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CD11d integrin blockade reduces the systemic inflammatory response syndrome after traumatic brain injury in rats

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Abstract

Traumatic CNS injury triggers a systemic inflammatory response syndrome (SIRS), in which circulating inflammatory cells invade body organs causing local inflammation and tissue damage. We have shown that the SIRS caused by spinal cord injury is greatly reduced by acute intravenous treatment with an antibody against the CD11d subunit of the CD11d/CD18 integrin expressed by neutrophils and monocyte/macrophages, a treatment that reduces their efflux from the circulation. Traumatic brain injury (TBI) is a frequently occurring injury after motor vehicle accidents, sporting and military injuries, and falls. Our studies have shown that the anti-CD11d treatment diminishes brain inflammation and oxidative injury after moderate or mild TBI, improving neurological outcomes. Accordingly, we examined the impact of this treatment on the SIRS triggered by TBI.

The anti-CD11d treatment was given at 2 h after a single moderate (2.5–3.0 atm) or 2 and 24 h after each of three consecutive mild (1.0–1.5 atm) fluid percussion TBIs. Sham-injured, saline-treated rats served as controls. At 24 h, 72 h, and 4 or 8 weeks after the single TBI and after the third of three TBIs, lungs of rats were examined histochemically, immunocytochemically and

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biochemically for downstream effects of SIRS including inflammation, tissue damage and expression of oxidative enzymes.

Lung sections revealed that both the single moderate and repeated mild TBI caused alveolar disruption, thickening of inter-alveolar tissue, hemorrhage into the parenchyma and increased density of intra-and peri-alveolar macrophages. The anti-CD11d treatment decreased the intrapulmonary influx of neutrophils and the density of activated macrophages and the activity of myeloperoxidase after these TBIs. Moreover, Western blotting studies showed that the treatment decreased lung protein levels of oxidative enzymes $gp91^{phox}$, inducible nitric oxide synthase and cyclooxygenase-2, as well as the apoptotic pathway enzyme caspase-3 and levels of 4-hydroxynonenal-bound proteins (an indicator of lipid peroxidation). Decreased expression of the cytoprotective transcription factor Nrf2 reflected decreased lung oxidative stress. Anti-CD11d treatment also diminished the lung concentration of free radicals and tissue aldehydes.

In conclusion, the substantial lung component of the SIRS after single or repeated TBIs is significantly decreased by a simple, minimally invasive and short-lasting anti-inflammatory treatment.

Keywords

Traumatic brain injury; Systemic inflammatory response; Lung damage; Anti-integrin treatment; Lung

1. Background

Traumatic or focal injury, or inflammatory challenge to the central nervous system triggers a systemic inflammatory response syndrome (SIRS), in which inflammatory cells from the circulation invade organs such as the liver, lung and kidney, potentially leading to damage of these organs (Campbell et al., 2003, 2005; Gris et al., 2008; Wilcockson et al., 2002). Central nervous system injury leads to a rapid release of brain-derived cytokines that initiate SIRS by promoting an influx of leukocytes into the liver, facilitated by hepatic chemokine expression that occurs within 2 h of the injury (Campbell et al., 2003, 2005; Wilcockson et al., 2002). The liver then produces acute-phase proteins as well as chemokines that are released into the circulation, initiating a widespread inflammatory response (Campbell et al., 2005; Wilcockson et al., 2002). Systemic inflammation thereby contributes to organ dysfunction after traumatic brain injury (TBI), spinal cord injury and other traumatic insults (Acosta et al., 1998; Baskaran et al., 2000; Bhatia et al., 2005; Gabay and Kushner, 1999; Ott et al., 1994). Indeed, a recent paper demonstrated significant liver accumulation of inflammatory cells as well as pro-inflammatory gene expression and histopathology for up to 21 days after a contusion spinal cord injury in rats (Sauerbeck et al., 2014).

The multiple organ dysfunction syndrome that can follow brain injury (Campbell et al., 2003; Gruber et al., 1999) results in a myriad of pathophysiologic complications affecting neurological, psychological, cardiovascular, pulmonary, and metabolic functions, which in turn impede the recovery process (Clifton et al., 1983; Kalsotra et al., 2003). Acute lung injury is a frequent but poorly understood complication of TBI (Holland et al., 2003). The

development of lung dysfunction is a critical independent factor affecting mortality in patients after brain injury and is associated with a worse long-term neurologic outcome in survivors (Holland et al., 2003; Hopkins et al., 2006). Lung-protective strategies can improve the outcome of TBI patients (Baigelman and O'Brien, 1981) as life-threatening lung dysfunction is extremely difficult to treat (Bhatia et al., 2005; Kyono and Coates, 2002; Ryugo et al., 2006).

The purpose of the present study was to ascertain whether a treatment that we have put forth to prevent the acute inflammatory response within the injured brain after fluid percussion TBI in rats would also reduce the SIRS after TBI. This treatment requires the intravenous delivery of a monoclonal antibody (mAb) against the CD11d subunit of the CD11d/CD18 integrin expressed by neutrophils and monocytes. This CD11d β2 integrin family member binds to VCAM-1 in rats and to ICAM-3 and VCAM-1 in humans, facilitating the firm adhesion of CD11d + leukocytes, in particular neutrophils and monocytes, to the cell surface of activated endothelial cells lining blood vessel walls (Grayson et al., 1998; Van der Vieren et al., 1995, 1999). In our previous studies, intravenous administration of the CD11d antibody, at 2 h after a single, moderate, fluid percussion TBI, decreased the numbers of neutrophils and macrophages in the injured brain with concomitant reductions in lipid peroxidation, astrocyte activation, amyloid precursor protein accumulations and neuronal loss. The reduced neuroinflammation in anti-CD11d-treated rats correlated with improved spatial cognition, sensorimotor performance and reduced anxiety-like behaviors (Bao et al., 2012b). In a study performed independently in a different institution (Utagawa et al., 2008) the anti-CD11d treatment reduced the density of macrophages and the contusion volume at a fluid percussion brain injury site in rats. More recently, in a model of three repeated mild lateral fluid percussion injuries, each separated by a 5-day interval, we have shown that intravenous anti-CD11d integrin treatment at 2 h and 24 h after each injury also reduced the neutrophil and macrophage presence in the injured brain with attendant decreases in lipid peroxidation, astrocyte activation, amyloid precursor protein accumulation, and neuronal loss (Shultz et al., 2013). Again, the anti-CD11d treatment also improved the outcome on cognitive tasks, sensorimotor ability, and anxiety. These positive findings led us to assess the effects of the anti-CD11d treatment on lung inflammation and oxidative injury in these models of acute moderate and repeated mild lateral fluid percussion TBI. The study was done using lung tissue from rats that had undergone assessment for brain inflammatory and oxidative responses, neuronal loss and behavioral deficits (Bao et al., 2012b; Shultz et al., 2013).

2. Materials and methods

2.1. Subjects

All procedures were in accordance with guidelines of the Canadian Council on Animal Care and approved by the University of Western Ontario Animal Use Subcommittee. Subjects were one hundred eighty-seven adult male Long-Evans hooded rats obtained from Charles River Laboratories (Quebec, Canada). Prior to surgery rats weighed between 250–300 g. After surgery rats were housed individually for the remainder of the study using a 12:12 light/dark cycle and were allowed access to food and water ad libitum. As described above,

in other studies (Bao et al., 2012b; Shultz et al., 2013) these same rats were also examined for brain inflammatory and injury responses as well as behavioral responses to TBI.

2.2. Treatment groups

Rats were randomly assigned to one of three treatment conditions: sham-injury with saline injection (sham), single moderate or repeated mild TBI and treatment with an isotype-matched control mAb designated as 1B7, or treatment with an anti-CD11d mAb (217 L). The mAb 217 and 1B7 were generously provided by the former ICOS Corporation (mAb currently owned by Eli Lilly & Co., Indianapolis, IN). Rats received their assigned treatment (saline, 1.0 mg/kg 1B7 or 1.0 mg/kg CD11d mAb 217L) either 2 h post-injury (single moderate TBI, 75 rats) or at 2 h and 24 h post-injury (repeated mild TBI, 92 rats) via tail vein injection. Accordingly, the rats undergoing the repeated mild TBI received a total of six doses of CD11d mAb whereas the rats that had a single injury received only one dose. In previous studies, this dosing regimen of CD11d mAb was highly effective in suppressing inflammation and oxidative damage in the brains of these same rats (Bao et al., 2012b; Shultz et al., 2013). Furthermore, a similar dosing regimen in spinal cord-injured rats reduces inflammation in their injured spinal cords as well as the SIRS response to cord injury (Bao et al., 2004, 2011; Gris et al., 2004). The response of the lung to TBI was assessed at 24 h and 72 h in the single injury group and at 72 h after the last injury in the repeated TBI group. Lungs were also examined in the chronic period post-injury, at 4 weeks after the single injury and at 8 weeks after the last treatment after the repeated injuries. Details of numbers of rats in these groups are in Table 1.

The lung samples in this study were obtained from rats that had been studied for effects of the anti-CD11d treatment on behavior and brain inflammatory responses after TBI (Bao et al., 2012b; Shultz et al., 2013). In those studies, saline was a suitable control treatment instead of the isotype-matched mAb because direct actions of the circulating mAb on the CNS were unlikely (Fleming et al., 2008; Gris et al., 2004; Oatway et al., 2005). However, the lungs of these rats were clearly exposed to the circulating antibody and, for that reason, an assessment of possible direct mAb effects on the lungs was necessary. Thus five additional control groups (n = 4 each, see Table 1) constituted a control study to examine possible direct effects of the circulating mAb on the lung that might initiate a SIRS response. Injections of the control 1B7 mAb were delivered as described above to uninjured rats, sham injured rats and rats injured with a single moderate TBI (see below). Comparisons also were made to untreated uninjured rats, and to untreated rats injured with the single moderate TBI. The most sensitive and reliable indicator of lung inflammation and the presence of a SIRS in our previous studies has been the activity of the oxidative enzyme myeloperoxidase (MPO) that is highly expressed by neutrophils and activated macrophages (Bao et al., 2011, 2012a; Gris et al., 2008), when measured between 12 and 24 h after a CNS injury. Therefore, in the control study, post-injury MPO analyses of the lungs were done at 24 h after the sham injury or TBI.

