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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2013

Link to publication in University of Groningen/UMCG research database

Traumatic Brain Injury in Rats Induces Lung Injury and Systemic Immune Suppression

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Abstract

Traumatic brain injury (TBI) is frequently complicated by acute lung injury, which is predictive for poor outcome. However, it is unclear whether lung injury develops independently or as a result of mechanical ventilation after TBI. Further, TBI is strongly associated with the development of pneumonia, suggesting a specific vulnerability for the development of nosocomial infections in the lung after TBI. In this study, we evaluated whether indeed pulmonary injury and immune suppression develop spontaneously in an animal model of mild TBI (mTBI). TBI was induced in male PVG rats by closed-head trauma using a weight-drop device. Subsequently, we evaluated the effects of this on the lungs as well as on the excitability of the systemic immune system. Finally, we performed an experiment in which TBI was followed by induction of pneumonitis and evaluated whether TBI affects the severity of subsequent pneumonitis induced by intratracheal instillation of heat-killed *Staphylococcus aureus*. mTBI resulted in significant lung injury, as evidenced by pulmonary edema, protein leakage to the alveolar compartment, and increased concentrations of interleukin-1 and -6 in broncho alveolar lavage fluid (all $p < 0.05$ vs. sham-treated animals). Further, after TBI, the release of tumor necrosis factor alpha was decreased when whole blood was stimulated *ex vivo* ($p < 0.05$ TBI vs. sham), indicating systemic immune suppression. When TBI was followed by pneumonitis, the severity of subsequent pneumonitis was not different in rats previously subjected to TBI or sham treatment ($p > 0.05$), suggesting that systemic immune suppression is not translated toward the pulmonary compartment in this specific model. We here show that during mild experimental TBI, acute pulmonary injury, as well as a decrease in the excitability of the systemic immune system, can be observed.

Key words: acute lung injury; immune suppression; neurogenic pulmonary edema; neuroimmunology; traumatic brain injury

Introduction

TRAUMA IS THE LEADING CAUSE OF DEATH and disability under the age of 45 in the Western world. Traumatic brain injury (TBI) is responsible for half of all trauma-associated mortality.[1] The incidence of head trauma requiring hospital admission has been estimated between 100 and 400 in 100,000 per year, with a death rate of 46.9 in 100,000 per year in North America.[2] Extracranial complications after TBI are a major determinant of outcome, because non-neurologic organ dysfunction contributes to approximately two thirds of all deaths after severe TBI (sTBI).[3] sTBI and increased intracranial pressure (ICP) increase the likelihood of the development of acute lung injury, which carries a higher risk of in-hospital death.[4,5] However, it is unclear whether lung injury is dependent of the severity of TBI and whether it is a direct effect of TBI on the lung or a result of "priming" of the lung to mechanical ventilation-associated lung injury. TBI is not only associated with sterile lung injury, but also with pulmonary infection.[6,7] The infection rate in isolated TBI patients exceeds 60% and results in an infection-related mortality rate of approximately 30%.[8,9] Although high incidences of infection are observed after major surgery, burn injury, and polytrauma, incidence of secondary infections in TBI patients is disproportionally higher.[10] Brain trauma patients develop infections primarily in the lung, frequently caused by specific organisms, of which *Staphylococcus aureus* is the most prominent.[11,12] The high incidence of infection after TBI is believed to be the result of a state of diminished capacity of immune cells to respond to infectious agents, also known as

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immune suppression.[13] Although this immune suppression is frequently assumed, its actual incidence after TBI and the underlying mechanism have not yet been identified.[14]

To study the development of lung injury and immune suppression after TBI and to characterize the cross-talk between the brain, lung, and the systemic immune system, a validated brain trauma model was implemented in our laboratory. In this model, closed-head trauma is mimicked by a weight falling freely by gravity from a designated height through a Plexiglas tube.[15–17] The severity of TBI in this model is mild because rats are usually free of any neurological sequelae within several hours. In this study, we used this mild TBI (mTBI) model in our laboratory and studied the development of lung injury and systemic immune suppression after TBI. Finally, we investigated whether preceding experimental TBI has an effect on the magnitude of the inflammatory response to pneumonitis.

