

MILD TRAUMATIC BRAIN INJURY ATTENUATES PNEUMONIA-INDUCED LUNG INJURY BY MODULATIONS OF ALVEOLAR MACROPHAGE BACTERICIDAL ACTIVITY AND M1 POLARIZATION

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ABSTRACT—Traumatic brain injury is one of the main causes of death and disability worldwide, and results in multisystem complications. However, the mechanism of mild traumatic brain injury (MTBI) on lung injury remains unclear. In this study, we used a murine model of MTBI and pneumonia (*Pseudomonas aeruginosa*) to explore the relationship between these conditions and the underlying mechanism. **Methods:** Mice (n = 104) were divided into control, MTBI, pneumonia, and MTBI + pneumonia groups. MTBI was induced by the weight-drop method. Pneumonia was induced by intratracheal injection with *P. aeruginosa* Xen5 strain. Animals were killed 24 h after bacterial challenging. Histological, cellular, and molecular indices of brain and lung injury were assessed using various methods. **Results:** Mice in both the MTBI and pneumonia groups had more Fluoro-Jade C-positive neurons than did the controls ($P < 0.01$), but mice in the MTBI + pneumonia group had fewer Fluoro-Jade C-positive cells than did the pneumonia group ($P < 0.01$). The MTBI + pneumonia mice showed decreased bacterial load ($P < 0.05$), reduced lung injury score and pulmonary permeability ($P < 0.01$), less inflammatory cells, and lower levels of proinflammatory cytokines (TNF- α and IL-1 β ; $P < 0.01$) when compared with the pneumonia group. Molecular analysis indicated lower levels of phosphorylated nuclear factor- κ B in the lung of MTBI + pneumonia mice compared with the pneumonia group ($P < 0.01$). Furthermore, alveolar macrophages from MTBI mice exhibited enhanced bactericidal capacity compared with those from controls ($P < 0.01$). Moreover, MTBI + pneumonia mice exhibited less CD86-positive M1 macrophages compared with the pneumonia group ($P < 0.01$). **Conclusions:** MTBI attenuates pneumonia-induced acute lung injury through the modulation of alveolar macrophage bactericidal capacity and M1 polarization in bacterial pneumonia model.

KEYWORDS—Alveolar macrophage, brain injury, lung injury, M1 polarization, bacterial pneumonia

INTRODUCTION

Acute respiratory distress syndrome (ARDS) involves diffuse inflammatory acute lung injury (ALI) that leads to decreased lung tissue ventilation, increased pulmonary vascular permeability, and decreased lung function (1). The incidence of ARDS is about 86.2 per 100,000 person-years, with pneumonia being the most common trigger (2). Although there are many treatments for ARDS, such as mechanical ventilation, prone position ventilation, manual lung inflation, and nitric oxide inhalation, the mortality rate of patients remains at around 30% (3). This study therefore attempts to identify a more effective treatment for ALI/ARDS.

Traumatic brain injury (TBI) is a serious global issue that results in about 2.5 million emergency hospital visits every year (4). More than 90% of TBI cases are classified as mild TBI (MTBI) (5). MTBI is especially common in the context of sports and military activities (3). Athletes using bicycles or participating in contact sports frequently suffer from MTBI (6). A survey of 3,952 US Army soldiers returning from Iraq showed that 14.9% of them had experienced MTBI (7).

Moderate and severe brain injuries have been shown to be associated with ALI/ARDS (8). Interestingly, previous studies have demonstrated that MTBI alleviates lung injury (9,10). Understanding the underlying mechanism why MTBI exerts protective

effects on ALI/ARDS may provide novel perspectives for the treatment of ALI/ARDS, especially in veterans and athletes. *Pseudomonas aeruginosa* (PA), a gram-negative bacterium, is one common cause of nosocomial pneumonia, especially in cases of brain injury-associated ventilator-associated pneumonia (11,12). In this study, we hypothesized that MTBI plays a role in modulating PA-induced ARDS/ALI. We have used the weight-drop method and PA pneumonia to induce MTBI and ALI/ARDS, respectively. We then studied the histological, cellular, and molecular mechanisms of MTBI on ALI/ARDS; we found that MTBI attenuates pneumonia-induced lung inflammation and ALI by modulating alveolar macrophage bactericidal capacity and M1 polarization in bacterial pneumonia model.

MATERIALS AND METHODS

Animals

Original FVB/N mice were purchased from Jackson Laboratory (Bar Harbor, ME). Adequate food and water were supplied throughout the entire study. Mice were maintained on a diurnal 12-h light/dark cycle under controlled temperature and humidity conditions. All animals were housed at the Animal Core Facility of SUNY Upstate Medical University under pathogen-free conditions. The mice were 8 to 12 weeks old and weighed about 25 g. All the related animal protocols were strictly adhered to according to ARRIVE guidelines and approved by the Institutional Animal Care and Use Committee of SUNY Upstate Medical University (No. 380). All mice (n = 104) including both male and female were assigned randomly to 4 groups: control, brain injury, pneumonia, and MTBI + pneumonia groups.

Mild brain injury and pneumonia model

Mild brain injury was induced via weight-drop method, and the mice were then intratracheally injected with PA Xen5 to induce pneumonia. The mice were anesthetized by intraperitoneal injection of ketamine/xylazine (90 mg/kg ketamine, 10 mg/kg xylazine). Then, the mice were placed prone on a foam bed and fitted with a steel helmet. This helmet is a metal disk that is used to prevent skull fracture

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when the mice are hit. An 82-g rod was dropped from a height of 10 cm through a vertical pipe onto the helmet (13). In our pilot experiments, we did not find significant changes of the brain in the histopathological hematoxylin and eosin (H/E) staining as well as by Evan blue test (data not shown) in this mild model. Thirty minutes later, one 0.5-cm incision was made to expose the trachea, and 50 μ L of bioluminescent PA Xen5 solution ($\sim 2 \times 10^4$ colony-forming units [CFU]/mouse) was injected into the trachea. In this study, 2×10^4 CFU/mouse of PA Xen5 was deemed appropriate for *in vivo* imaging analysis, and about 50% of mice were still alive 72 h after bacterial challenge (14).

