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ORIGINAL ARTICLE

Exosome-mediated inflammasome signaling after central nervous system injury

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Abstract

Neuroinflammation is a response against harmful effects of diverse stimuli and participates in the pathogenesis of brain and spinal cord injury (SCI). The innate immune response plays a role in neuroinflammation following CNS injury via activation of multiprotein complexes termed inflammasomes that regulate the activation of caspase 1 and the processing of the pro-inflammatory cytokines IL-1β and IL-18. We report here that the expression of components of the nucleotide-binding and oligomerization domain (NOD)-like receptor protein-1 (NLRP-1) inflammasome, apoptosis speck-like protein containing a caspase recruitment domain (ASC), and caspase 1 are significantly elevated in spinal cord motor neurons and cortical neurons after CNS trauma. Moreover, NLRP1 inflammasome proteins are present in exosomes derived from CSF of SCI and traumatic brain-injured patients following trauma. To investigate whether exosomes could be used to therapeutically block inflammasome activation in the CNS, exosomes were isolated from embryonic cortical neuronal cultures and loaded with short-interfering RNA (siRNA) against ASC and administered to spinal cord-injured animals. Neuronal-derived exosomes crossed the injured blood–spinal cord barrier, and delivered their cargo *in vivo*, resulting in knockdown of ASC protein levels by approximately 76% when compared to SCI rats treated with scrambled siRNA. Surprisingly, siRNA silencing of ASC also led to a significant decrease in caspase 1 activation and processing of IL-1β after SCI. These findings indicate that exosome-mediated siRNA delivery may be a strong candidate to block inflammasome activation following CNS injury.

Keywords: brain injury, caspase 1, exosomes, inflammasome, spinal cord injury.

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Spinal cord injury (SCI) and traumatic brain injury (TBI) are complex and devastating clinical conditions characterized by neuronal loss, axonal destruction, and demyelination during the secondary injury cascade (Popovich 2000; Beattie *et al.* 2002; Bramlett and Dietrich 2004, 2014; Hutchinson *et al.* 2007). Both types of injuries involve multiple factors, including systemic humoral pathophysiological factors in addition to direct CNS injuries. Since CSF is in contact with many areas of the brain and spinal cord, it has been suggested that the humoral factors that mediate signaling after CNS injury may be carried and transported in CSF or other body fluids (Street *et al.* 2012). Recently, it has been shown that CSF contains exosomes that may play a role in humoral signaling in the CNS (Harrington *et al.* 2009). In the context of CNS pathology, pathogenic proteins such as β-amyloid, prion protein, α-synuclein, tau, and superoxide dismutase are released into the CSF in association with exosomes (Fevrier *et al.* 2004; Rajendran *et al.* 2006; Gomes *et al.* 2007; Emmanouilidou *et al.* 2010; Saman *et al.* 2012). It has been proposed that exosomes containing pathological proteins mediate the spread of cell damage or

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Abbreviations used: DAB, diaminobenzidine; GOS, Glasgow Outcome Scores; MHC, major histocompatibility; NLRP-1, NOD-like receptor protein-1; SCI, spinal cord injury; TBI, traumatic brain injury.

alter the microenvironment in various metabolic and nervous system disorders (Pant *et al.* 2012; Fruhbeis *et al.* 2013). However, it remains unclear whether exosomes secreted into CSF contribute to molecular signaling in the pathology after CNS injury.

In the CNS, neurons, astrocytes, microglia, and oligodendrocytes have been reported to secrete exosomes into the extracellular environment (Chivet *et al.* 2012; Fruhbeis *et al.* 2013). Exosomes contain a distinct set of proteins conserved across different cell types and species, e.g., cytoskeletal proteins such as tubulin and actin, heat-shock proteins (Hsp 70 and 90), metabolic enzymes of glucose metabolism, flotillin-1, signal transduction proteins (kinases, heterotrimeric G proteins), major histocompatibility (MHC) molecules, clathrin, proteins involved in transport and fusion (annexins, Rab proteins), and translation elongation factors. In terms of the mechanisms responsible for the activation of the immune response, recent reports suggest that exosomes carry bioactive cytokines such as IL-1β as well as inflammasome components (Qu *et al.* 2007) and that exosomes regulate Toll-like receptor signaling and IL-1β production by the NLRP3 inflammasome (Haneklaus *et al.* 2013).