Methods

Animals

The animal ethical committee of the Academic Medical Center, University of Amsterdam (Amsterdam, the Netherlands) approved all experiments. Experiments were conducted using male PVG rats (8–10 rats per group), weighing 171–294 g (Harlan Laboratories, Horst, the Netherlands). Animals were kept in collective cages (4 animals per cage) and maintained in a room under controlled temperature and a 12-h light/dark interval with free access to food and water.

TBI model

TBI was induced according to the protocol by Marmarou and colleagues.[15] Briefly, rats were anesthetized using 2.5% isoflurane (1 L/min O_2) and buprenorphine hydrochloride 0.05 mg/kg subcutaneously and were intubated because of transient hypoventilation that is observed after induction of TBI. A metallic helmet was fixed to the central portion of the skull vault of the rat by simple tape. Rats were placed in a prone position on a foam bed. The lower end of a Plexiglas tube was positioned directly above the helmet. A 300-g weight contained within the Plexiglas tube was dropped from a height of 2 m. This height was determined in initial pilot experiments (data not shown). After induction of TBI, rats were transiently (usually 5–15 min) subjected to mechanical ventilation using lung-protective settings (tidal volume, 6 mL/kg body weight) until spontaneous ventilation was observed. Sham animals underwent a similar procedure, but were not subjected to the actual trauma. Animals were euthanized 24 h after TBI.

Pneumonitis model

We specifically chose to develop a pneumonitis (sterile pneumonia) model using heat-killed bacteria, and not a live bacterial pneumonia model, because we wanted to be able to investigate the effects of immune suppression on inflammatory response without the potential confounding effects of accelerated bacterial growth or reduced bacterial clearance induced by changes in activity of the inflammatory response. For this, rats were anesthetized using 2.5% isoflurane (1 L/min O_2). A total of 1×10^9 colony-forming units of heat-killed *S. aureus* (InvivoGen, San Diego, CA) was dissolved in 250 μL of saline, and rats were inoculated intratracheally using a trans-oral miniature nebulizer. Sham animals were inoculated with saline. Animals were sacrificed 24 h thereafter. The optimal dose of the inoculum, as well as the time point of euthanization, was determined in initial dose- and time-finding experiments (data not shown).

TBI and pneumonitis experiment

In a separate experiment, TBI induction or sham treatment, as described above, was followed 24 h later by induction of pneu-

monitis. These time points were determined using initial pilot experiments that showed severe lethality of pneumonitis, if a shorter interval between TBI and pneumonia was used (data not shown). Animals were euthanized 24 h after induction of pneumonitis.

Euthanization

All rats were euthanized by intraperitoneal injections of 80 mg/kg of ketamine (Nimatek; Eurovet, Bladel, the Netherlands) combined with 0.5 mg/kg of medetomidine (Domitor, Novartis, Arnhem, The Netherlands). The thorax was opened and blood from the heart was drawn, resulting in exsanguination of animals.