2.3. Surgery and injury

Rats were anesthetized with 4% isoflurane and, under aseptic conditions, underwent a circular window craniotomy centered with reference to Bregma at anterior/posterior −3.0

mm and medial/lateral 6.0 mm (Paxinos and Watson, 1986). A hollow plastic cap was sealed over the craniotomy and filled with sterile saline. The rat was then attached to the fluid percussion injury device [for details see Shultz et al. (Shultz et al., 2011)]. For the single moderate injury paradigm, rats in the anti-CD11d group or 1B7 control group received a single fluid percussion pulse of 2.5–3.0 atm (a moderate injury). Sham-injured rats were treated similarly but were removed from the FPI device without receiving fluid percussion. This fluid percussion force was based values used in previous rodent studies (Passineau et al., 2001; Pillay et al., 2007; Shojo and Kibayashi, 2006; Thompson et al., 2005). Rats received tail vein injections of the assigned treatments at 2 h post-injury. After treatment the rats were assigned to a 24 h, 72 h or 4 week recovery group.

For the repeated mild injury paradigm, rats in the CD11d and 1B7 groups were treated as above and received a single fluid percussion pulse with a force of 1.0–1.5 atm (a mild injury). Again, sham-injured rats were treated identically but were removed from the injury device without receiving the percussion pulse. Rats received tail vein injections of the assigned treatments 2 h and 24 h after each injury and then were given a 5-day inter-injury recovery period before undergoing the identical injury procedure as described above for the remaining two injuries. After their final drug injection, rats were assigned to either a 72 h or 8 week recovery group.

2.4. Tissue preparation for morphological examination and for Western blotting analysis

For morphological examination, sham-injured and TBI rats were anesthetized at their assigned time after injury and perfused transcardially into the aorta with cold saline, followed by 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2–7.4. At the same time the lungs were perfused separately via the pulmonary artery with saline and then paraformaldehyde. The left lung was removed, post-fixed for 24 h at 4 °C and cryoprotected in increasing concentrations of sucrose. A piece, approximately 0.5 cm thick, was sampled from the center of the lung and sectioned into 25 μm thick sections which were placed in buffer for immunohistochemical staining. The pieces of lung sampled from each rat were as identical as possible.

Approximately five lung sections sampled from each of four sham-injured rats and four single moderate TBI rats at 24 h after the injury were stained by hematoxylin and eosin. Likewise, lung sections from each of four sham-injured rats and four rats at 72 h after the third of three mild TBIs were stained by hematoxylin and eosin. The sections were viewed and photographed using an Olympus microscope and imaging system as described below.

For biochemical and Western blotting analyses, the various groups of rats were perfused only with cold 0.9% NaCl, via the aorta and pulmonary artery, as above. The left lungs were then removed and stored at −80 °C until they were used. For the assays, tissue samples were taken from the approximate center of the lobes of lung and each sample was then divided into three parts for different biochemical analyses. All homogenization steps were done with a glass homogenizer on ice. For Western blotting, lung (350 mg) samples were homogenized, centrifuged and the supernatant was used as described previously (Bao et al., 2004). For measurement of myeloperoxidase (MPO) activity, different samples from the lung (130 mg) were homogenized with the inclusion of hexadecyltrimethylammonium

bromide (to extract the MPO from the leukocyte granules), centrifuged and the supernatant was used for MPO assay as described previously (Bao et al., 2004). Thiobarbituric acid reactive substances (TBARS) were measured to assess lipid peroxidation and a 2′-7′-dichloroflorescin-diacetate (DCFH-DA) assay was used to detect free radicals. The lung samples (170 mg) were homogenized, centrifuged and the supernatant used for TBARS and DCFH-DA assays as described previously (Bao et al., 2004, 2005). The protein concentration of samples were determined using the modified Bradford method (Bio-Rad, Protein assay kit II; Bio-Rad, Hercules, CA) with bovine serum albumin as standard.

2.5. Assessing infiltration of neutrophils and phagocytic macrophages

Infiltration of neutrophils and phagocytic macrophages was detected by an MPO assay, by immunohistochemical staining of tissue sections and by Western blotting. For the MPO assay, the lung tissue homogenate was incubated in a 96-well plate in a developing buffer that consisted of 100 μl of K-PBS and 100 μl of o-dianosinisidine (12.5 mg per 10 ml distilled water and 9 μl of 30% H_2O_2). The reaction was stopped by addition of 100 μl of 1% NaH_3 in each well. The plate was scanned using a 96-well plate reader (Multiskan Ascent, Thermo Fisher Scientific, Waltham, MA) at a wavelength of 450 nm. For every plate, one standard curve in triplicate was performed using MPO from human leukocytes (Sigma Chemical Company, St. Louis, MO) (Bao et al., 2004).

For immunohistochemical staining, randomly selected sections from the pools of lung sections (generated as described above) were processed free-floating for staining as described previously (Bao et al., 2012a). A rabbit anti-rat neutrophil polyclonal antibody diluted 1:20,000 (generous gift of Dr. Daniel Anthony (Anthony et al., 1998), Oxford University, Oxford, UK) was used to identify neutrophil infiltration and a mouse monoclonal anti-rat ED-1 antibody (1:500, Serotec, Raleigh, NC; analogous to CD68 in humans) was used to identify phagocytic macrophages in the sections. Sections were next incubated overnight with biotinylated anti-rabbit antibody made in donkey (1:500 dilution; Jackson Laboratories, West Grove, PA). The immunoreactivity was visualized using diaminobenzidine [(DAB) Sigma Chemical Company, as above] as a chromogen. The slides were viewed using an Olympus microscope (BX50, Olympus America Inc., Center Valley, PA) and photomicrographs were acquired using a digital camera (Retiga, Quantitative Imaging Corporation, Burnaby, BC, Canada) and Image Pro software version 5.1 (Media Cybernetics, Silver Spring, MD). The sections were examined only qualitatively to confirm the density of inflammatory cells in the lungs in each treatment group. Quantitative analyses of the neutrophils and macrophages were done by Western blotting (details below).

The anti-neutrophil antibody was used for Western blotting of proteins in the lung homogenates, to quantify the neutrophil presence. This experiment was successful, yielding a particularly strong band at 56 kDa with about five more faint bands at lower molecular weights. Because of the consistency and clarity of the 56 kDa band, we analyzed this band quantitatively, considering it a good example of neutrophil protein expression. Macrophage ED-1 protein expression in the lung was quantified by Western blot analysis (ED-1 antibody as above). Details of the Western blotting can be found in Bao et al. (Bao et al., 2012a). All Western blots were also stained for β-actin. This β-actin staining was done to assure equal

protein loading of the gels; the similar bands from the sham-injured, 1B7 control-treated TBI and anti-CD11d-treated TBI samples consistently demonstrated equal protein loading. Quantification of the blots was done by densitometric analysis of the bands of interest as well as the β-actin bands. Quantitative data presented in the figures and tables are expressed as the ratios of the optical density (per mm^2) of the bands of interest to the optical density of the β-actin bands (per mm^2). Accordingly, all quantification of Western blot data in figures and tables are expressed as ratios, normalized to β-actin.

2.6. Assessing oxidative enzymes

Oxidative enzymes in lung were detected by Western blotting for the catalytic subunit ($gp91^{phox}$) of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase with a mouse anti-rat $gp91^{phox}$ antibody (1:500, Upstate Biotechnology, Lake Placid, NY) using methods described previously (Bao et al., 2004). Two other oxidative enzymes, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were also detected by Western blot analysis with polyclonal rabbit anti-iNOS (1:500, Oxford Biomedical Research, Oxford, MI) and polyclonal rabbit anti-COX-2 (1:500, Cayman Chemical, Ann Arbor, MI) antibodies using the methods described above.

2.7. Assessing free radical production (DCFH-DA assay)

To assess free radical production in the lung, we used 2′-7′-dichlorofluorescin diacetate (DCFH-DA) as a probe for free radical detection (Bao et al., 2005). DCFH-DA is hydrolyzed to DCFH that then is oxidized by reactive oxygen species to form the fluorescent compound, 2′-7′-dichlorofluorescein (DCF). An aliquot (25 μl) of the lung homogenate that was used for MPO assays was incubated with 0.1 mM DCFH-DA at 37 °C for 30 min. The formation of the oxidized fluorescent derivative DCF was monitored using a fluorescence spectrophotometer as described previously (Bao et al., 2005).

2.8. Assessing lipid peroxidation and expression levels of Nrf2 and caspase-3

Malondialdehyde (MDA), a marker for lipid peroxidation, can be associated with cell membrane damage (Ohkawa et al., 1979). MDA and other aldehydes were quantified in the homogenates of the lung as described previously (Bao et al., 2004), using a thiobarbituric acid reactive substances (TBARS) assay (Ohkawa et al., 1979). A standard curve was established using MDA bis(dimethyl acetal) (Sigma-Aldrich), and lipid peroxidation was expressed as nmol of TBARS/g tissue. Lipid peroxidation in lung was also detected by the presence of 4-hydroxynonenal (HNE)-bound proteins by Western blots, using a mouse anti-HNE monoclonal antibody (1:5000, Alpha Diagnostic International, San Antonio, TX) and 10% polyacrylamide gels. To assess the cellular response to the oxidative stress in the lung, we used Western blotting to examine expression of the transcription factor Nrf2 that recognizes an antioxidant response element found in the promoters of cytoprotective genes that are upregulated by oxidative or chemical stress signals (Lee and Johnson, 2004). To detect Nrf2 we used an antibody from Santa Cruz Biotechnology, Santa Cruz, CA (diluted 1:1000). Finally, to assess activity in the apoptotic cell death pathway in the lung, we examined the expression of the pro-apoptotic protein caspase-3 by Western blotting (1:500, anti-caspase-3, Upstate, Lake Placid, NY). We quantified the full 32 kDa pro-caspase-3 as our goal was to identify initial changes in gene expression in this apoptotic pathway.

