasma from patients with ARDS in

human endothelial cells. This suggests that although blocking the nAChR α 1 receptor by CIS plays a dominant role, mechanisms other than through the nAChR α 1 receptor may be involved in the pharmacologic effects of CIS.

Prolonged use of heavy sedation and NMBAs may be associated with atrophy and muscular weakness.^{20,21} In our model, there were no significant differences in the expression

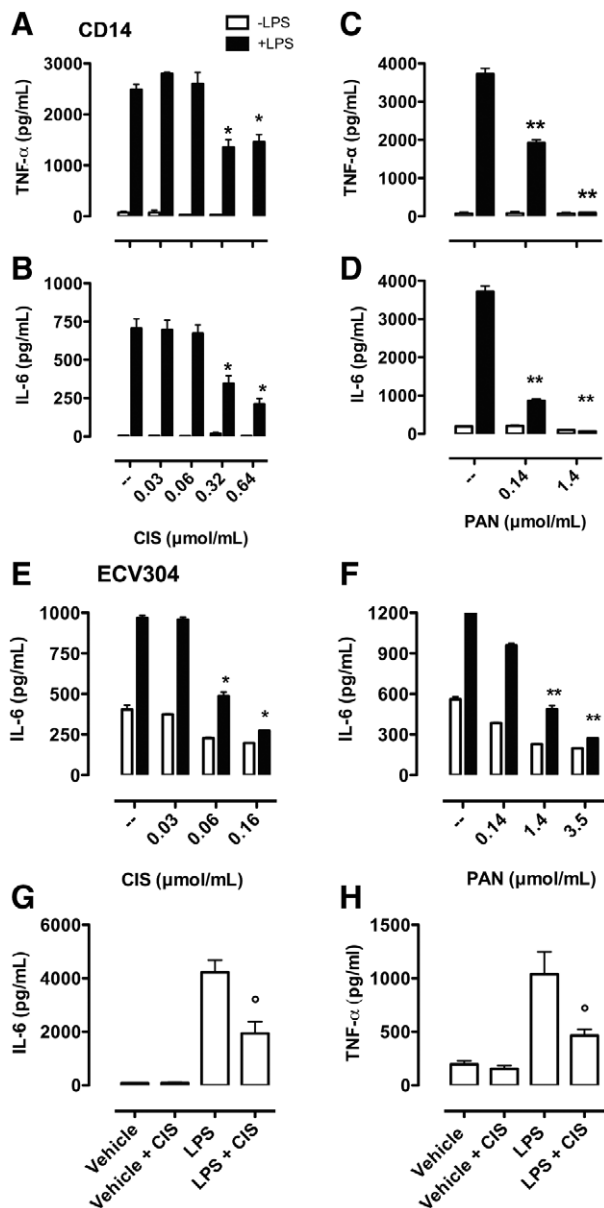


Fig. 5. TNF- α and IL-6 production in response to different stimulation in the presence and absence of CIS and PAN. (A–D) Levels of TNF- α and IL-6 released from CD14⁺ cells after LPS stimulation for 4 h. (E and F) Levels of IL-6 released from human endothelial cells (ECV304) after LPS stimulation for 4 h in the presence or absence of CIS or PAN. (G and H) Levels of IL-6 and TNF- α in lung lavage fluids from the perfused mouse lung model after LPS challenge in the presence or absence of CIS. CIS = cisatracurium; ECV304 = human endothelial cells; IL-6 = interleukin-6; LPS = lipopolysaccharide; PAN = pancuronium; TNF- α = tumor necrosis factor- α . * P < 0.05 CIS 0.32 and 0.64 μ M versus control. ** P < 0.05 PAN 1.4, 3.5 μ M versus control. ° P < 0.05 LPS + CIS versus LPS.

of known mediators of muscle atrophy among groups as demonstrated by similar diaphragm mRNA changes expression of the muscle-specific ubiquitin ligases MuRF1 and Atrogin-1. This is not surprising given the relatively short period of time that our animals were studied.