Tissue handling

Blood was collected in heparin-coated vacutainer tubes, spinned, and supernatant was kept at $-20°C$ until further assays. Relative lung weights were calculated by weighing the right lung, and the relative lung weight of the rat was calculated by dividing the weight of the right lung with the total weight of the rat at baseline and expressed as mg lung/g rat. Subsequently, the right lung was dissected and one lobe was used for histologic examination. For this, parts were fixed in 10% buffered formalin, embedded in paraffin, and 4-μm-thick sections were stained with hematoxylin and eosin. Remaining parts of the right lung were homogenized in four volumes of sterile saline and five volumes of lysis buffer (pH 7.4) containing 150 mM of NaCl, 15 mM of Tris, 1 mM of $MgCl(H_2O)_6$, 1 mM of $CaCl_2(H_2O)_2$, 1% Triton X-100, and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, ethylenedinitrilote-traacetic acid, pepstatin, and leupeptin. After each homogenization, the homogenizer was carefully cleaned and disinfected with 70% alcohol. Homogenates were centrifuged at $1500g$ at $4°C$ for 15 minutes, and supernatants were stored at $-20°C$ until assays were performed. The left lung was used for bronchoalveolar lavage (BAL). BAL was performed by instilling two 2-mL aliquots of sterile saline. Approximately 3.0–3.5 mL of BAL fluid (BALF) was retrieved per rat and stored at $-20°C$ until assays were performed.

Assays

The phosphorylated axonal from of the high-molecular-weight neurofilament subunit, NF-H (pNF-H), has been validated extensively as a biomarker that represents the extent of injury severity in TBI.[21] pNF-H was measured using a phosphorylated Neurofilament H ELISA kit (RD191138300R; Biovendor GmbH, Heidelberg, Germany), according to the instructions of the manufacturer. For *ex vivo* full-blood stimulation and cytokine measurements, blood was diluted in an equal volume of RPMI 1640 (Invitrogen, Breda, the Netherlands) or with RPMI 1640 containing heat-killed *S. aureus* (InvivoGen, San Diego, CA). Blood was incubated at $37°C$ in 5% CO_2 for 24 h, and supernatants were stored at $-20°C$ until assays were performed. As readout for *ex vivo* excitability of the immune system, tumor necrosis factor alpha (TNF-α) levels were measured in the supernatant. Cytokine levels were also measured in lung homogenates and BALFs (Interleukin [IL]-1β and IL-6) by enzyme-linked immunosorbent assays (ELISAs), according to the manufacturer's instructions (all DuoSets; R&D systems, Mineapolis, MN), as were protein levels in BALF (Oz Biosciences, Marseille, France).

Histopathology

A pathologist who was blinded for group identity analyzed all pulmonary specimens. To score lung inflammation and damage, lung samples were screened for the following variables: interstitial inflammation; intra-alveolar inflammation; edema; endothelialitis; bronchitis; pleuritis; and thrombi formation. Each variable was graded on a scale of 0–3 (0, absent; 1, mild; 2, moderate; 3, severe), and a total histology score was subsequently calculated.

Statistical analysis

All data are presented as means ± standard deviation. A Student's *t*-test or Mann-Whitney's U test was used, where appropriate. Throughout, a *p* value < 0.05 was considered statistically significant. All statistical analyses were carried out using GraphPad Prism (version 5.01; GraphPad Software Inc., San Diego, CA).

Results

Plasma pNF-H is increased after TBI in rats

Compared to the sham group, rats exposed to TBI showed significantly higher plasma levels of pNF-H (Fig. 1), indicating extensive neuronal damage after brain trauma. Rats recovered without neurological sequelae within several hours of induction of closed-head injury.

mTBI induces pulmonary edema, protein leakage, and inflammation

Twenty-four hours after mTBI, no significant histological changes were observed in the lung (Fig. 2; $p > 0.05$). However, several markers of lung injury were present in rats subjected to TBI. First, pulmonary edema was present in animals subjected to TBI, because relative lung weight was significantly increased, as compared to sham-treated animals ($p < 0.05$). Further, as a marker of vascular permeability, protein content of the bronco alveolar lavage fluid was increased in TBI versus sham-treated animals ($p < 0.05$ vs. sham). Finally, as a marker of an inflammatory response in the pulmonary compartment, as compared to sham rats, increased levels of IL-1 ($p < 0.05$ vs. sham) as well as IL-6 ($p < 0.05$ vs. sham) were observed in the BALF of TBI rats.