2.9. Statistical analyses

Mean values are expressed ±standard error (S.E.). Results were subjected to parametric statistical analysis using one-way analysis of variance (ANOVA) (Snedecor and Cochran, 1989). These analyses included data from the sham-injured rats. Although some of the data appear to be suitable for a two-way ANOVA, the sham-injured group did not have two levels of treatment and, because comparisons between the sham-injured, 1B7 control TBI and anti-CD11d TBI groups were essential, it was necessary to use a one-way ANOVA. Differences between means were determined by the post hoc Student Neuman Keuls test if the one-way ANOVA revealed significant differences within the groups. Significance was accepted at $P \leq 0.05$. The control studies designed to test possible direct effects of the mAb treatment on the lung were analyzed by the Student Neuman Keuls test and also by a Holm Sidak test for multiple comparisons (Glantz, 2012). The power of the performed tests always approximated or exceeded 0.80 when statistically significant differences were detected. Details of the ANOVA for the variables measured are provided in Table 2 and Supplemental Table 1. Probability values presented in the text refer to the Student Neuman Keuls test unless indicated otherwise.

3. Results

3.1. Fluid percussion traumatic brain injury leads to lung damage

Lung sections from rats at 24 h after the single sham or 72 h after the third of three sham injuries had normal histological appearance. Alveoli and alveolar sacs were well defined in a lacework-like pattern (Fig. 1A,C) with clear alveolar septa composed of epithelial cells with darkly stained round nuclei. In the illustrated fields are also areas of more dense epithelial cells and connective tissue that are part of the normal lung parenchyma. The normal resident intra- and peri-alveolar macrophages are present in the sham-injury samples shown in panels A and C.

At 24 h after the moderate TBI, regions were found in the lung sections that had disrupted cytoarchitecture (Fig. 1B). The lacework-like pattern was lost due to thickening of the peri-alveolar tissue, apparent alveolar wall damage and increased density of peri-alveolar macrophages. In some areas, clusters of clearly defined erythrocytes were present indicating frank hemorrhage into the lung parenchyma. Other regions of the lung sections from the moderate TBI rats displayed normal cytoarchitecture. At 72 h after the third of three mild TBIs, the lungs generally appeared more compact, with thickened alveolar septa and an abundant distribution of peri-alveolar macrophages (Fig. 1D). Occasionally little clusters of erythrocytes were noted, indicating that small hemorrhages also occurred in the lungs after the repeated mild TBIs. Some regions of these lung sections also had normal cytoarchitecture.

3.2. Anti-CD11d treatment reduces lung neutrophils and macrophages at 24 and 72 h after TBI

A single moderate TBI caused an influx of neutrophils into the lung at 24 h and 72 h after the injury (Fig. 2A–C). MPO activity in lung homogenates, generated largely by neutrophil activity, increased significantly from that in sham-injured rats after the single TBI (Fig. 2A

left and middle panels). Details of the ANOVA for all figures are in Supplemental Table 1. MPO activity increased by 200% in the lungs of 1B7 controls at 24 h ($P = 0.001$) and by ~130% at 72 h after the injury ($P = 0.017$). These increases were significantly attenuated by anti-CD11d treatment (24 h $P = 0.027$; 72 h $P = 0.049$, respectively). At 72 h after the completion of three repeated mild TBI, MPO activity changed significantly (Fig. 2A, right panel), increasing significantly by ~175% ($P = 0.006$) in the 1B7 controls compared to that in sham-injured rats. Although the values in the anti-CD11d-treated group were not different from the sham-injured group, they only tended to be different from those of the 1B7 group ($P = 0.089$). A portion of the increase in MPO activity in all of the aforementioned groups may also be attributed to activation of phagocytic macrophages.

The effects of the anti-CD11d treatment on the neutrophil infiltrate in the lung after TBI were also studied by Western blotting for a 56 kDa neutrophil protein. At 24 h after the moderate single TBI, the 56 kDa neutrophil protein expression in the 1B7 controls increased by more than 500% when compared to values in the sham-injured rats (Fig. 2B left panel, $P < 0.001$). The anti-CD11d treatment reduced this 56 kDa protein expression significantly, by almost half ($P = 0.002$). At 72 h after the moderate single injury, the 56 kDa protein expression in the 1B7 controls increased by ~200% compared to the sham-injured group (Fig. 2B middle panel, $P < 0.001$) and the anti-CD11d treatment reduced this expression by more than one-third ($P = 0.003$). At 72 h after the third mild TBI, the 56 kDa protein was significantly increased (Fig. 2B, right panel) with ~200% greater expression in the 1B7 controls than in the sham-injured rats ($P = 0.002$). Treatment with the CD11d mAb reduced resulted in almost one-third reduction in the neutrophil protein levels, yielding a tendency for statistical significance between the 1B7 control and anti-Cd11d-treated groups ($P = 0.075$).

Photomicrographs of lung tissue sections stained immunocytochemically for the 56 kDa neutrophil protein are shown in Fig. 2C to illustrate the cellular infiltrate qualitatively. The upper panels illustrate sections from a rat at 24 h after a moderate single injury and the lower panels show sections from a rat at 72 h after three mild injuries. In the sham-injured rats, a few neutrophils stained with the anti-neutrophil antibody were present in the lung (Fig. 2C, left panels). The inset in the upper sham-injury panel shows the morphology of a cell with a multi-lobed nucleus typical of neutrophils. After the single injury or repeated injuries, an infiltrate of neutrophils appeared within the alveoli and extravascular tissue in the 1B7 controls (Fig. 2C middle panels). In contrast, the rats treated with the anti-CD11d mAb had visibly fewer neutrophils within the lung (right panels).

Expression of ED-1, a marker primarily associated with macrophages, in lung homogenates increased significantly by ~150% in control 1B7 rats compared to sham-injured rats at 24 h after a single moderate TBI (Fig. 3A left panel, $P < 0.001$). The anti-CD11d treatment reduced this expression significantly (by one-third, $P = 0.001$). At 72 h after this moderate TBI, ED-1 expression was almost 300% greater in the 1B7 controls than in the sham-injured rats (Fig. 3A, middle panel, $P < 0.001$). The anti-CD11d treatment reduced the ED-1 expression by one-third ($P = 0.002$). At 72 h after the third of three mild TBIs, ED-1 expression was significantly changed Fig. 3 (A, right panel) with 260% greater expression in

the 1B7 controls than in the sham-injured rats ($P < 0.001$). In the anti-CD11d treatment group, ED-1 expression was about one-third less than in the 1B7 control group ($P < 0.01$).

The lung of a sham-injured rat contained a normal population of resident ED-1-immunoreactive macrophages within the tissue parenchyma surrounding the alveoli as illustrated in Fig. 3B (left panels). An example of a large irregular macrophage is shown in the inset of the upper sham-injury panel. The density of these macrophages was greatly increased in examples taken from 1B7 control rats at 24 h after the single moderate TBI (upper panel) and at 72 h after the third of the three repeated mild TBIs (lower panel). The macrophages were large and abundantly distributed throughout the peri-alveolar tissue. Consistent with the Western blots, after anti-CD11d treatment, the macrophage numbers in the lungs appeared to be less than in the control 1B7 rats (Fig. 3B right panels) in both the single moderate or repeated mild injury groups.

3.3. Treatment with the monoclonal antibody causes no direct inflammatory response within the lung

We examined inflammatory responses in the lung to the control 1B7 mAb, delivered at 2 h after a single moderate TBI or sham-injury. Lung MPO activity was assessed at 24 h after these injuries and in uninjured rats. We compared the following groups of rats: uninjured untreated, uninjured 1B7-treated, sham-injured saline-treated (from Fig. 2), sham-injured 1B7-treated, TBI untreated, TBI 1B7-treated (Fig. 4). One way ANOVA revealed significant differences among these groups [$P < 0.0001$, $F(5,20) = 14.88$]. The differences and similarities can be expressed as follows: TBI untreated = TBI 1B7-treated ($P = 0.894$) ≠ all uninjured and sham-injury groups ($P < 0.0001$). Furthermore, untreated uninjured = 1B7-treated uninjured ($P = 0.894$); saline-treated sham-injured = 1B7-treated sham-injured ($P = 0.894$). The lack of MPO response to 1B7 mAb in the uninjured, sham-injured and TBI groups and the clear MPO response of the untreated TBI rats demonstrated that the SIRS response observed in the lungs in this study was not related to the mAb treatment.

3.4. Anti-CD11d treatment reduces expression of oxidative enzymes and the concentration of free radicals in lung at 24 h and 72 h after TBI

Having ruled out direct mAb treatment effects on the lungs, further studies of the SIRS response and treatment effects of the CD11d mAb on the lungs were undertaken. Enzymes associated with oxidative injury to the brain were evaluated in the lung homogenates using Western blotting. Expression of the oxidative enzyme $gp91^{phox}$ (the catalytic subunit of NADPH oxidase) was significantly increased by 270% in the lung homogenates of 1B7 controls compared to the sham-injured rats at 24 h after a single moderate TBI (Fig. 5A, left panel, $P < 0.001$). Treatment with the anti-CD11d mAb reduced the $gp91^{phox}$ expression by one-third ($P = 0.009$). At 72 h after the single moderate TBI, $gp91^{phox}$ expression continued to remain significantly changed (Fig. 5A middle panel), with an almost 200% increase in the 1B7 control group compared to the sham-injured group ($P < 0.001$). The anti-CD11d treatment decreased this expression by more than one-third ($P = 0.002$). Three repetitive mild TBI also increased the expression of $gp91^{phox}$ at 72 h after the third injury (Fig. 5A, right panel). Expression of this oxidative enzyme was ~200% greater in the lungs of 1B7

controls than in the sham-injured rats ($P < 0.001$) and anti-CD11d treatment decreased it by one-third ($P = 0.015$).