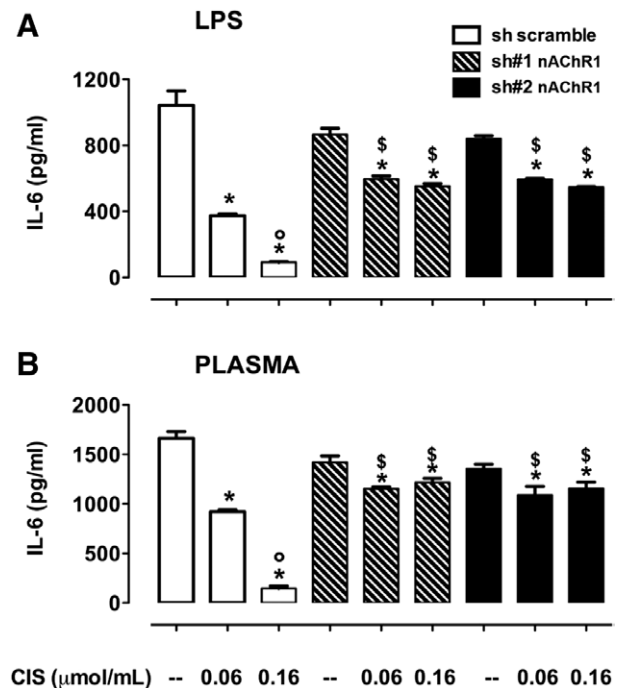


Fig. 6. Concentration of IL-6 released from human endothelial ECV304 cells transfected with nAChR α 1 shRNAs after stimulation with LPS (A) or plasma (B) from patients with acute respiratory distress syndrome. Small hairpin scrambled (sh scramble), small hairpin #1 (sh#1), and small hairpin #2 (sh#2) for nAChR α 1. CIS = cisatracurium; IL-6 = interleukin-6; LPS = lipopolysaccharide; nAChR α 1 = nicotinic acetylcholine receptor α 1. * P < 0.05 CIS 0.06 and 0.16 μ M versus no CIS. \$ P < 0.05 sh#1 and sh#2 versus sh scramble at CIS 0.06 and 0.16 μ M. ° P < 0.05 CIS 0.06 versus 0.16 μ M.

Some limitations of this study should be addressed. First, we did not record esophageal pressure traces to detect the signs of asynchrony in experimental groups without NMBA. However, we verified the occurrence of inspiratory efforts during hold inspiratory maneuver. Second, rats were ventilated only for 3 h, and this short period of time may account for small difference in Pao₂ between groups. Third, in the whole organism, CIS has a half-life of half an hour depending on temperature and pH. In *in vitro* studies, we did not replace CIS, and this might have affected the duration of nAChR α 1 blockade and the antiinflammatory effect.

In conclusion, this study demonstrates that NMBA, including CIS and PAN, can attenuate lung injury largely by antiinflammatory effects acting *via* interaction with the nAChR α 1 receptor.

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Competing Interests

The authors declare no competing interests.

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Address correspondence to Dr. Zhang: Room 619, LKSKI, 209 Victoria Street, Toronto, Ontario, Canada M5B 1W8. zhangh@smh.ca. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References