In vivo imaging analysis

PA Xen5 produces bioluminescence signals that can be detected *in vivo* and is positively associated with the bacterial load as described previously (14). Bioluminescence was assessed at 24 h after bacterial challenge to determine bacterial quantity using an *in vivo* imaging system (IVIS-200; Caliper Life Science, Hopkinton, MA), as described previously (14). A cooled charge-coupled device camera (Xenogen-200; Caliper Life Sciences) was used to take photographs. Mice were anesthetized with isoflurane (2% in oxygen) in the induction chamber and then transferred into the imaging chamber of the IVIS-200 with continuous isoflurane anesthesia. The bioluminescent signals were spatially localized by merging pseudo-colored images of photon emissions and gray-scale images of the mice.

Specimen collection

Lung tissues and bronchoalveolar lavage fluid (BALF) were collected as described previously (14). To collect the specimens, mice were anesthetized by intraperitoneal injection of the ketamine/xylazine cocktail described previously and were then exsanguinated to collect or lavage lungs. BALF was obtained by lavaging the lung 3 times with 0.5 mL saline.

Histopathological analysis

Lung tissues were fixed with 10% formalin for at least 24 h and embedded in paraffin. Five-micrometer lung sections were prepared for H/E staining using standard procedures. Two investigators then examined multiple randomly chosen microscopic fields from 5 mice in each group, in a blinded manner. Twenty high-power fields were chosen for each slide. A lung injury scoring system was used to estimate the severity of lung injury (15).

Fluoro-Jade C staining

Fluoro-Jade C (FJC) staining was performed using the Fluoro-Jade C Staining Kit (Biosensis, Thebarton, Australia) in accordance with the manufacturer's instructions. Paraffin-embedded brain tissue sections were dewaxed and rehydrated by xylene and graded alcohol solutions. The sections were then incubated in 0.06% KMnO_4 for 10 min, transferred into 0.0001% FJC in 0.1% acetic for a further 10 min, washed, dried, and mounted using a neutral mounting medium-DPX (Sigma-Aldrich). Sections from 5 mice in each group were observed using the TE2000-U microscope (Nikon, Tokyo, Japan), and the number of FJC-positive neuron axons from 20 high-power fields were analyzed statistically among groups.

Evans blue dye

To evaluate the pulmonary permeability, the extravasation of Evans blue (EB) dye was assessed as described previously (16). EB dye binds with albumin and other plasma proteins immediately after injection into the vein and serves as a marker for plasmexudrosis. EB dye (2% in phosphate-buffered saline; Sigma, St. Louis, MO) was injected into the mice through the tail vein (4 mL/kg). One and a half hours after injection, mice were exsanguinated and perfused through the left ventricle with 30 mL of phosphate-buffered saline. The lungs from 5 or 6 mice in each group were collected for analysis. To evaluate EB dye extravasation, each lung was homogenized with 1 mL of formamide. The solutions were incubated at 55°C for 24 h. The homogenates were centrifuged at 21,130g for 25 min, and the supernatants were transferred into a new tube and detected spectrophotometrically at optical density 620 nm (OD620).

Inflammatory cells in BALF

To assess the inflammatory response within the lungs, we quantified macrophages and neutrophils in BALF as described previously (14). Five or 6 mice from each group were killed at 24 h after bacterial challenge, and the tracheas were cannulated; 0.5 mL of saline was injected into the lungs, aspirated, and collected. Alveolar lavage was repeated 3 times, and about 1.2 mL of BALF was collected. Cell pellets were collected by centrifugation, and cells were sedimented by cytospin onto glass slides. The cells were stained using a Hema-3 staining kit (Thermo Fisher Scientific, Waltham, MA). Cells on the slides were identified and quantified using the TE2000-U microscope (Nikon).

Concentrations of cytokines in lung tissues

Proinflammatory mediators reflect the intensity of pulmonary inflammation. As shown in our previous publication (14), lung tissues from 5 mice in each group were homogenized to measure the concentrations of IL-1 β , IL-6, and TNF- α using enzyme-linked immunosorbent assay kits following the manufacturer's instructions (Super Array Bioscience, Frederick, MD).

Western blotting analysis

The transcription factors of the nuclear factor (NF)- κ B family play an important role in inflammatory responses. NF- κ B is found in the cytoplasm but is activated by phosphorylation in the nucleus to regulate inflammatory (and other) responses. We assessed the expression of phosphorylated NF- κ B (p-NF- κ B) by Western blotting as described previously (14). Lung tissues from 5 mice in each group were ground into tissue homogenates with phosphatase inhibitor and protease. Homogenates were centrifuged at 13,000 g for 10 min to collect supernatants. The protein concentrations of the supernatants were determined using Micro-BCA protein assay kits (Thermo Scientific, Rockford, IL). A total of 50 μ g of protein was loaded onto reducing 12% sodium dodecyl sulfate–polyacrylamide gel for electrophoresis and then transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked in 3% skim milk in Tris-buffered saline, incubated with a primary antibody against p-NF- κ B p65 (No. 3033; Cell Signaling Technology, Danvers, MA), and subsequently incubated with a secondary horseradish peroxidase–conjugated antibody (Bio-Rad). The bands were detected using Pierce ECL Western Blotting detection reagent (Thermo Scientific, Rockford, IL) and developed by X-film. The membrane was stripped and incubated with β -actin antibody (ab16039; Abcam, Cambridge, United Kingdom). The bands on the films were analyzed using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD).