Expression of the pro-inflammatory oxidative enzyme iNOS also changed significantly in lung homogenates at 24 h after a single moderate TBI (Fig. 5B, left panel), increasing by 450% in the 1B7 controls compared to the sham-injury group ($P < 0.001$). The anti-CD11d treatment reduced iNOS expression by almost half ($P = 0.008$). At 72 h after the single moderate TBI, iNOS expression in the 1B7 controls was still 260% greater than in the sham-injured group (Fig. 5B, middle panel, $P < 0.001$). Treatment with the CD11d mAb reduced this expression by almost one-third ($P = 0.048$). iNOS expression also increased significantly at 72 h after the third of three repetitive mild TBI (Fig. 5B, right panel); expression in the 1B7 controls was ~300% greater than in the shams ($P < 0.001$). Anti-CD11d treatment reduced the iNOS expression by more than one-third ($P = 0.005$).

COX-2 expression in the lung homogenates also increased, following the patterns of change of $gp91^{phox}$ and iNOS. Significant increases of 360% ($P < 0.001$) occurred at 24 h and of 220% ($P < 0.001$) at 72 h after the single moderate TBI and of 300% ($P < 0.001$) after the repeated mild TBIs (Supplemental Fig. 1A). After treatment with the CD11d antibody these responses were significantly smaller than in 1B7 controls, by more than one-quarter to almost one-half ($P = 0.049$; $P = 0.004$ and $P = 0.007$, respectively).

Free radicals in the lungs produced by oxidative activity were assessed by a quantitative DCF assay in the lung homogenates. Free radicals increased by 57% ($P < 0.001$) and 38% ($P < 0.001$) at 24 h and 72 h, respectively, after the single moderate TBI, and by 27% ($P = 0.013$) after the third of the repeated mild TBIs (Supplemental Fig. 1B). Anti-CD11d treatment caused small but significant decreases in these responses (by one-quarter to one-fifth; $P < 0.001$, $P = 0.005$ and $P = 0.040$, respectively).

3.5. Anti-CD11d treatment reduces lipid peroxidation, cytoprotective gene expression and apoptotic cell death in lung at 24 and 72 h after TBI

Lung damage resulting from oxidative activity and the presence of reactive oxygen species was evaluated by examining lipid peroxidation of cell membranes, levels of the oxidative stress responsive transcription factor Nrf2 and expression of the pro-apoptotic enzyme caspase-3. Lipid peroxidation was first estimated by assaying relative levels of malondialdehyde (MDA) and other aldehydes by the TBARS assay. This TBARS product of lipid peroxidation increased by 130% at 24 h after the single moderate TBI (Fig. 6A left panel, $P < 0.001$). After anti-CD11d treatment the TBARS value was reduced by almost half from that in the 1B7 controls, a significant reduction ($P = 0.001$) and the post-treatment TBARS was not different from that in sham-injured rats. At 72 h after the single injury, TBARS remained increased (Fig. 6A middle panel), with values in the 1B7 controls 80% greater than in the sham-injured group ($P = 0.004$). After anti-CD11d treatment, the TBARS concentration was reduced by one-third ($P = 0.011$) to values not different from those in sham-injured rats. At 72 h after the third of three mild repeated TBIs, the TBARS estimate of lipid peroxidation was 56% greater in the 1B7 group than in the sham injury group (Fig. 6A right panel, $P = 0.018$). After anti-CD11d treatment, TBARS at 72 h were reduced by

one-quarter compared to the 1B7 controls ($P = 0.050$) and were not different from those in the sham-injured rats.

Western blotting for the presence of hydroxynonenol (HNE)-bound proteins also revealed increases in lipid peroxidation at 24 h after the single moderate TBI (Fig. 6B left panel). Lung HNE in 1B7 controls increased significantly at 24 h by ~200% compared to sham-injured rats ($P < 0.001$) and anti-CD11d treatment reduced HNE by one-quarter ($P = 0.030$) compared to 1B7 controls. At 72 h after the single injury, HNE remained significantly changed (Fig. 6B middle panel). Lung HNE values in the 1B7 control rats were 260% greater than in the sham-injury group ($P < 0.001$). Treatment with the anti-CD11d mAb reduced HNE by one-quarter compared to that in the 1B7 group ($P = 0.050$). At 72 h after the three mild repeated TBIs, lipid peroxidation assessed by HNE was 160% greater in 1B7 controls than in sham-injured rats (Fig. 6B right panel, $P = 0.001$). Anti-CD11d treatment caused a one-third reduction in HNE compared to the 1B7 controls ($P = 0.033$).

The cellular response to the oxidative stress in the lung was evaluated by examining expression of the transcription factor Nrf2, a protein critical to the up-regulation of cytoprotective genes during oxidative stress (Marzec et al., 2007; Tsitsopoulos and Marklund, 2013). At 24 h after the single moderate TBI, expression of Nrf2 increased by almost 200% in the 1B7 controls compared to that in the sham-injured rats (Fig. 7A, left panel, $P = 0.001$). Treatment with the anti-CD11d mAb decreased Nrf2 expression by more than one-third ($P = 0.003$) compared to 1B7 controls. At 72 h after this single injury, Nrf2 expression was still increased by ~200% in the 1B7 group (Fig. 7A, middle panel, $P = 0.001$). After the anti-CD11d treatment, this expression was more than one-third lower than in the 1B7 controls ($P = 0.006$). Nrf2 expression was also increased at 72 h after the third of three mild repeated TBIs (Fig. 7A, right panel); expression in the 1B7 controls was 250% greater than in the sham-injured group ($P = 0.001$). Values in the anti-CD11d-treated group were one-third less than in the 1B7 controls ($P = 0.031$).

Expression of caspase-3 was examined as a critical protein in the apoptotic cell death pathway. Quantification of expression of the full 32 kDa caspase-3 molecule by Western blotting revealed very limited expression of this enzyme in lungs of sham-injured rats but significant 300% increases in expression were detected in the 1B7 controls at 24 h after a single moderate TBI (Fig. 7B, left panel, $P < 0.001$). After anti-CD11d treatment caspase-3 expression was reduced by one-third ($P = 0.024$). At 72 h after moderate TBI, caspase-3 expression was still increased by 240% in 1B7 controls compared to that in sham-injured rats (Fig. 7B, middle panel, $P < 0.001$). Treatment with the anti-CD11d mAb caused a one-third reduction in expression compared to the 1B7 controls at 72 h ($P = 0.013$). Caspase-3 expression also was altered at 72 h after the three repeated mild TBI (Fig. 7B, right panel) as it increased by 150% in the 1B7 controls ($P < 0.001$). The anti-CD11d treatment diminished this response, with values in the treated group reduced by one-quarter compared to the 1B7 controls ($P = 0.038$).

3.6. Inflammatory and cellular responses: effects of anti-CD11d treatment at 4 and 8 weeks after TBI

By 4 weeks after the single moderate TBI or 8 weeks after the three repeated mild TBI, the SIRS response in the lungs was markedly reduced compared to that in the 24–72 h period (Table 2 see below). For example, at the later times the increases in MPO activity and in neutrophil and macrophage protein expression in the 1B7-treated animals compared to sham-injured rats ranged from 40% to ~115% in contrast to the 130% to ~500% increases observed in the acute period. In these more chronic periods, changes in oxidative enzymes, estimates of free radicals and lipid peroxidation, and expression of the cytoprotective transcription factor Nrf2 were also small compared to those in the acute periods. One exception was the 160% increase in expression of the pro-apoptotic enzyme caspase-3 at 4 weeks after the single moderate injury (compared to 150% and ~300% increases at 24 and 72 h).

If only the values in the sham-injured rats and those in the 1B7 controls were compared by a one-way ANOVA, seven of the small changes in the 4 week and 8 week groups would be statistically significant and eight had P values less than 0.07. These responses demonstrate a small residual SIRS within the lungs at these times.

When the small differences between all three groups listed in Table 2 were compared by the one-way ANOVA, no significant group effects were detected. One exception to this was the significant P value for the neutrophil protein Western at 8 weeks that clearly related to the difference between shams and 1B7 controls. Thus, no significant effect of anti-CD11d treatment was detected by 4 or 8 weeks after TBI. However, comparing the three columns of Table 2, the sham injury values were always the lowest, the 1B7 controls were the highest and the anti-CD11d values were always intermediate between the other two, sometimes approaching the value in the sham group (for example, see TBARS at 4 weeks after injury). In half of the cases P values for the ANOVA were between 0.078 and 0.099, showing a tendency for differences within these groups in many of the measures examined. Subjecting the rank order of the values in the three columns of Table 2 (1B7 > CD11d > sham) to a nonparametric Kruskal–Wallis one-way ANOVA, the groups differed statistically with $P < 0.001$.

4. Discussion

Acute lung injury occurs frequently after TBI and is a serious complication. In a twenty-year study of in-hospital mortality after TBI, acute respiratory distress and acute lung injury secondary to TBI significantly increased the risk of in-hospital death (Rincon et al., 2012). In accord with this study, respiratory dysfunction as a complication of TBI was reported to account for 34% of the deaths after severe TBI (Kemp et al., 2008). Studies in rats revealed that acute increased intracranial pressure associated with TBI correlates positively with increased risks for the development of lung injury and the acute respiratory distress syndrome (Lou et al., 2013). In a rat model of mild TBI, significant lung injury was evidenced by pulmonary edema, plasma protein leakage into the alveolar compartment, and increased concentrations of interleukin-1β and interleukin-6 in bronchoalveolar lavage fluid (Vermeij et al., 2013).

The purpose of this investigation was to examine a peripheral consequence of TBI that is not highly recognized, namely, the SIRS that accompanies it. We evaluated the impact of the SIRS on the lungs of rats that had experienced either a single moderate fluid percussion TBI or three repeated mild fluid percussion injuries. The analysis was done using rats that were undergoing assessment for brain inflammatory and oxidative responses, neuronal loss and behavioral deficits (Bao et al., 2012b; Shultz et al., 2013). The study of the SIRS in these lungs revealed cytoarchitectural disruption and intrapulmonary hemorrhage, an infiltrate of neutrophils, the presence of activated macrophages, up-regulation of oxidative enzymes, of a cytoprotective transcription factor Nrf2, of an enzyme in the apoptotic cell death pathway and finally the presence of free radicals and lipid peroxidation after both of these paradigms of brain injury, at the same time as these processes were occurring in the injured brain. Moreover, the lung responses were ongoing at the time that the animals were displaying deficits in spatial cognition and motor function as well as heightened anxiety. The acute anti-CD11d treatment significantly decreased the lung responses to TBI which are an important component of the SIRS.