Evidence suggests that exosomes trigger an innate immune response that amplifies such response via the cargo of proteins, RNA and miRNA that transfer immune responsiveness to neighboring cells. Exosome content and functions depend on the precise maturation stage, cell lineage, and stimulation state of the parent immune cell (Pant *et al.* 2012). In fact, immune functions of exosomes can be tailored to be immunogenic or tolerizing, depending on the presence of specific molecular cargo. Moreover, exosomes have been used in creating highly specialized therapeutics termed 'designer dexosomes' that are utilized in cancer therapy, vaccine development, and transplant tolerance induction (Viaud *et al.* 2010).

Here, we show that exosomes derived from neurons can deliver short-interfering RNA (siRNA) into the CNS to significantly decrease inflammasome activation after injury. We also found that inflammasome protein expression in exosomes derived from CSF in TBI and SCI subjects was increased after CNS trauma. Thus, exosomes offer a new therapeutic approach to deliver RNA-based drugs to block inflammation after CNS injury.

Materials and methods

Neuropathological procedures

For immunohistochemical analysis of inflammasome proteins, spinal cord sections were obtained from The Miami Project to Cure Paralysis' Human Spinal Cord Bank. Spinal cords from nine cases of SCI (eight males and one female with ages ranging from 20 to 77 years) who sustained vertebral fractures were used in this study (Table 2). SCI was classified on the basis of histological appearance as 'contusion/cyst', massive compression or laceration as described (Fleming *et al.* 2006) (Table 1). Brain sections used for immunohistochemistry correspond to brains from healthy decedents.

In all cases, tissue samples from the center of SCI and at various distances above and below the injury were obtained. In this study, we analyzed inflammasome protein expression of diaminobenzidine (DAB) immunostained sections at the epicenter, penumbra, and an area distal from the epicenter.

All tissue samples were removed within 24 h of death and fixed in neutral buffered formalin as described previously. Blocks from the spinal cords were dehydrated, embedded in paraffin wax, cut into 6-μm-thick sections and placed on positively charged glass slides. One set of sections was stained with hematoxylin and the remaining sets were used for immunohistochemistry.

Immunohistochemistry

Paraffin-embedded sections were stained with anti-NLRP1 (Bethyl Laboratories as described (de Rivero Vaccari *et al.* 2008), anti-caspase 1 (Upstate, Novus Biologicals, Littleton, CO, USA), and anti-ASC (Chemicon, Santa Cruz Biotechnology, Dallas, TX, USA) using DAB as the chromophore along with hematoxylin. Negative controls included sections in which the primary antibody was omitted, sections incubated with isotype-matched antibodies (1 : 200) and controls using secondary antibodies alone. These negative controls were processed with every batch of immunohistochemical slides.

Neuropathological assessment

The cord and brain were assessed microscopically, using brightfield optics, by examining H&E or H&E/DAB-stained sections from the epicenter, penumbra, and uninvolved area distal from the epicenter.

CSF samples

The study was approved by the University of Miami Miller School of Medicine Institutional Review Board (Protocol #20090655 for SCI sample collection and protocol #20030154 for TBI sample collection). The CSF of seven patients with SCI was used to analyze the levels of inflammasome protein expression after injury. The American Spinal Cord Injury Association (ASIA) scale of these patients at admission to the emergency department ranged from Association impairment scale (AIS) A to B. Information regarding the diagnosis, procedures, and outcomes are shown in Table 1. CSF from uninjured individuals was obtained as a control from patients ranging from 29 to 91 years old. Patients in the control group required a ventriculostomy for non-traumatic pathology. SCI CSF samples were obtained from patients ($n = 6$) at different time points after injury. CSF was collected from patients who suffered a severe, traumatic SCI that revealed a dural laceration as determined at the time of surgical decompression and stabilization. These patients underwent therapeutic diversion of CSF using a silastic catheter drain placed after surgery. Informed consent was obtained directly from the patient and family after all the potential risks and benefits were fully explained, and patients were only considered for study enrollment if a lumbar drain was already clinically necessary. CSF was collected every 12 h as the clinical status and drain function permitted for a total of up to 6 days following injury. Five cc