Macrophage killing of bacteria

Alveolar macrophages recognize and eliminate pathogens in airways. To evaluate the bactericidal capacity of alveolar macrophages, bacteria were phagocytosed, and after a certain period, the residual bacteria were sprayed onto agar plates. The bacterial colonies were counted after incubation (17). Thirty minutes after challenge with weight drop, the macrophages of 5 mice from the brain injury group were collected. Macrophages from the brain injury and control groups were co-cultured with PA Xen5 for 30 min; the ratio of macrophages to PA Xen5 was 1:10. The PA Xen5 on the surface of the macrophages was killed and removed using gentamicin (50 μ g/mL). The gentamicin was washed off using saline, and macrophages containing PA were resuspended in distilled water to release the swallowed bacteria. Distilled water containing the released bacteria was sprayed onto agar plates. The plates were incubated at 37°C overnight to count the bacterial colonies.

Immunofluorescence analysis

Immunofluorescence analysis of specific M1 macrophage biomarker CD86 was performed as described previously (14). In brief, paraffin-embedded sections with lung tissues were deparaffinized with xylene and graded ethanol after incubation at 60°C for 1 h. Antigen were retrieved over 10 min with boiling sodium citrate buffer (pH 6.0). Sections were permeabilized with 0.2% Triton X-100 and then blocked with 10% donkey serum (ab7475; Abcam). Sections from 5 mice in each group were incubated with CD86 antibodies (14086282; eBioscience, San Diego, CA) or CD16 antibodies (sc-53376; Santa Cruz Biotech, Dallas, TX) and then incubated with secondary antibodies (ab150073; Abcam). The nuclei were counterstained with DAPI (ab104139; Abcam). Sections were examined using the TE2000-U microscope (Nikon).

Statistical analysis

All data are expressed as mean $\pm$ SEM. The normality of the data was first examined using Kolmogorov-Smirnov test. Statistical analyses including 1-way ANOVA followed by the Student-Newman-Keul test or Student *t* test for 1 pair were performed using SPSS software (version 20.0; SPSS Inc., Chicago, IL). *P* < 0.05 was considered statistically significant.

RESULTS

Reduced brain injury in MTBI + pneumonia mice compared with either MTBI or pneumonia mice

FJC, a polyanionic fluorescein, was used to examine neuronal changes/injury in MTBI (18). FJC-positive myelin/cells were found in both the brain injury and pneumonia groups (Fig. 1, A–D). However, the MTBI + pneumonia group had significantly fewer positive myelin/cells (Fig. 1E, *P* < 0.01). Hence, the combined effects of

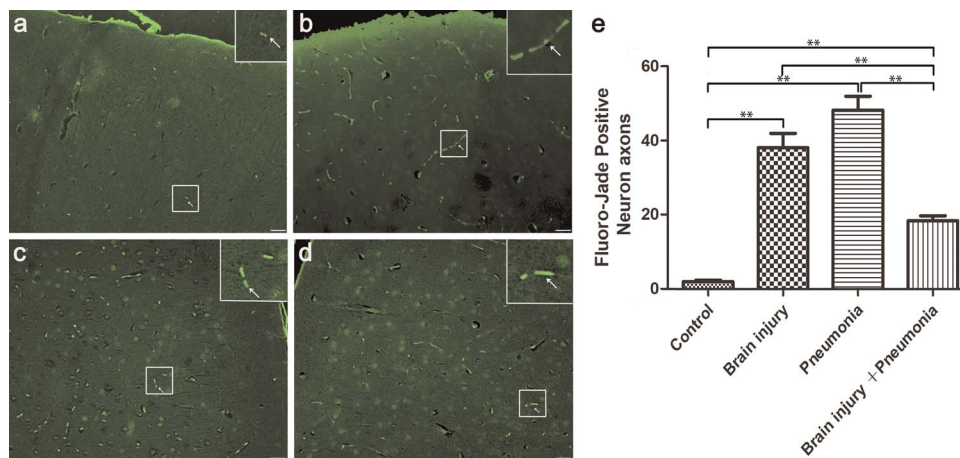


FIG. 1. MTBI-attenuated neuronal changes caused by pneumonia of PA Xen5. Mice were separated into 4 groups: control, brain injury, pneumonia, and brain injury + pneumonia. The brain tissue was embedded by paraffin, and the section of brain tissue was stained by FJC staining kit. Damaged myelin was detected by FJC staining. A–D, FJC-positive neurons from the control, brain injury, pneumonia, and MTBI + pneumonia groups, respectively. E, The statistical data for the FJC-positive neuron axons. Both MTBI and PA Xen5 infection increased FJC-positive neuron axons in the brains of the mice. Interestingly, MTBI + pneumonia together reduced FJC-positive neurons compared with MTBI or pneumonia alone. At least 3 independent experiments were performed for this study. Graphs show the mean ± SEM. N = 5 mice/group. ** $P < 0.01$. Scale bar represents 10 μ m. FJC indicates Fluoro-Jade C; MTBI, mild traumatic brain injury; PA, *Pseudomonas aeruginosa*.

MTBI and pneumonia seem to reduce the brain damage and injury compared with only either MTBI or PA pneumonia.

Decreased bacterial load in the lung of MTBI + pneumonia mice compared with pneumonia mice

To study the effect of MTBI on bacterial growth, the bacterial load of the mice was determined by *in vivo* imaging analysis (Fig. 2A). As expected, no signal was detected in the control and MTBI groups. The bioluminescence signal of the MTBI + pneumonia group was significantly lower than that of the pneumonia groups (Fig. 2B; $P < 0.05$).