4.1. Evidence for SIRS after traumatic brain injury

Brain injury leads to the very rapid production within the brain of the pro-inflammatory chemokines CINC-1 and CCL-2(Campbell et al., 2003, 2005) and cytokines such as interleukin (IL)-β1 and tumor necrosis factor α (Fan et al., 1995; Feuerstein et al., 1994; Galea and Brough, 2013). The CINC-1 and CCL-2 chemokines appear in the circulation within 2 h, and trigger the liver (Koj, 1996; Kushner, 1993) to produce acute phase proteins such as the complement protein C3, C-reactive protein and more CINC-1 and CCL-2 chemokines (Campbell et al., 2003, 2005; Wilcockson et al., 2002). These proteins and chemokines can attract leukocytes from the circulation and marginal pools into the liver, amplifying this acute phase reaction. The leukocytes, activated in the liver, can return to the circulation to enter the parenchyma of other organs including the brain, hence augmenting the inflammatory response at the site of brain injury. Entry of leukocytes into organs such as the lungs, with consequent inflammatory reactions within those organs, constitutes the SIRS.

Some details of the lung response to TBI have been studied. At 24 h after TBI in rats, the influx of neutrophils and monocyte/macrophages into the lungs is associated with lung production of the leukotriene (LT)-B4, a chemoattractant and significant activator of granulocytes, and a source of aggravation of the intrapulmonary inflammatory response (Kalsotra et al., 2007). As in our study, these investigators also noted significant increases in the prostaglandin-synthesizing enzyme COX-2 at 24 h after the injury. Moreover, IL-1β and IL-6 levels in the lung were markedly increased whereas those in the serum were only slightly changed or not changed at all at 24 h after injury, demonstrating amplification of the inflammatory response within the lung. This pattern is consistent with the fact that the initial triggers of the SIRS within the circulation appear within hours of the TBI (reviewed above) and probably abate by 24 h. In this model, the decline of LT-B4 levels at 2 weeks after the injury was associated with an increasing lung concentration of cytochrome p450, a molecule that catalyzes the inactivation of leukotriene B4. The lung inflammation in this study of inflammatory mediators was diminishing within weeks of the TBI, similar to the time course that we observed.

4.2. The potential role of Nrf2 after TBI

The transcription factor Nrf2 is considered to be essential in mediating protection of the lung from oxidative stress (Lee and Johnson, 2004; Yan et al., 2008). In the lung, Nrf2 is found primarily in the tracheobronchial epithelium and in alveolar macrophages where it translocates to the cell nucleus in response to inflammation or oxidative stress and binds the antioxidant responsive element, initiating gene expression of cytoprotective proteins (Cho et al., 2004; Dinkova-Kostova et al., 2005). Nrf2-regulated gene products such as heme oxygenase-1 and NAD(P)H:quinone oxidoreductase-1 protect cells from inflammatory or oxidative damage (Chan and Kan, 1999; Mochizuki et al., 2005). Nrf2 expression has been considered to be a susceptibility gene that reflects risk for development of acute lung injury in humans as increased expression implies the need for cytoprotection (Marzec et al., 2007). For these reasons we examined the expression of this transcription factor and found it significantly increased at the acute time points after the single moderate or repeated mild TBIs. Expression tended to remain increased in the more chronic period only after the repeated mild injuries. The reduction of Nrf2 expression after the anti-CD11d treatment was likely secondary to the lowered inflammatory activation and oxidative stress because the treatment reduced the influx of pro-inflammatory leukocytes. The reduced expression of the full caspase-3 as a consequence of this treatment would also diminish progression toward apoptotic cell death in the lung.

4.3. Sustained SIRS in the chronic weeks after TBI?

The inflammatory and oxidative responses in the lungs at 4 and 8 weeks after the single and repeated injuries, respectively, were only a fraction of those in the acute period (compare values in Table 2 to those of Figs. 2–7). Despite the small responses, values in some of the 1B7 isotype control groups were significantly greater than those in the sham-injured rats and, in other groups, a tendency for increases was noted. These results suggested the presence of sustained changes in the lung at 4 and 8 weeks after TBI. However, when the ANOVA included the anti-CD11d data, differences between the three groups were small and inconsistent, failing to reveal significant anti-CD11d treatment effects. Again, tendencies were apparent, suggesting that a small residual or secondary response persists in the lungs at chronic times after the TBI and that the response is less evident in the anti-CD11d treated group. The acute, short-lasting blockade of intra-pulmonary inflammatory cell influx and activation may have sustained effects to limit the more chronic lung reactions to the TBI. This interpretation is supported by data from our previous study in spinal cord-injured rats revealing that the sustained presence of macrophages in the lesion site at 3 weeks after cord injury was significantly reduced by an intravenous treatment with the CD11d mAb completed at 48 h after the injury (Gris et al., 2009).

In contrast to the small sustained inflammatory response in the lungs after TBI, a robust inflammatory response occurs in the injured brain in which the density of macrophages remains greatly increased in the chronic period and lipid peroxidation and cell death continues after the acute period (Bao et al., 2012b; Shultz et al., 2013). Some of these responses were reduced by acute treatment with the CD11d mAb (like the reductions observed after spinal cord injury described above). The later inflammatory response within the injured brain may relate to chronic activation of microglia and their production of pro-

inflammatory molecules and generation of oxidative damage. Axonal injury and proteinopathies are also common secondary injury mechanisms that occur in the brain after TBI and that may contribute to sustained neuroinflammation (Tsitsopoulos and Marklund, 2013). The absence of such processes in the lung may be advantageous to its recovery from a SIRS.

4.4. Comparison of brain and lung responses in the same rats after TBI

Some of the findings obtained from the brains and lungs in our studies of this cohort of rats were comparable (Bao et al., 2012b; Shultz et al., 2013). At 24 h after the single moderate TBI, the neutrophil influx into the lungs and brain was similar (~500–600%) as was the increased expression of the macrophage marker ED-1 (~150–250%). At this acute time point, the degree of lipid peroxidation in the lung (130% increase) was greater than in the brain (60% increase), despite a smaller increase in MPO activity in the lung (200% increase) than in the brain (700% increase). The changes in MPO activity were similar to those of the other oxidative enzymes that we measured and appear to be a good indicator of the oxidative stress. Despite similar inflammatory responses of the brain and lung to the TBI, the impact of this inflammation on the generation of oxidative stress appeared to differ between the two organs. At 72 h after the three repeated mild fluid percussion brain injuries, neutrophil influx and macrophage presence were within the same range in the brain and in lungs (150–250% increases), and lipid peroxidation in the two organs was similar (~60% increases). In contrast, MPO in the brain was again substantially more activated (~500% increase) compared to that in the lung (175% increase). In summary, these comparisons reveal that injury to the brain has a substantial acute effect on at least one other vulnerable organ of the body, making lung damage part of the secondary complications during the recovery period after the injury. Moreover, the inflammation and injury in the lung might enhance feedback to the brain, exacerbating the injury and contributing to the behavioral issues.

4.5. Anti-CD11d integrin treatment limits the SIRS after TBI or spinal cord injury

The intensity of the inflammatory response in the lung and resultant lung oxidative activity and membrane damage at 24 to 72 h after the single TBI and after the third of the repeated injuries was substantially reduced by intravenous treatment of the rats with the integrin-blocking anti-CD11d mAb. By interfering with the mechanism by which the β2 integrin CD11d/CD18 facilitates diapedesis of neutrophils and monocyte/macrophages through the vascular wall, the CD11d mAb treatment permits fewer leukocytes to enter the lung parenchyma during a SIRS. This contention is consistent with earlier published results demonstrating that an antibody to CD11d blocked leukocyte infiltration into the lungs of rats that had lung injury induced with IgG immune complexes (Shanley et al., 1998).

In our study of SIRS after TBI, the anti-CD11d treatment also decreased the inflammatory response, oxidative injury and cell death in the brain of these same rats, while improving behavioral outcomes (Bao et al., 2012b; Shultz et al., 2013). Comparing the effect of treatment on the lung and brain in the acute period of injury, all parameters of lung or brain inflammation, oxidative activity and tissue injury were reduced by 30–40%. A small difference was a slightly more robust effect of the anti-CD11d treatment in the brain to decrease the inflammatory infiltration by 40–55% after the single moderate injury. As the

CD11d antibody concentrations in the circulation would not have persisted more than a few days, any effects of this acute treatment in the chronic period of recovery must have been due to indirect effects on the inflammatory and oxidative responses in lungs and brains.

Spinal cord injury also instigates a robust SIRS that significantly impacts the lungs (Bao et al., 2011, 2012a; Gris et al., 2008). In studies of rats after mid-thoracic spinal cord injury, anti-integrin treatments also significantly decreased the impact of the injury on the SIRS. We have tested effects on the lung of blocking the CD11d/CD18 integrin and also of blocking the α4β1 integrin. Comparisons of our own data reveal that the intensity of the lung inflammatory and oxidative responses after spinal cord injury are similar to those after TBI, considering the constraints of comparing data from different studies. Lung responses can be detected readily within 12 h of a spinal cord injury and are sustained for at least 7 days (Bao et al., 2011; Gris et al., 2008). The anti-integrin treatments blocked the influx of leukocytes and ensuing oxidative injury in the lung after cord injury by 30–50%, similar to the efficacy after TBI. These comparisons demonstrate that an anti-integrin treatment that blocks the influx of neutrophils and monocytes into the lungs after CNS trauma, whether the injury is sustained in the brain or spinal cord, leads to reduced impact of an ensuing SIRS on the lungs.