Table 1 Data of spinal cord decedents used for immunohistochemistry

Case	Age	Gender	Pathology report
8	77	Female	African American female involved in a motor vehicle accident (MVA). The injury was contusive at the level of C2 characterized by central white/gray matter necrosis, hemorrhage, edema, and axonal swelling. The cerebellum and pons showed anoxic changes. There was a radiculopathy associated with posterior column degeneration (remote and unrelated to trauma). The subarachnoid spaced showed sloughed off cerebellar tissue at C1–C2 associated with tonsillar herniation) and central gray microglial proliferation was present at C4–C7
9	26	Male	African American decedent who sustained multiple gunshot wounds to the neck, chest, abdomen, and lower and upper extremities. The injury was cuntission type with the epicenter at C7 and extending from C2 to T1 with multifocal gray and white hemorrhages associated with parenchymal fragmentation, axonal spheroids, neuronal necrosis, and mononuclear inflammatory infiltrates. A subarachnoid hemorrhage was present, C7 to T1. A cervical CT scan showed intact cervical vascular structures, fracture of the left pedicle and the spinous process at C5, fractures of bilateral lamina/pedicles/facets at C6 and C7, spinous process fracture at C7 (associated with bullet fragments), fractures of the right lamina transverse process at L3 and of the right transverse process at L1/L2. No bony or bullet fragments within the lumbar spinal cord were found. The calvarium, brain, dura and leptomeninges were unremarkable
10	20	Male	Caucasian male involved in a MVA. Decedent had a dislocation of the third thoracic vertebra with associated pink discoloration of the spinal cord parenchyma, and mild subarachnoid hemorrhage was present at T1, T2 and T8
11	39	Male	Hispanic man involved in a MVA. Findings included atlanta-occipital dislocation with stretching of the spinal cord. C7, T11 and L1 fractures and mild multiple contusion at C8, slight subarachnoid hemorraghes of the ventral sulcus at T2, distortion of the ventral tract at T11
12	29	Male	Decedent suffered multiple gunshot wounds, including one that penetrated the spinal canal and lacerated the spinal cord at C7. Laceration at C7 was associated with myelopathic changes from CS to T1 (white matter fragmentation, hemorrhage and edema)
13	35	Male	Caucasian shot multiple times. One bullet penetrated the T9 vertebra in a dorsoventral direction. Injury was contusive from T7 to T12 with the epicenter at T9-T10. Petechial hemorrhages in the gray and white matter with focal subarachnoid and subpial hemorrhages

aliquots of CSF were obtained at each time interval and immediately spun in a centrifuge to remove cellular material and debris before protein analysis. There were no complications as a result of drain placement or fluid removal (Table 2).

For CSF collection of brain-injured patients, samples were collected from patients who suffered severe TBI (Glasgow Coma Scale (GCS) 8 or less) every 6 h as the clinical status and drain function permitted for a total of up to 5 days following injury. Two cc aliquots of CSF were obtained at each time interval and immediately spun in a centrifuge to remove cellular material and debris before protein analysis. There were no complications as a result of drain placement or fluid removal (Table 2).

Immunoblotting

For detection of inflammasome proteins, CSF samples were mixed with Laemali buffer. In all experiments, 5 μg of protein was loaded. Immunoblot analysis was carried with the Criterion system (Bio-Rad Laboratories, Hercules, CA, USA) as described (de Rivero Vaccari *et al.* 2008) using antibodies (1 : 1000 dilution) to NLRP1 (Millipore Corporation, Billerica, MA, USA), Caspase 1 (Novus Biologicals, Littleton, CO, USA), and ASC (Santa Cruz Biotechnology, Dallas, TX, USA). Proteins were resolved in 14–20% TGX Criterion precasted gels (Bio-Rad Laboratories), transferred to polyvinylidene difluoride transfer membranes (Tropifluor; Applied Biosystems, Grand Island, NY, USA) and placed in blocking buffer (phosphate-buffered saline, 0.1% Tween-20, 0.4% I-Block (Applied Biosystems), and then incubated for 1 h with primary antibodies. Membranes were then incubated for 1 h with anti-mouse, anti-rat, or anti-rabbit horseradish peroxidase-linked antibodies. Signal visualization was performed by enhanced chemiluminescence (Cell Signaling Technology, Beverly, MA, USA). All images analyzed were done with the same film exposure of 7 min to maintain all patients under the same conditions for comparison purposes.