Decreased pulmonary microvascular permeability in the lung of MTBI + pneumonia mice compared with pneumonia mice

The inflammation associated with pneumonia-induced pulmonary microvascular permeability (19). The amount of EB dye in the lung was taken to reflect pulmonary microvascular permeability. As expected, there was no increase of lung microvascular permeability in and MTBI group compared with the controls (Fig. 3, A and B).

The pneumonia group showed greater leakage of EB dye than the MTBI + pneumonia group (Fig. 3, C and D). The EB dye was quantified using a spectrophotometer method and was significantly higher in the pneumonia and MTBI + pneumonia groups compared with the Control and MTBI groups ($P < 0.01$). There was also a statistical difference between the pneumonia and MTBI + pneumonia groups (Fig. 3B; $P < 0.01$).

Reduced lung injury in MTBI + pneumonia mice compared with pneumonia mice

To evaluate the effects of MTBI on PA Xen5-induced pneumonia, we examined the lung injury using H/E-stained sections. Infection with PA Xen5 caused significantly pulmonary histopathological changes in the pneumonia group, whereas the brain injury group showed only mild-moderate histopathological changes in the lungs (Fig. 4, A–D). Of note, quantitative analysis indicated that the MTBI + pneumonia group showed less lung injury than the pneumonia group (Fig. 4E; $P < 0.01$).

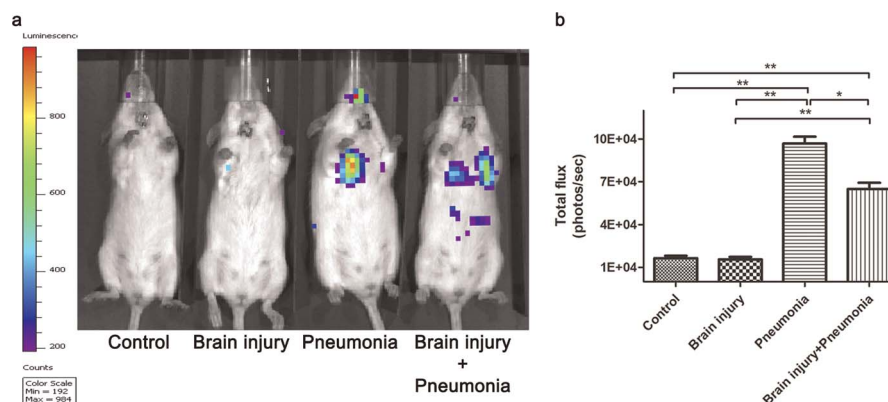


FIG. 2. MTBI damped bacterial growth of PA Xen5 in the lung of infected mice. The growth of PA Xen5 in the lung was assessed by its bioluminescence with *in vivo* imaging system. A, A representative image of bacterial proliferation in the infected mice. As expected, no bacteria exist in the control group and the brain injury group. B, Statistical data for bioluminescent signal levels of the 4 groups. The MTBI + pneumonia group shows a lower level of bacteria compared with the pneumonia group ($P < 0.05$). At least 3 independent experiments were performed for this study. Graphs represent the mean ± SEM. N = 5 mice/group. * $P < 0.05$, ** $P < 0.01$. MTBI indicates mild traumatic brain injury; PA, *Pseudomonas aeruginosa*.

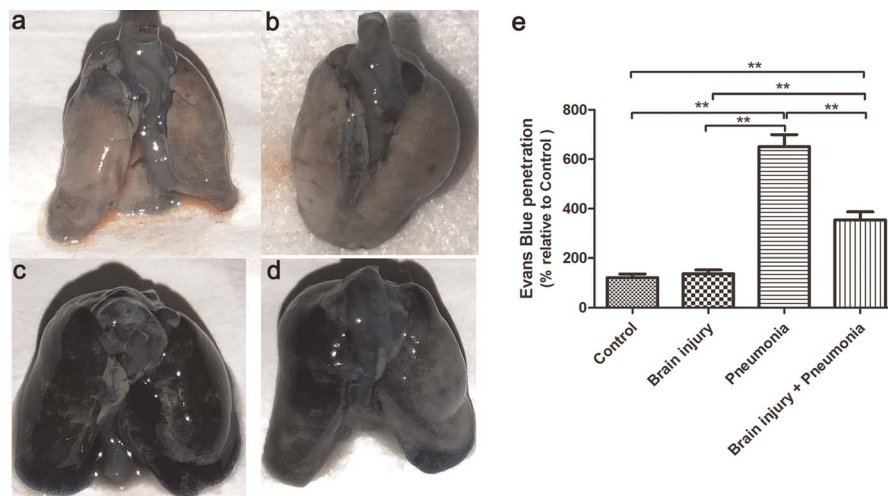


FIG. 3. MTBI reduced lung vascular permeability in mice infected with PA Xen5. EB dye was used to assess pulmonary vascular permeability. Mice were injected with EB, exsanguinated, and perfused through the left ventricle 1.5 h after injection. Lungs in each group were collected for analysis. A–D, Gross views of the lungs. E, Results of quantitative examination of EB in the 4 groups. The lungs of the pneumonia group contained significantly more EB compared with the other groups ($P < 0.01$). The EB content of the MTBI + pneumonia group was lower compared with the pneumonia group but higher than in the control and brain injury groups ($P < 0.01$). At least 3 independent experiments were performed for this study (graphs represent the mean $\pm$ SEM; $N = 5$ –6 mice/group; * $P < 0.05$, ** $P < 0.01$). EB indicates Evan blue; MTBI, mild traumatic brain injury; PA, *Pseudomonas aeruginosa*.