5. Conclusion

The lung component of SIRS after TBI clearly can have a devastating impact on morbidity and survival. Moreover, the lung and brain appear to have strong communication regarding their respective conditions of inflammation and injury. ‘Dangerous lung–brain crosstalk’ has been - associated with neurological dysfunction following primary injury to the lung (Pelosi and Rocco, 2011). In the case of TBI, feedback from the inflamed lung may exacerbate the ongoing inflammation in the brain, in part by release of pro-inflammatory molecules that enter the injury site. For this reason, a treatment like the CD11d mAb, that limits SIRS and the involvement of the lung in this response, may also indirectly limit the injury to the brain caused by a traumatic event, leading to a positive impact on morbidity and even mortality after TBI. We have revealed details of the lung inflammatory and oxidative responses in two models of fluid percussion TBI that are highly relevant to the human injury. Lung injury as a complication of concussion is a documented and serious problem. The reduction of lung inflammation and oxidative activity by a simple intravenous treatment with the anti-integrin antibody is a straightforward solution to this problem and our data show that the treatment is consistently efficacious in both TBI models. This treatment that reduces the brain injury also has an excellent impact on the serious complications of that injury on the lung.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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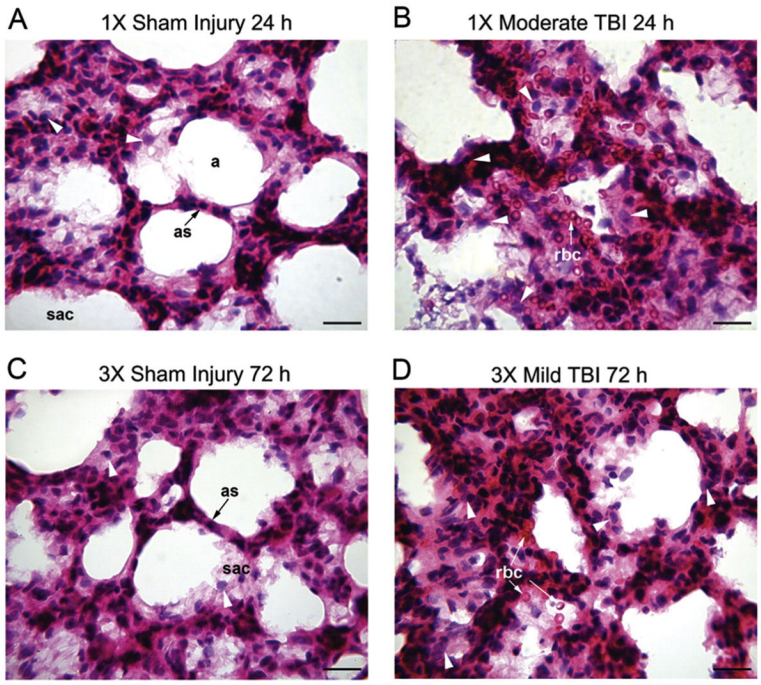

Fig. 1.

Moderate or mild TBI leads to disruption of normal lung structure. Photomicrographs (60×) illustrate 25 μm thick sections of hematoxylin and eosin-stained lung sampled 24 h after a sham or a single moderate TBI (A, B) and 72 h after the third of three sham or mild TBIs (C, D). A) Normal alveoli (a) and alveolar sacs (sac) are shown bordered by alveolar septa (as) and inter-alveolar epithelial and connective tissue. Alveolar and peri-alveolar epithelial cells have dark round nuclei and pink cytoplasm. Peri-alveolar macrophages (examples indicated by white arrowheads) are large cells with blue cytoplasm and dark blue nuclei. B) At 24 h after a single moderate TBI, the peri-alveolar tissue is disrupted, containing many erythrocytes (examples indicated by white rbc with arrows) demonstrating intrapulmonary hemorrhage, and a large number of macrophages. C) Normal lung structure at 72 h after the third sham TBI. D) At 72 h after the third mild TBI, the peri-alveolar tissue is compacted, alveolar walls appear disrupted, alveolar septa are thickened, small areas of hemorrhage are present and the density of peri- and intra-alveolar macrophages is greater than in the examples from the lung after sham injury. Scale bars = 20 μm.

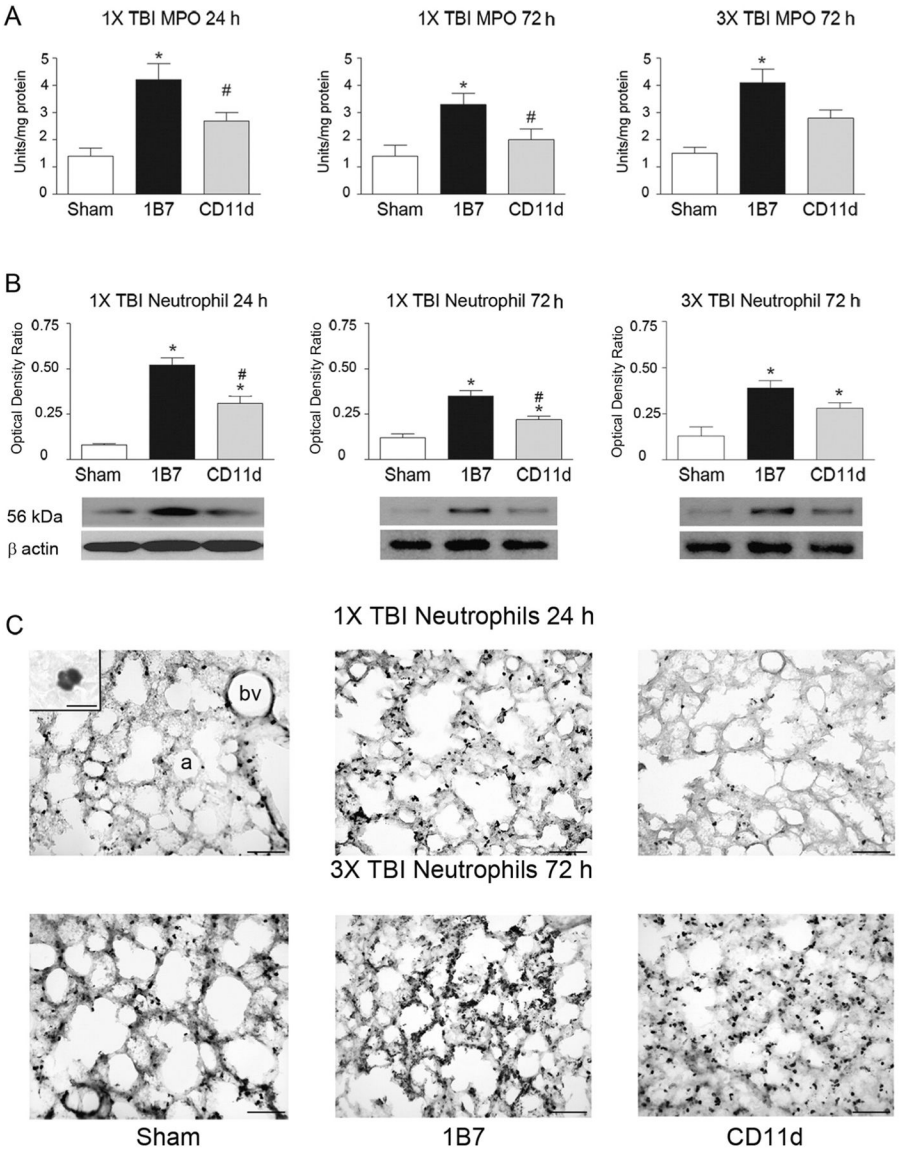

Fig. 2.

The anti-CD11d treatment decreases neutrophils in the lung at 24 and 72 h after a single moderate TBI and 72 h after the third of three mild repeated TBIs. A) MPO activity in lung homogenates from sham-injured (sham), 1B7-antibody treated control TBI rats (1B7) and anti-CD11d-treated TBI rats (CD11d). Numbers of rats per group are in Table 1. B) Neutrophil protein, identified by Western blotting in lung homogenates from sham-injured and TBI rats, expressed as a ratio of the optical density/mm^2 of the 56 kDa neutrophil protein band to the optical density/mm^2 of the corresponding β-actin band. A representative autoradiogram of a Western blot of the 56 kDa protein is shown below the bar graphs. In this and all other figures: values are means ± S.E.; *significantly different from sham-injured; #significantly different from IB7 control; $P \leq 0.05$ after Student Neuman Keul's test for all comparisons. C) Photomicrographs (20×) of lung sections immunostained by an anti-neutrophil antibody from sham-injured rats (left panels), IB7 control TBI rats (middle

panels) and TBI rats treated with the anti-CD11d mAb (right panels). The top row illustrates examples at 24 h after a single moderate TBI and the bottom row shows examples at 72 h after the third of three mild TBIs. Upper sham injury panel shows a high power (100×) detail of a stained cell (inset). An alveolus (a) and a blood vessel (bv) are indicated in the top sham injury panel. Scale bar is 100 μm and applies to all photomicrographs. Scale bar in the inset is 10 μm.