TBI induces systemic immune suppression

To investigate the effect of mild experimental TBI on the excitability of the systemic immune system, *ex vivo* stimulation of blood after TBI was performed. *Ex vivo* stimulation of whole blood with heat-killed *S. aureus* resulted in systemic immune suppression, as

evidenced by a significant reduction in TNF-α release after *ex vivo* stimulation of whole blood with heat-killed *S. aureus* (Fig. 3; $p < 0.005$, TBI vs. sham).

*Intratracheal installation of heat-killed* S. aureus *leads to pneumonitis*

To study the effects of previous TBI on subsequent pulmonary inflammation, a sterile lung injury model was implemented in our laboratory. Rats were subjected to the inhalation of heat-killed *S. aureus*. These rats showed extensive histological pulmonary inflammation, as well as lung damage, 24 h thereafter, as compared to animals that were treated by inhalation of saline (Fig. 4A; $p < 0.05$). Further, wet dry-weight ratio was increased as evidence of pulmonary edema (Fig. 4A; $p < 0.05$). Levels of IL-1 and IL-6 showed no statistically significant difference between both groups at this time point (Fig. 4A).

*Previous TBI does not affect the severity of subsequent pneumonitis*

In a final experiment, rats were subjected to TBI or sham, followed by pneumonitis, 24 h thereafter. When sham/pneumonitis and TBI/pneumonitis groups were compared, no significant alterations in pulmonary inflammation and damage were observed (Fig. 4B; $p > 0.05$). Although relative lung weights were decreased in TBI/pneumonitis rats ($p < 0.05$ vs. sham/pneumonitis), this finding coexisted with an increase in weight loss during this experiment in this group versus sham/pneumonitis rats of approximately 5–10% (data not shown) and is therefore difficult to interpret properly. Finally, no difference was observed in pulmonary cytokine levels between both groups (Fig. 4B; $p > 0.05$).