Reduced inflammatory cells in BALF of MTBI + pneumonia mice compared with pneumonia mice

The number of inflammatory cells in BALF is known to accurately reflect pathological changes in cases of ALI. We determined inflammatory cells (macrophages and neutrophils) in BALF from the 4 groups. As shown in Figure 5, the cells in BALF from Control and MTBI mice were almost alveolar macrophages (Fig. 5, A and B). Bacterial infection significantly increased neutrophils in the lung of infected mice (Fig. 5F; $P < 0.01$). However, the MTBI + pneumonia mice had less abundant macrophages and neutrophils when compared with the mice in the pneumonia group (Fig. 5, E and F; $P < 0.05$).

Decreased proinflammatory cytokines in the lungs of MTBI + pneumonia mice compared with pneumonia mice

The proinflammatory cytokines (IL-1 β , IL-6, and TNF- α) were measured in lung tissues via enzyme-linked immunosorbent assay.

There was no statistical difference in the concentrations of IL-1, IL-6, and TNF- α between the control and brain injury groups (Fig. 6). The levels of all 3 cytokines were higher in the pneumonia than control and brain injury groups ($P < 0.01$). Of note, the concentrations of IL-1 β and TNF- α were significantly lower in the MTBI + pneumonia mice when compared with the pneumonia group ($P < 0.05$). However, no statistical difference of the concentration of IL-6 was found between the MTBI + pneumonia and pneumonia groups (Fig. 6B).

Reduced p-NF- κ B level in the lungs of MTBI + pneumonia mice compared with pneumonia mice

Phosphorylation of NF- κ B activates the expression of a series of proinflammatory cytokines and is emerging as an important determinant of inflammation. Western blotting analysis with p-NF- κ B–specific antibody showed increased p-NF- κ B level in the lung

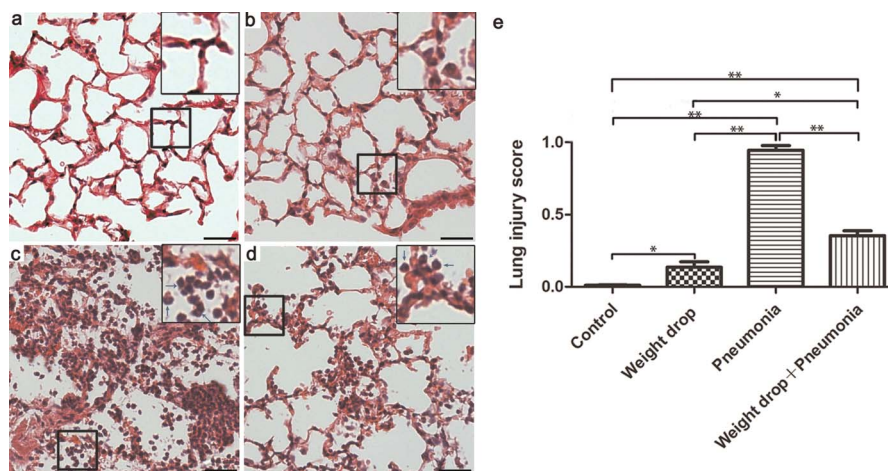


FIG. 4. MTBI attenuated lung injury caused by infection of PA Xen5. Lungs were fixed with 10% formalin solution and then embed with paraffin. The paraffin-embedded sections were prepared and stained with HE staining. Histological changes in the lung were evaluated by HE staining. A–D, Changes in lung histopathology in the 4 groups. E, Lung injury scores of the groups. The lungs of the pneumonia group mice exhibited obvious inflammatory cell infiltration and tissue edema. Tissue edema and inflammatory cell infiltration were also apparent in mice in the MTBI + pneumonia group but were less severe than in the pneumonia group ($P < 0.01$). At least 3 independent experiments were performed for this study. Graphs show the mean $\pm$ SEM. $N = 5$ mice/group. ** $P < 0.01$. Scale bar = 10 μ m. HE indicates hematoxylin and eosin; MTBI, mild traumatic brain injury; PA, *Pseudomonas aeruginosa*.

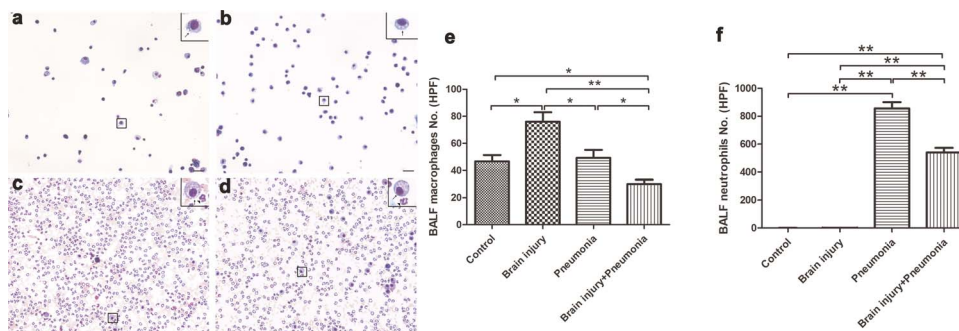


FIG. 5. **MTBI changed inflammatory cells in BALF of infected mice.** The cells in BALF were mounted on slides by cytospin centrifugation. The slides were stained using the Hema-3 staining kit. A–D, Inflammatory cells were greatly increased in the pneumonia group, of which a great majority were neutrophils. E and F, Results of the quantitative analyses of BALF macrophages (E) and neutrophils (F) in all 4 groups. At least 3 independent experiments were performed for this study. Graphs show the mean $\pm$ SEM. $N = 5$ –6 mice/group. $**P < 0.01$. Scale bar represents 10 μ m. BALF indicates bronchoalveolar lavage fluid; MTBI, mild traumatic brain injury.

tissue of 3 treated groups but not in controls (Fig. 7A). The level of pNF- κ B 65 in the lung tissue of MTBI + pneumonia group was significantly lower than that of the pneumonia group (Fig. 7B; $P < 0.01$). However, MTBI mice had a significantly lower level of p-NF- κ B than that of either the MTBI + pneumonia or pneumonia group (Fig. 7B; $P < 0.01$).