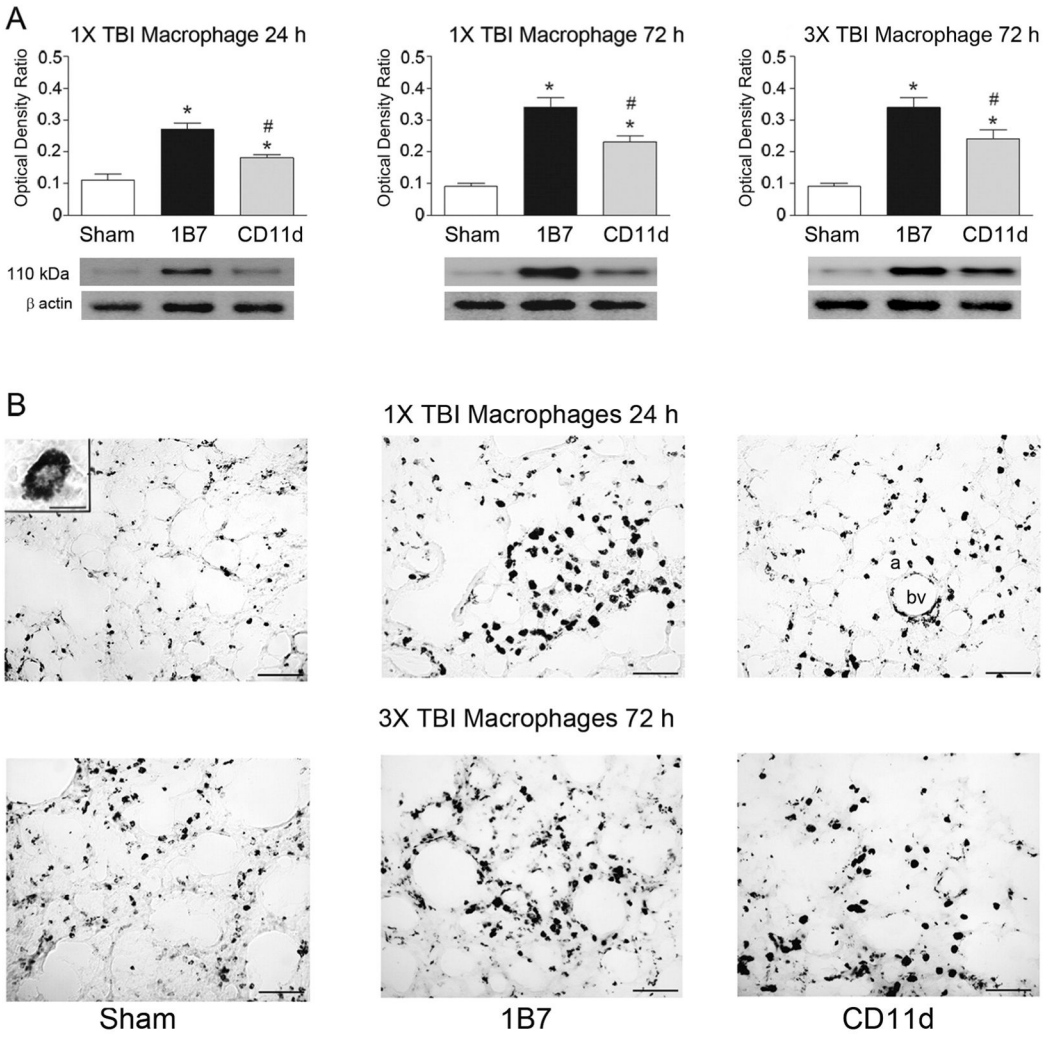

Fig. 3.

The anti-CD11d treatment decreases macrophages in the lung at 24 and 72 h after a single moderate TBI and 72 h after the third of three mild repeated TBIs. A) Macrophage protein (ED-1) expression (Western blotting) in lung homogenates from sham-injured rats (sham, n = 5), 1B7-antibody treated control TBI rats (1B7, n = 5) and anti-CD11d-treated TBI rats (CD11d, n = 5). Figure format is as in Fig. 2B. *Significantly different from sham-injured; #significantly different from 1B7 controls. B) Photomicrographs (20×) of lung sections immunostained by an anti-ED-1 antibody to detect macrophages in lungs from sham-injured rats (left panels), IB7 control TBI rats (middle panels) and TBI rats treated with the anti-CD11d mAb (right panels). The top row illustrates examples at 24 h after a single moderate TBI and the bottom row shows examples at 72 h after the third of three mild TBIs. The inset in the upper sham panel shows a high power (100×) detail of a stained cell. In the upper CD11d panel an alveolus (a) and a blood vessel (bv) are indicated. Scale bar in the upper sham panel is 100 μm and applies to all photomicrographs. Scale bar in the inset is 10 μm. Numbers of rats per group studied by immunostaining are in Table 1.

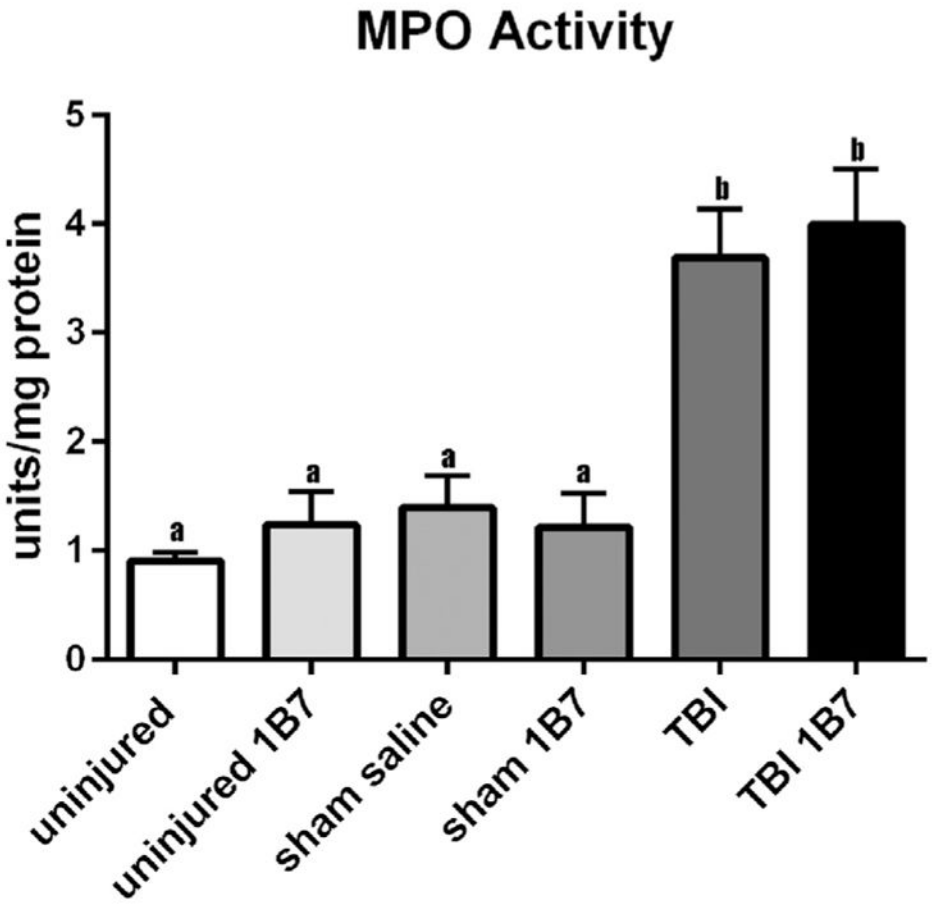

Fig. 4.

Treatment with the monoclonal antibody causes no direct inflammatory response within the lung. Comparisons are made between MPO activity in the lungs of untreated, 1B7 control mAb-treated and saline-treated rats that were uninjured, sham-injured or injured by a single moderate TBI. Numbers of rats per group are in Table 1. The saline treated sham-injured group (n = 6) is from Fig. 2. Groups designated with an ‘a’ are not different from each other, groups with a ‘b’ are not different from each other but all groups ‘a’ differ from both groups ‘b’ ($P < 0.0001$).

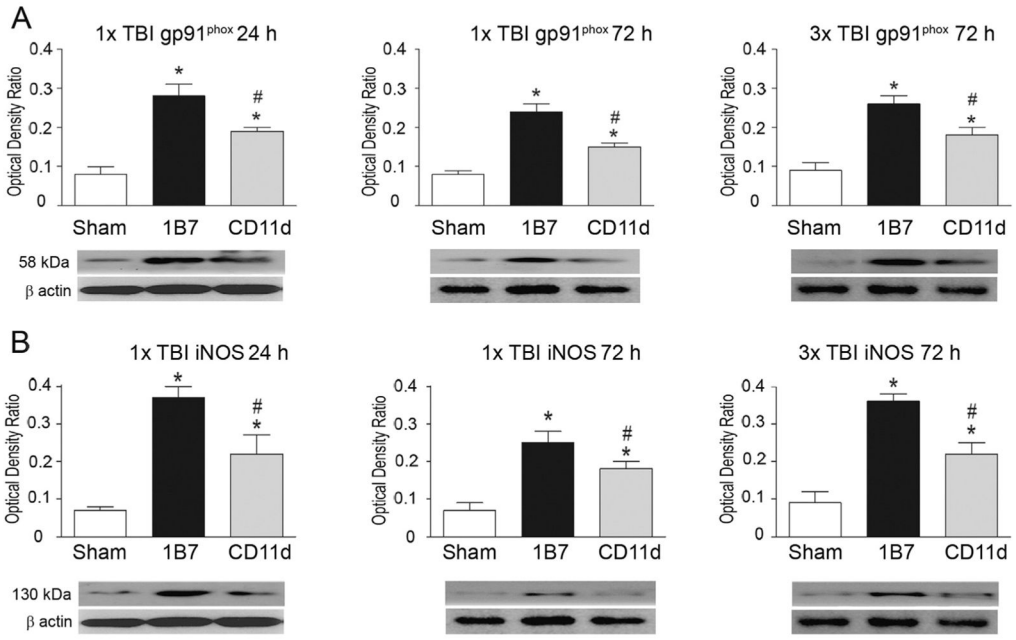

Fig. 5.

The anti-CD11d treatment decreases expression of gp91^{phox} and iNOS in the lung at 24 and 72 h after a single moderate TBI and 72 h after the third of three mild repeated TBIs. gp91^{phox} (A) and iNOS (B) expression were identified by Western blotting in lung homogenates from sham-injured, 1B7-treated TBI and anti-CD11d treated TBI rats (n = 5 per group) and are expressed as ratios of the optical density/mm^2 of the gp91^{phox} and iNOS bands to the optical density/mm^2 of the corresponding β-actin bands. Representative autoradiograms of Western blots of the gp91^{phox} and iNOS proteins are shown below the bar graphs. Figure format is as in Fig. 2B. *Significantly different from sham-injured; $^{\#}$significantly different from 1B7 controls.