Discussion

Extracranial complications after TBI are a major determinant of outcome. Clinically, sTBI is associated with the development of acute lung injury as well as infectious complications in the
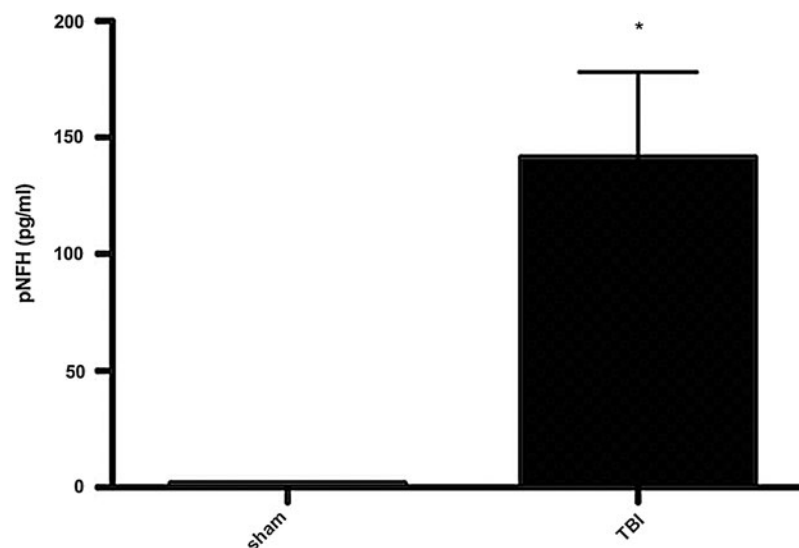
FIG. 1. pNF-H levels in plasma after TBI. Rats were subjected to TBI or sham treatment and sacrificed 24 h thereafter. Open bar represents sham-treated and closed bar represents animals subjected to TBI. Blood pNF-H ELISA data are shown. Data are means ± standard error of 8 rats per group. Asterisk indicates statistical significance ($p < 0.05$). TBI, traumatic brain injury; pNF-H, high-molecular-weight neurofilament subunit, NF-H; ELISA, enzyme-linked immunosorbent assay.
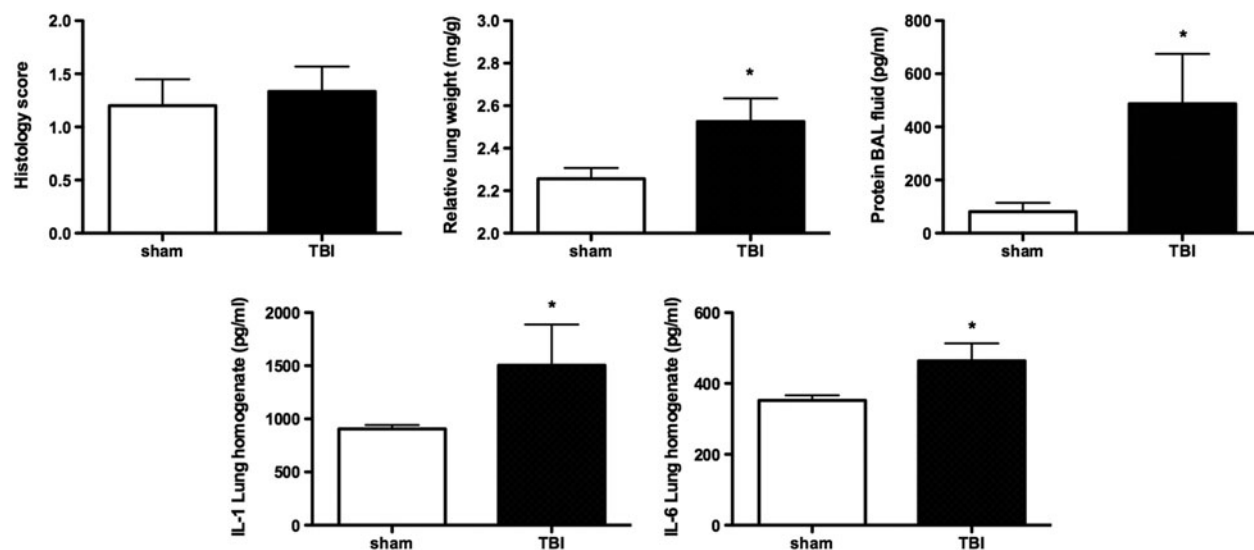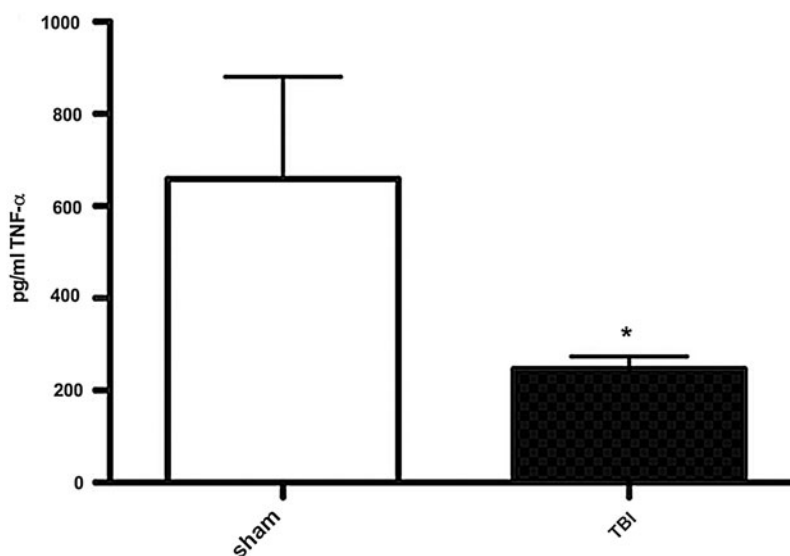
FIG. 2. Lung injury after mTBI. Rats were subjected to TBI or sham treatment and sacrificed 24 h thereafter. Open bars represent sham-treated and closed bars represent animals subjected to TBI. Histology, relative lung weight, and BALF protein content (upper panels) as well as lung IL-1 and IL-6 (lower panels) levels are shown. Data are means ± standard error of 8 rats per group. Asterisks indicate statistical significance ($p < 0.05$). mTBI, mild traumatic brain injury; BALF, bronchoalveolar lavage fluid; IL, interleukin.