- Papazian L, Forel JM, Gacouin A, Penot-Ragon C, Perrin G, Loundou A, Jaber S, Arnal JM, Perez D, Seghboyan JM, Constantin JM, Courant P, Lefrant JY, Guérin C, Prat G, Morange S, Roch A; ACURASYS Study Investigators: Neuromuscular blockers in early acute respiratory distress syndrome. *N Engl J Med* 2010; 363:1107–16
- Forel JM, Roch A, Marin V, Michelet P, Demory D, Blache JL, Perrin G, Gannier M, Bongrand P, Papazian L: Neuromuscular blocking agents decrease inflammatory response in patients presenting with acute respiratory distress syndrome. *Crit Care Med* 2006; 34:2749–57
- Zhang G, Kernan KA, Thomas A, Collins S, Song Y, Li L, Zhu W, Leboeuf RC, Eddy AA: A novel signaling pathway: Fibroblast nicotinic receptor $\alpha 1$ binds urokinase and promotes renal fibrosis. *J Biol Chem* 2009; 284:29050–64
- Albuquerque EX, Pereira EF, Alkondon M, Rogers SW: Mammalian nicotinic acetylcholine receptors: From structure to function. *Physiol Rev* 2009; 89:73–120
- Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, Li JH, Wang H, Yang H, Ulloa L, Al-Abed Y, Czura CJ, Tracey KJ: Nicotinic acetylcholine receptor $\alpha 7$ subunit is an essential regulator of inflammation. *Nature* 2003; 421:384–8
- Tracey KJ: Physiology and immunology of the cholinergic antiinflammatory pathway. *J Clin Invest* 2007; 117:289–96
- Zhang G, Thomas AL, Marshall AL, Kernan KA, Su Y, Zheng Y, Takano J, Saido TC, Eddy AA: Nicotinic acetylcholine receptor $\alpha 1$ promotes calpain-1 activation and macrophage inflammation in hypercholesterolemic nephropathy. *Lab Invest* 2011; 91:106–23
- Abraham E, Gyetko MR, Kuhn K, Arcaroli J, Strassheim D, Park JS, Shetty S, Idell S: Urokinase-type plasminogen activator potentiates lipopolysaccharide-induced neutrophil activation. *J Immunol* 2003; 170:5644–51
- Zhang G, Marshall AL, Thomas AL, Kernan KA, Su Y, LeBoeuf RC, Dong XR, Tchao BN: *In vivo* knockdown of nicotinic acetylcholine receptor $\alpha 1$ diminishes aortic atherosclerosis. *Atherosclerosis* 2011; 215:34–42
- Fink H, Yasuhara S, Blobner M, Martyn JA: Up-regulation of acetylcholine receptors during subchronic infusion of pancuronium is caused by a posttranscriptional mechanism related to disuse. *Crit Care Med* 2004; 32:509–13
- Itoh H, Shibata K, Matsumoto T, Nitta S, Nishi M, Kobayashi T, Yamamoto K: Effects of neuromuscular-blocking drugs in rats *in vivo*: Direct measurements in the diaphragm and tibialis anterior muscle. *Acta Anaesthesiol Scand* 2004; 48:903–8
- Fanelli V, Puntorieri V, Assenzio B, Martin EL, Elia V, Bosco M, Delsedime L, Del Sorbo L, Ferrari A, Italiano S, Ghigo A, Slutsky AS, Hirsch E, Ranieri VM: Pulmonary-derived phosphoinositide 3-kinase gamma (PI3K γ) contributes to ventilator-induced lung injury and edema. *Intensive Care Med* 2010; 36:1935–45
- Terragni PP, Filippini C, Slutsky AS, Birocco A, Tenaglia T, Grasso S, Stripoli T, Pasero D, Urbino R, Fanelli V, Faggiano C, Mascia L, Ranieri VM: Accuracy of plateau pressure and stress index to identify injurious ventilation in patients with acute respiratory distress syndrome. *ANESTHESIOLOGY* 2013; 119:880–9
- Cappello P, Tomaino B, Chiarle R, Ceruti P, Novarino A, Castagnoli C, Migliorini P, Perconti G, Giallongo A, Milella M, Monsurro V, Barbi S, Scarpa A, Nisticò P, Giovarelli M, Novelli F: An integrated humoral and cellular response is elicited in pancreatic cancer by alpha-enolase, a novel pancreatic ductal adenocarcinoma-associated antigen. *Int J Cancer* 2009; 125:639–48
- Slutsky AS: Neuromuscular blocking agents in ARDS. *N Engl J Med* 2010; 363:1176–80
- Tremblay L, Valenza F, Ribeiro SP, Li J, Slutsky AS: Injurious ventilatory strategies increase cytokines and c-fos mRNA expression in an isolated rat lung model. *J Clin Invest* 1997; 99:944–52
- Belperio JA, Keane MP, Burdick MD, Londhe V, Xue YY, Li K, Phillips RJ, Strieter RM: Critical role for CXCR2 and CXCR2 ligands during the pathogenesis of ventilator-induced lung injury. *J Clin Invest* 2002; 110:1703–16
- Kalamida D, Poulas K, Avramopoulou V, Fostieri E, Lagoumintzis G, Lazaridis K, Sideri A, Zouridakis M, Tzartos SJ: Muscle and neuronal nicotinic acetylcholine receptors. Structure, function and pathogenicity. *FEBS J* 2007; 274: 3799–845
- Robbie LA, Dummer S, Booth NA, Adey GD, Bennett B: Plasminogen activator inhibitor 2 and urokinase-type plasminogen activator in plasma and leucocytes in patients with severe sepsis. *Br J Haematol* 2000; 109:342–8
- Batt J, dos Santos CC, Cameron JI, Herridge MS: Intensive care unit-acquired weakness: Clinical phenotypes and molecular mechanisms. *Am J Respir Crit Care Med* 2013; 187: 238–46
- Herridge MS, Cheung AM, Tansey CM, Matte-Martyn A, Diaz-Granados N, Al-Saidi F, Cooper AB, Guest CB, Mazer CD, Mehta S, Stewart TE, Barr A, Cook D, Slutsky AS; Canadian Critical Care Trials Group: One-year outcomes in survivors of the acute respiratory distress syndrome. *N Engl J Med* 2003; 348:683–93