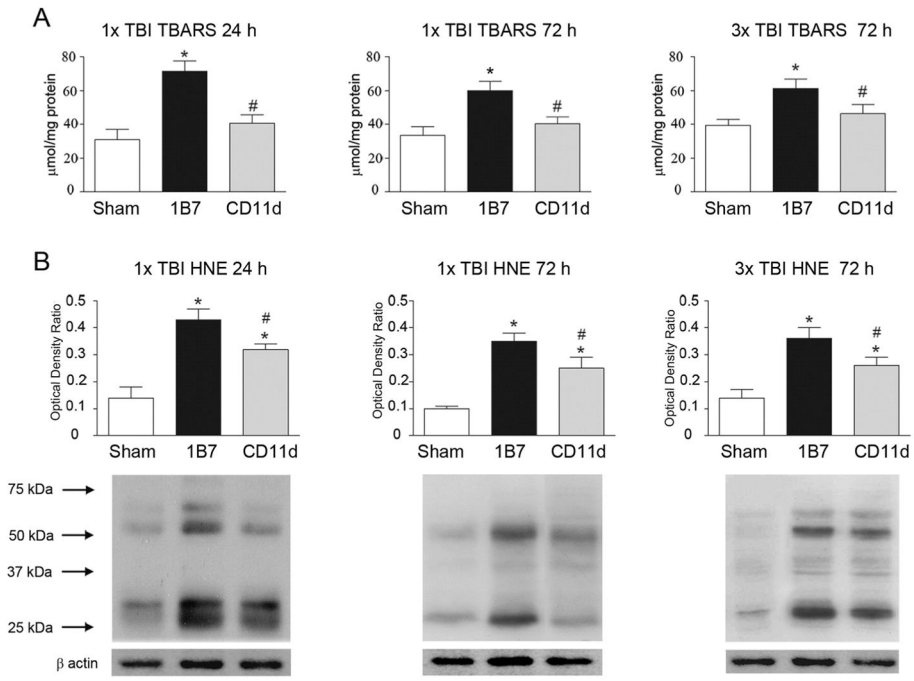

Fig. 6.

The anti-CD11d treatment decreases lipid peroxidation in the lung at 24 and 72 h after a single moderate TBI and 72 h after the third of three mild repeated TBI. A) Lipid peroxidation was assessed by the TBARS assay for malondialdehyde (MDA) and other aldehydes in lung homogenates from sham-injured, 1B7-treated TBI and anti-CD11d treated TBI rats. Numbers of rats per group are in Table 1. Figure format is as in Fig. 2A. B) Lipid peroxidation was also assayed by Western blotting for 4-hydroxynonenol (HNE)-bound proteins in these groups of rats (n = 5 per group). Format is as in Fig. 2B. Western blot illustrates an example of HNE-bound protein expression at several molecular weights. *Significantly different from sham-injured; #significantly different from 1B7 controls.

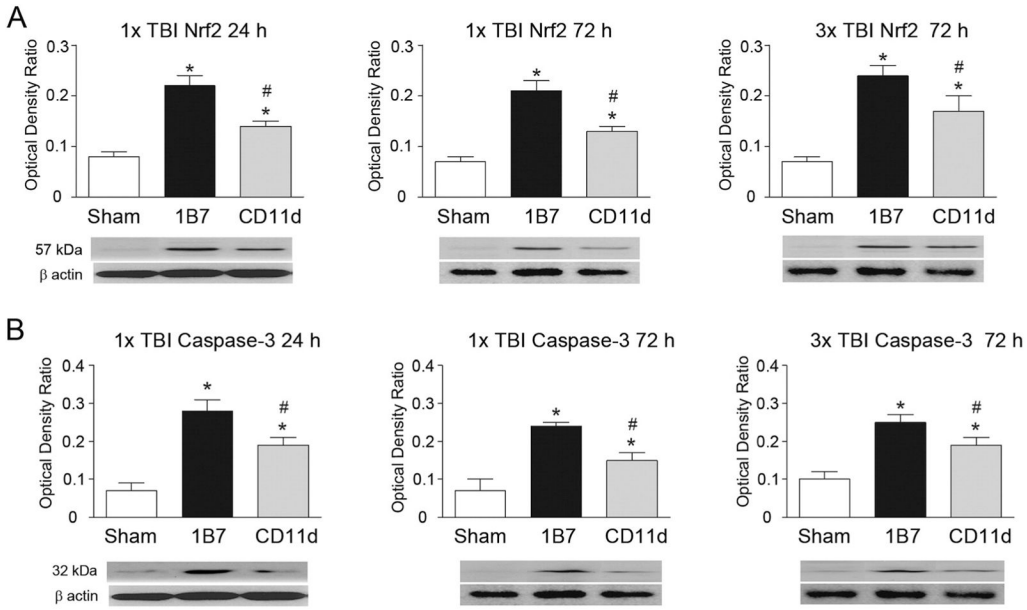

Fig. 7.

The anti-CD11d treatment decreases expression of the cytoprotective transcription factor Nrf2 and of caspase-3 expression in the lung at 24 and 72 h after a single moderate TBI and 72 h after the third of three mild repeated TBI. Nrf2 (A) and caspase-3 (B) expression were identified by Western blotting in lung homogenates from sham-injured, 1B7-treated TBI and anti-CD11d treated TBI rats (n = 5 per group). Numbers of rats studied by immunostaining are in Table 1. Figure format is as in Fig. 2B. *Significantly different from uninjured; #significantly different from T4 SCI control.

Table 1

Number of rats used in the study.

	Uninjured	Sham-injured saline			TBI 1B7			TBI anti-CD11d		
Single moderate TBI	**24 h**	**24 h**	**72 h**	**4 weeks**	**24 h**	**72 h**	**4 weeks**	**24 h**	**72 h**	**4 weeks**
Control studies	4[a], 4[b]	4[b]			4[a], 4					
Biochemistry and Western blotting		6	7	5	7	7	6	7	8	5
Immunohistochemistry		5	5	9	4	5	9	5	5	9
Repeated mild TBI		72 h	8 weeks		72 h	8 weeks		72 h	8 weeks	
Biochemistry and Western blotting		6	6		7	7		6	6	
Immunohistochemistry		6	6		6	6		6	6	

[a]No treatment.

[b]Treated with 1B7 mAb.

Table 2

Biochemical and Western blot analyses at chronic time points after traumatic brain injury.

Single moderate injury 4 weeks	**Sham**	**1B7 control**	**Anti-CD11d**	**N**	**F (df)**	***P***
Myeloperoxidase	1.10 ± 0.179	2.00 ± 0.367	1.60 ± 0.313	5, 6, 5	2.160 (2,13)	0.155
Neutrophil Western	0.11 ± 0.024	0.16 ± 0.028	0.13 ± 0.019	5	1.111 (2,12)	0.361
ED-1 Western	0.09 ± 0.018	0.14 ± 0.011	0.11 ± 0.019	5	2.360 (2,12)	0.137
gp91 Western	0.08 ± 0.013	0.13 ± 0.018	0.11 ± 0.013	5	2.541 (2,12)	0.120
iNOS Western blot	0.08 ± 0.016	0.14 ± 0.023	0.11 ± 0.011	5	3.094 (2,12)	0.082
COX2 Western	0.07 ± 0.019	0.13 ± 0.017	0.09 ± 0.017	5	2.870 (2,12)	0.096
DCF	887.1 ± 87.25	1074 ± 34.17	1043 ± 50.85	5, 6, 5	2.875 (2,13)	0.093
TBARS (MDA)	32.9 ± 4.875	43.8 ± 2.776	35.0 ± 1.923	5, 6, 5	3.122 (2,13)	0.078
HNE Western	0.11 ± 0.017	0.17 ± 0.021	0.14 ± 0.019	5	2.857 (2,12)	0.097
Nrf2 Western	0.06 ± 0.014	0.10 ± 0.020	0.09 ± 0.018	5	1.512 (2,12)	0.260
Caspase Western	0.07 ± 0.024	0.18 ± 0.039	0.14 ± 0.024	5	3.000 (2,12)	0.088

Repeated mild injury 8 weeks	**Sham**	**1B7 control**	**Anti-CD11d**	**N**	**F (df)**	***P***
Myeloperoxidase	0.70 ± 0.122	1.50 ± 0.340	1.2 ± 0.327	6, 7, 6	1.962 (2,16)	0.173
Neutrophil Western	0.11 ± 0.026	0.17 ± 0.012	0.16 ± 0.010	5	3.825 (2,12)	0.050
ED-1 Western	0.10 ± 0.012	0.14 ± 0.016	0.13 ± 0.018	5	1.185 (2,12)	0.339
gp91 Western	0.10 ± 0.027	0.17 ± 0.020	0.13 ± 0.021	5	2.445 (2,12)	0.129
iNOS Western	0.08 ± 0.013	0.13 ± 0.015	0.10 ± 0.015	5	3.135 (2,12)	0.080
COX2 Western	0.08 ± 0.019	0.12 ± 0.019	0.09 ± 0.015	5	1.725 (2,12)	0.220
DCF	997.8 ± 62.99	1064 ± 52.20	1014 ± 23.68	6, 7, 6	0.503 (2,16)	0.614
TBARS (MDA)	34.4 ± 3.144	45.3 ± 3.099	37.8 ± 2.694	6, 7, 6	3.408 (2,16)	0.058
HNE Western	0.08 ± 0.020	0.14 ± 0.026	0.11 ± 0.028	5	1.384 (2,12)	0.288
Nrf2 Western	0.07 ± 0.013	0.11 ± 0.009	0.09 ± 0.015	5	3.247 (2,12)	0.075
Caspase Western	0.09 ± 0.020	0.14 ± 0.016	0.11 ± 0.007	5	2.815 (2,12)	0.099

Values from analyses are expressed as mean ± standard error. Myeloperoxidase units: activity/mg protein; Western = Western blot; Western blot units: optical density ratios; TBARS units: µmol/mg protein; N = number of rats per group (5, 6, 5, etc., indicates different numbers of rats in the three treatment groups); F (degrees of freedom numerator/denominator) and *P* from 1-way ANOVA.