pulmonary compartment. Our study shows that even mild experimental TBI induces acute lung injury, as evidenced by pulmonary edema, protein leakage, and an inflammatory response in the lung. Further, experimental mTBI results in a diminished systemic innate immune response, although no effects on pulmonary inflammation or damage could be demonstrated after subsequent pneumonitis.

Mechanical ventilation practices advocating normo- or even hypocapnia for extensive periods of time in TBI patients are, although unproven and potentially harmful, still widely used.[18] The use of high tidal volumes required to permit tight control of CO_2

might contribute to the development of acute lung injury in TBI.[19] It is unclear whether the association of acute lung injury with TBI is independent of mechanical ventilation or a result of (pulmonary priming toward) ventilation-induced lung injury.[19] Further, it is unclear whether acute lung injury after TBI is related to the severity of TBI and/or associated with increased ICP.[4,20] To evaluate whether acute lung injury develops independently of mechanical ventilation in a model in which ICP is not increased, we investigated the effects of mTBI on development in an experimental model without the need for prolonged mechanical ventilation. We

FIG. 3. Systemic immunosuppression after TBI. Rats were subjected to TBI or sham treatment and sacrificed 24 h thereafter. Open bar represents sham-treated and closed bar represents animals subjected to TBI. TNF-α release after *ex vivo* stimulation of whole blood (using heat-killed *S. aureus*) is shown. Data are means ± standard error of 8 rats per group. Asterisks indicate statistical significance ($p < 0.05$). TBI, traumatic brain injury; TNF-α, tumor necrosis factor alpha.
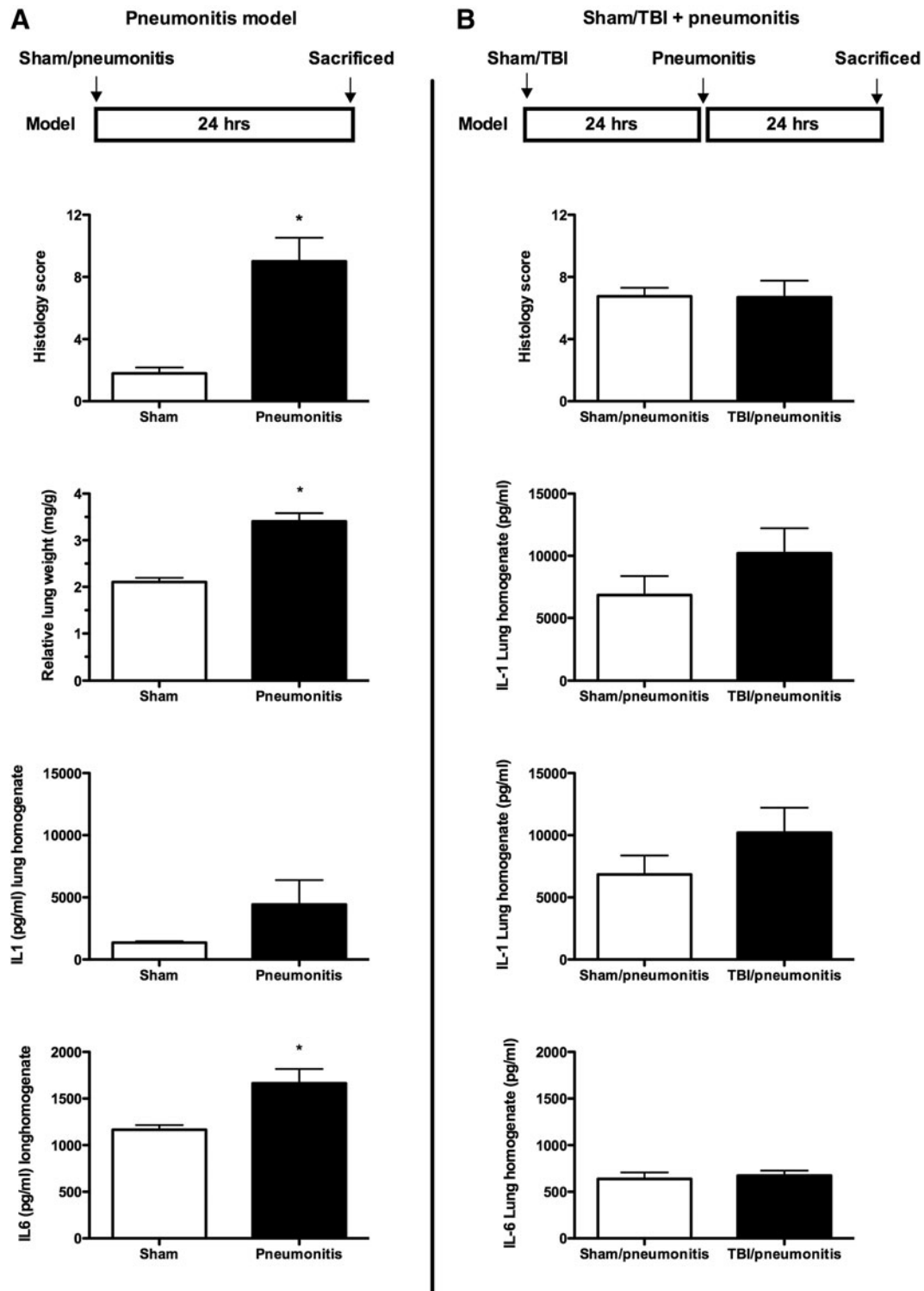