Enhanced bactericidal capacity of alveolar macrophages from MTBI mice

We collected alveolar macrophages to evaluate their bactericidal capacity. Alveolar macrophages were collected from mice with or without MTBI challenge. After mixing macrophages and PA xen5 bacteria (ratio, 1:10) and co-culturing for 30 min, bacteria that were associated with the surface of the cells were killed by gentamicin; bacteria inside the cells were released and cultured onto Luria broth (LB) agar plates. Bacteria colonies on the Luria broth agar plates were counted. The results showed significantly less CFUs in the macrophages from MTBI mice compared with those from control mice (Fig. 8, $P < 0.01$), suggesting that the macrophages from MTBI mice had increased bactericidal capacity compared with those from control mice without MTBI challenge.

Reduced M1 macrophages in the lungs of MTBI + pneumonia mice compared with pneumonia mice

Macrophages play an important role in pathogen clearance and inflammatory modulation in the lung. Alveolar macrophages are activated by many stimuli, a process known as polarization. M1 macrophages are known to be proinflammatory and cytotoxic,

whereas M2 macrophages are anti-inflammatory “prorepair” macrophages (20). CD86 expression is a specific biomarker for the identification of M1 macrophages (21). We found increased expression of CD86-positive cells in the lung of both the MTBI and pneumonia groups compared with the control group (Fig. 9, A–D), whereas expression of CD86 was decreased significantly in the MTBI + pneumonia group compared with the pneumonia group (Fig. 9E). In addition, similar pattern of the changes of CD16 biomarker was observed in the lung of this model. These results indicate that M1 polarization was induced by bacterial infection, but that this process may be inhibited in MTBI mice. The inhibition of M1 polarization of macrophages in MTBI mice may explain the protective effects of MTBI in pneumonia-induced ALI/ARDS.

DISCUSSION

Lung infection after severe TBI is common, and severe TBI is one of major risk factors to cause other organ injuries, including ALI/ARDS (12). The relationship between lung infection and MTBI, however, has not been explored extensively. In this study, our results revealed that MTBI can attenuate inflammation and ALI in one murine model with PA Xen5 lung infection, which has been found to be associated with the enhanced bactericidal capacity of alveolar macrophages and the inhibition of M1 macrophage polarization after MTBI.

The lung is involved in complex cross-talk with many other organs, including the brain, gut, and kidney (22–24). The brain can affect the lung via complex pathways (25), such that, in many

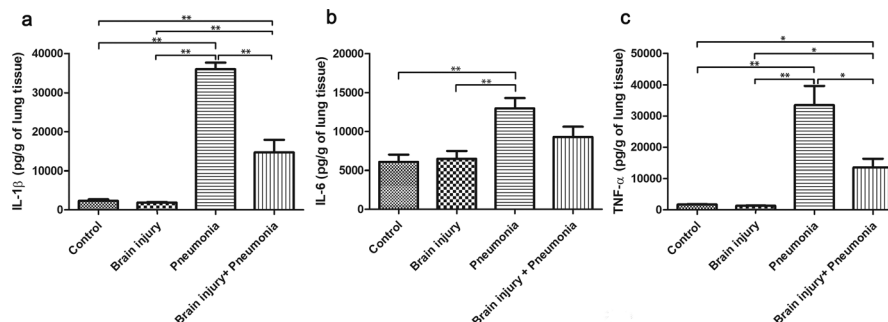


FIG. 6. **MTBI inhibited the elevation of inflammatory cytokines in mice infected with PA Xen5.** Lung tissues were homogenized after being harvested at 24 h after infection to measure the concentrations of IL-1 β , IL-6, and TNF- α . The ratios between cytokine levels and total lung tissue weight were calculated. A, IL-1 β levels of the 4 groups. The levels of IL-1 β in the control and brain injury groups were similarly low. The pneumonia group had a significantly higher level of IL-1 β than the other groups. The MTBI + pneumonia groups showed lower levels of IL-1 β than the pneumonia group but higher levels than the control and brain injury groups. B and C, Levels of IL-6 and TNF- α , respectively. The levels of these cytokines were similar among the 4 groups. Three independent experiments were performed for this study. Graphs show the mean $\pm$ SEM. $N = 5$ mice/group. $**P < 0.01$. MTBI indicates mild traumatic brain injury; PA, *Pseudomonas aeruginosa*.