FIG. 4. Pneumonitis model and effect of previous TBI on subsequent pneumonitis. Data from our pneumonitis model are shown in (**A**). Rats were subjected to inhalation of heat-killed *S. aureus* or sham and sacrificed 24 h thereafter. Open bars represent sham-treated and closed bars represent animals subjected to induction of pneumonitis. Histology and wet dry weight (upper panels) as well as lung IL-6 and IL-6 (lower panels) are shown. Data are means ± standard error of 5 rats per group. Asterisks indicate statistical significance ($p < 0.05$). (**B**) Effect of previous TBI on pneumonitis severity is shown. Rats were subjected to TBI or sham treatment and 24 h thereafter to aspiration of heat-killed *S. aureus*. Animals were euthanized 24 h after induction of pneumonitis. Open bars represent sham-treated and closed bars represent animals subjected to TBI. Histology and relative lung (upper panels) as well as lung IL-1 and IL-6 (lower panels) levels are shown. Data are means ± standard error of 8 rats per group. Asterisks indicate statistical significance ($p < 0.05$). TBI, traumatic brain injury; IL, interleukin.

here show that pulmonary injury, inflammation, and edema develop even in mild experimental TBI and independently of mechanical ventilation.

Risk for aspiration is increased in patients with acute neurological injury, such as TBI and stroke, as a result of the absence of protective reflexes. A systematic review by Martino and colleagues showed that dysphagia occurs in approximately half of stroke patients and increases the risk for pneumonia in patients with confirmed aspiration.[21] However, up to half of stroke patients do not aspirate and still are at higher risk for the development of pneumonia, which implies that also other mechanisms are involved (e.g., stroke-induced immunodepression).[22] Further, we previously showed, in a mouse model of acid aspiration, that aspiration of acid primes the host for an exaggerated inflammatory response to subsequent pneumonia.[23] Taken together, the high incidence of pneumonia after TBI likely results from increased aspiration of oropharyngeal contents and/or gastric acid, combined with an increased vulnerability for the development of pneumonia resulting from immune suppression.