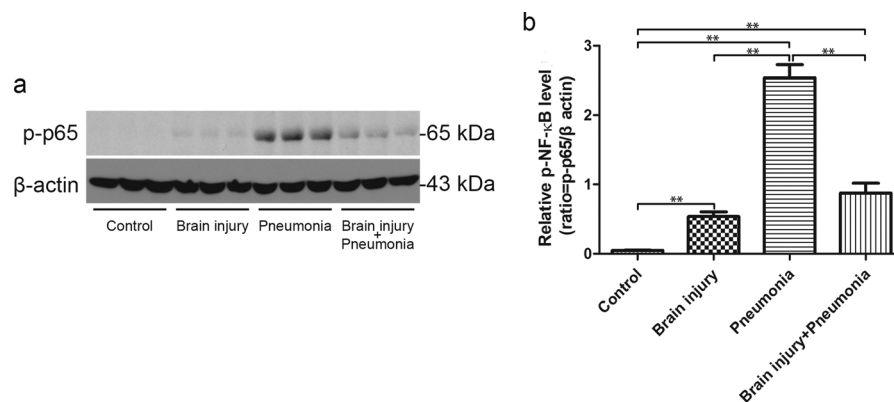


FIG. 7. **MTBI reduced the NF- κ B activation in the lung of mice infected with PA Xen5.** Lung tissues were homogenized after being harvested at 24 h after infection. A, Western blots of p-NF- κ B and β -actin. B, Quantitative analysis of p-NF- κ B expression was performed. Both MTBI and infection by PA Xen5 increased the expression of p-NF- κ B in the lungs of the mice. However, the combination of MTBI and infection by PA Xen5 attenuated the expression of p-NF- κ B compared with only pneumonia ($P < 0.01$). Three independent experiments were performed for this study. Graphs represented as the mean $\pm$ SEM; $N = 5$ mice/group. * $P < 0.05$, ** $P < 0.01$. MTBI indicates mild traumatic brain injury; PA, *Pseudomonas aeruginosa*; p-NF- κ B, phosphorylated nuclear factor- κ B.

cases, brain damage/injury is often associated with lung injury. This interrelation is linked to impaired airway protection, cytokine release via neuronal activation, pulmonary vasoconstriction, and higher capillary permeability due to increased sympathetic activity (22). Similarly to previous studies, this study revealed that MTBI mice also suffered from acute, albeit mild, lung injury. Paradoxically, we found that MTBI attenuated the neurological damage and pulmonary acute injury caused by bacterial infection with PA Xen5. Altered macrophage phagocytosis and polarization may be major reasons underlying the MTBI-related protective effect against bacterial pneumonia. These observations in this study are consistent with the recent findings by Hsieh et al. (10), in which increased substance P expression was found in MTBI mice. Further understanding of the cellular and molecular mechanisms related to protective effects of MTBI against pneumonia may facilitate the treatment of bacterial induced ALI/ARDS.

For clinical diagnosis of MTBI, one or more of the following criteria must be met: confusion or disorientation, loss of consciousness

for up to 30 min, posttraumatic amnesia for less than 24 h, and/or other transient neurological abnormalities such as focal signs, seizure, and intracranial lesions not requiring surgery, as well as a Glasgow Coma Scale score of 13 to 15 at least 30 min after injury upon presentation to a healthcare facility (26). In general, MTBI is not associated with obvious pathological changes in routine radiological tests or based on H/E staining. Although many animal models have been developed to replicate human TBI (13), we adopted the weight-drop mouse model because it is closer to the clinical condition of human TBI (9) and has been proven to be consistent by Vaickus et al. (9,27). The general view and H/E staining of the brain between mice with weight-drop injury and the normal mice showed no significant difference. We also examined the leakage of EB dye in the brain of mice with and without weight drop, and no difference was found (data not shown). The damage degree was mild as previously described (27). Although there was no obvious histological change in the brain of mice belonging to the MTBI group, we found positive signals in the MTBI mice by FJC staining, which

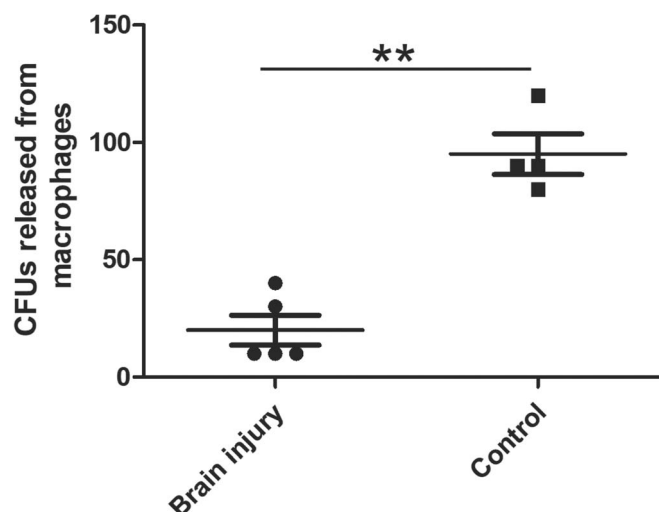


FIG. 8. **MTBI enhanced the bactericidal capacity of alveolar macrophages.** Alveolar macrophages were obtained from the lungs of brain injury mice and controls. The macrophages were then co-cultured with PA Xen5 for 30 min. Co-culture with macrophages and PA Xen5 was performed at a ratio of 1:10 for 30 min. Gentamicin (50 μ g/mL) was used to kill outside bacteria around the macrophages and then washed off using saline. Macrophages containing PA were resuspended in distilled water to release bacteria. Bacteria in distilled water were sprayed onto agar plates and incubated at 37°C overnight to count the bacterial colonies. The CFUs per 50 macrophages of mice with brain injury were significantly less abundant compared with the control group ($P < 0.01$), suggesting that MTBI enhanced the bactericidal capacity of alveolar macrophages in the lung. Three independent experiments were performed for this study. $N = 5$ mice/group. ** $P < 0.01$. CFUs indicates colony-forming units; MTBI indicates mild traumatic brain injury; PA, *Pseudomonas aeruginosa*.