One of the main findings of our study is that experimental TBI indeed results in a significant decrease of innate immune response in the systemic compartment. This immunoparalysis was evidenced by a highly significant decrease of *ex vivo* excitability of the innate immune system in the systemic compartment after TBI induction. This study adds to the field by not only showing that TBI-induced immune suppression is a real and relevant phenomenon, but also by providing a model to study TBI-induced immune suppression in an experimental setting. Clearly, the descriptive nature of our study is one of its shortcomings, and follow up studies are mandatory to evaluate the underlying pathways involved. Further, because we analyzed animals 24 h after TBI and did not perform time-curve experiments, we cannot evaluate the kinetics of the observed immunological phenotype in time. In follow-up experiments, it would be interesting to investigate at what time after TBI maximal immune suppression develops. It is tempting to speculate what mechanisms might be responsible. Although immune suppression is associated with a variety of diseases states, neurological injury, such as TBI and stroke,[24] have been associated with disproportionally increased infection rates, suggesting that a final common pathway linking central nervous system (CNS) injury and infection might be involved. Several lines of evidence indicate that important connections between the CNS and the nervous system exist that might explain why immune suppression after CNS injury is especially predominant. One of the involved pathways might be the vagus nerve, because the vagus nerve and acetylcholine, its main neurotransmitter, have been identified as crucial mediators of inflammatory response.[25] *In vivo* studies showed that electrical stimulation of the efferent vagus nerve inhibits proinflammatory cytokine release and prevents endotoxic shock,[26] as well as the severity of experimental peritonitis and pancreatitis.[27,28] Indeed, a clinical observational study using heart-rate variability as a marker of vagal activity showed that conditions associated with increased ICP, such as brain injury, but, especially, intracerebral hemorrhage, were associated with increased vagal tone as well as immune suppression.[29]

The sympathetic nervous system might be involved as well. In mouse models of increased ICP as well as stroke administration of the *β*-adrenoreceptor antagonist, propranolol, reduced the incidence of immune suppression as well as secondary infection.[30] Taken together, injury to the CNS might result in an activation of one, or both, of these pathways, resulting in systemic immune suppression. Future studies will evaluate whether one of the pathways is involved in the TBI-induced immune suppression that we

describe here. Because the model we used results in transient injury to the brain stem, as evidenced by the transient impact apnea observed, it is conceivable that injury to specific areas in the brain stem (e.g., the dorsal motor nucleus of the vagus nerve, among others) is responsible for the immunological phenotype observed in our report. Follow-up experiments should focus on repeating our study in a dissimilar model, such as the controlled cortical impact model that is not associated with brain-stem injury.

Although we here show that systemic immune suppression after TBI is clearly established, the occurrence of this immune suppressive state does not affect the severity of subsequent pneumonia. Several explanations exist for this finding. First, one should realize that the severity of TBI in this model is very mild. Rats usually recover completely in a matter of hours free of any neurological sequelae. Therefore, it might be possible that our model is simply not severe enough to induce neurological damage, and therewith immune suppression, of enough gravity to affect a subsequent infectious insult. A second explanation is that the proposed systems involved (e.g., the autonomic nervous system) interact with the primary and secondary lymphoid organs, inducing a systemic immune suppression after activation. Because this neurogenic effect is limited to the lymphoid organs, one could postulate that after TBI, only a systemic effect is observed, as was the case in the *ex vivo* stimulation in our experiment, which is not translated to the pulmonary compartment during subsequent secondary intrapulmonary injury.

Taken together, we here show that TBI not only induces a systemic immune suppression, but also results in lung injury, as evidenced by protein leakage, lung edema, and an inflammatory response in the alveolar compartment, although an increase in secondary pulmonary damage after pneumonitis could not be demonstrated. Subsequent studies should focus on the underlying mechanisms involved.

Author Disclosure Statement

No competing financial interests exist.

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