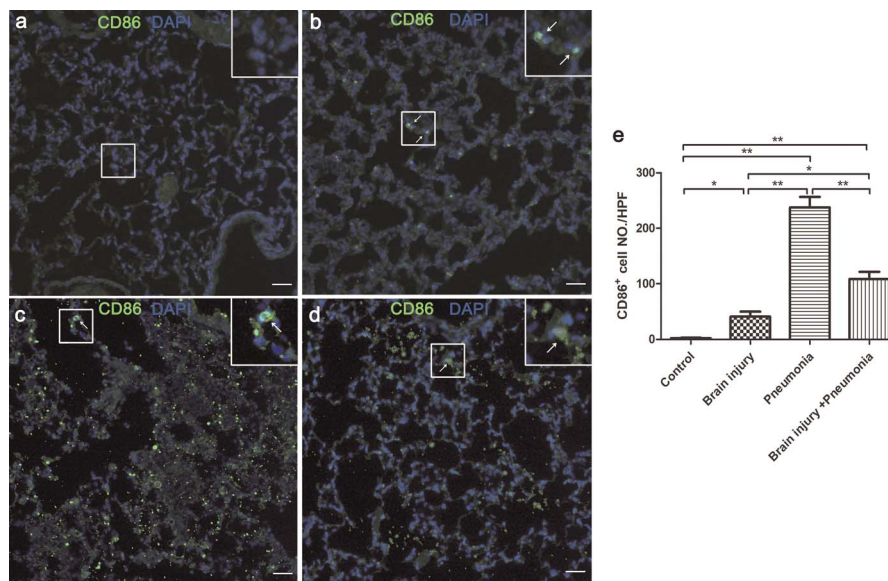


FIG. 9. MTBI modulated M1 polarization of alveolar macrophages in the lung of mice infected with PA Xen5, thus alleviating excessive inflammation. M1 macrophages are known to be proinflammatory and cytotoxic macrophages. CD86 was as a specific biomarker of M1 macrophages. A–D, Lung tissue sections from the 4 groups were deparaffinized and incubated with CD86 antibody. Nuclei were counterstained with DAPI. E, Results of quantitative analysis of CD86-positive cells. CD86-positive cells were higher in the pneumonia group compared with the brain injury group or the MTBI + pneumonia group ($P < 0.01$), indicating that MTBI influenced M1 polarization after PA Xen5 infection. At least 3 independent experiments were performed for this study. Graphs represented as the mean $\pm$ SEM; $N = 5$ mice/group. * $P < 0.05$, ** $P < 0.01$. MTBI indicates mild traumatic brain injury; PA, *Pseudomonas aeruginosa*.

suggested that more testing methods should be adopted to access the MTBI. Functional magnetic resonance imaging detected myelin in MTBI and may imply that more research is required in the study of MTBI (28).

In this study, both FJC-positive cells and lung injury were found in the MTBI group mice, consistent with previous studies (22,29). MTBI attenuated the myelin injury associated with pneumonia, which may be via the expression and regulation of glucocorticoids. Previous studies demonstrated that serum glucocorticoid levels increased immediately after MTBI (30). Hormone secretions, including those of adrenocorticotropin, among others, are altered in the acute phase of brain injury. These hormones are important in trauma and bacterial infection (31). Clinical studies showed that plasma cortisol levels were markedly elevated in the acute phase of brain injury. In one pilot study, we also observed an increased level of serum cortisol 30 min post-MTBI (data not shown). Therefore, it is warranted to examine the change of glucocorticoid level in the blood of MTBI/pneumonia mice in the future. Previous studies have demonstrated that glucocorticoids have been shown to attenuate lung injury by modulating macrophage polarization. Tu et al. (32) found that methylprednisolone induced M2 polarization of macrophages to ameliorate ALI. We found that MTBI enhanced bacterial clearance by alveolar macrophages in lung tissue of mice and reduced CD86-positive cells in the lungs. MTBI may regulate macrophage polarization through cortisone secretion, resulting in protection against pneumonia. Although the use of cortisol to prevent lung injury in patients suffering from brain injuries has been controversial, it seems that the results from this study support the use of cortisol in MTBI cases. However, further studies are necessary to assess the role of cortisol and the protective effects of MTBI against bacterial pneumonia.

The bacterial PA Xen5 used in this study is high toxicity because of its specific type III secretion system, and the bacterial dose used in this study could cause severe lung injury and ARDS based on our previous observation (14,33). Of interesting, MTBI mice exhibited

reduced bacterial load and pulmonary permeability and decreased proinflammatory cytokine expression in the lung after bacterial infection compared with without MTBI-infected mice. Further analysis of *ex vivo* alveolar macrophages revealed enhanced bactericidal capacity of the alveolar macrophages from MTBI mice compared with those from control mice. Altered activation/status of alveolar macrophages that play a critical role in the lung innate immunity and host defense could influence outcome of bacterial pneumonia, which has been observed in other conditions (34). Furthermore, it has been found that NF- κ B signaling pathway is involved in the pathogenesis of ALI/ARDS induced by bacterial infection or other conditions (33,35). Decreased NF- κ B activation and less lung injury was observed in the MTBI + pneumonia group in comparison with only-pneumonia group, which is consistent with the published observations (33,35).

Although the findings in this study are exciting, there are several limitations. First, all experiments were performed with an FVB/N mouse strain that are relatively T helper-2-prone and anti-inflammatory property in their immune response to infection; this property may have a degree of impact on the response of bacterial infection after MTBI. Second, there is a limited mouse number of each group to analyze sex difference for the output measures, although both male and female animals were used in the study. Third, this design is to address the situation of MTBI/lung infection simultaneously, so animals were infected with PA 30 min after MTBI treatment, which is not related to clinically ventilated-induced pneumonia of MTBI. Fourth, we examined a few biomarkers of alveolar macrophages and used a classic M1/M2 model to explain the effect of MTBI-induced changes of alveolar macrophage phenotypes. It should be helpful to analyze the profile of many gene expressions in alveolar macrophages with modern sc-RNA sequencing method or other techniques because there exists a more complex profile of gene expression in alveolar macrophages after MTBI and pneumonia.

In summary, this study used a mouse model of weight-drop-induced MTBI combined with bacterial-induced pneumonia

to explore the relationships of MTBI and pneumonia-induced ALI/ARDS. We found that MTBI attenuates pneumonia-induced ALI, and the mechanism may involve the modulation of alveolar macrophage bactericidal activity and M1 polarization in the lung after MTBI and bacterial challenges.

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