Isolation of exosomes from CSF and neuronal cell cultures

Exosomes were isolated from CSF samples using ExoQuick (System Biosciences, Mountain View, CA, USA) according to the manufacturer's instructions. Approximately 2 μg of protein from CSF input (IN) and isolated exosome fractions (EX) were analyzed by immunoblotting for exosome-enriched protein expression (Fig. 3). Glasgow Outcome Scores (GOS) for each TBI patient are shown to the left of the immunoblots. CD9, an exosomal marker, was used as a marker

Table 2 Subjects used in this study (CSF)

Patient	Age	Gender	Race	Mechanism of injury	Spinal injury	AIS Grade	Level	Surgery	Hypothermia	Exam at Rehab D/C
Spinal cord injury										
1	21	M	White	Diving accident	C5/6 Fracture dislocation	A	C5	C5/6 Anterior discectomy & fusion	Yes	C5 ASIA C
2	19	M	Black	Motor vehicle accident	C4/5 Bilateral jumped facets	B	C4	C4/5 Anterior discectomy & fusion	No	C4 ASIA D
Exosomes experiment	22	M	White	Rugby accident	C5/6 Bilateral jumped facets	A	C5	C5/6 Anterior discectomy & fusion	Yes	C6 ASIA A

Patient	Age	Gender	Mechanism of injury	Type of injury	GCS	ISS	Motor	Surgery	Hypothermia	Notes
Traumatic brain injury										
1	23	M	Motor vehicle accident	Subdural and Epidural hematoma, refractory high ICP	5	25	3	Cranial decompression and craniotomy	Yes	Developing vegetative state, Infarcts on CT
2	29	M	Motor vehicle accident	DAI suspected	7	25	5	None	No	Following Commands

for exosome-positive fractions. Caspase 1 and NLRP1 are enriched in the exosomal fractions from patients with poor outcome (GOS-3). ASC is enriched in the exosomal fraction from two of the three patients with poor outcome. Molecular weights in kilodaltons are shown beneath each protein. To obtain neuronal exosomes, primary neuronal cell cultures were grown from E-18 rat brains as described (Adamczak *et al.* 2014). Primary rat cortical neuronal cultures were grown for 7 days and culture medium was collected and exosomes were extracted using ExoQuick (System Biosciences) following the manufacturer's instructions.

siRNA and exosome loading

psiRNA labeled with green fluorescent protein (GFP) against ASC was purchased from Invitrogen, (Grand Island, NY, USA). Exosome loading of psiRNA was carried out as described (Alvarez-Erviti *et al.* 2011). Briefly, exosomes at a total protein concentration of 12 μg (measured by Bradford assay) and 400 nmol (for cell culture) or 12 μg (for *in vivo* injections) of psiRNA were mixed with 400 μL of electroporation buffer (1.15 mM potassium phosphate pH 7.2, 25 mM potassium chloride, 21% Optiprep) and electroporated in a 4 mm cuvette. For *in vivo* experiments, electroporation was performed in 400 μL and pooled together before resuspension in a volume of 5% glucose corresponding to 80 μL per injection.

In vitro delivery of siRNA by targeted exosomes

To assess whether neuronal-derived exosomes loaded with siRNA can be used to deliver their cargoes *in vitro*, we used primary rat cortical neuronal cultures grown for 7 days. Culture medium was harvested and exosomes were extracted using ExoQuick (System Biosciences, Mountain View, CA, USA). Exosomes at a total protein concentration of 12 μg (measured by Bradford assay) and 400 nmol siRNA-GFP against ASC or scramble siRNA-GFP were mixed with 400 μL of electroporation buffer (1.15 mM potassium phosphate pH 7.2, 25 mM potassium chloride, 21% Optiprep) and electroporated in a 4 mm cuvette. Neurons were treated for 72 h with exosomal preparations. High delivery efficiency was confirmed by immunofluorescence microscopy (data not shown). The knockdown of ASC protein was specific with a knockdown efficiency of approximately 32%.

Spinal cord injury

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Miami. Adult female Fischer rats (180–200 g) were used in these studies. The rat model of moderate contusive SCI employed was as described (Keane *et al.* 2001) with minor modifications. Briefly, animals were anesthetized with isoflurane (2%), and then a laminectomy was performed at vertebral level T8 and the cord was exposed without disrupting the dura. A moderate injury was induced using the New York University weight drop device (10 g, 12.5 mm). After injury, muscles were closed in layers, and the incision was closed with wound clips. Rats were returned to their cages and placed on computer-controlled warmed blankets, with access to water and food *ad libitum*. Gentamycin (5 mg/kg intramuscular) was given once a day for a week following surgery to control infection, while buprenorphine (0.01 mg/kg subcutaneous) was given twice a day for 3 days after injury to relieve pain. The rats' bladders were

manually voided twice a day until they were able to regain normal bladder function. Rats were killed 24 h after SCI. Sham rats were used as control.

Determination of effect of siRNA to injured spinal cord by systemic injection of targeted exosomes

Exosomes for *in vivo* experiments were spun down and resuspended in 80 μL of 5% glucose immediately before femoral artery injection. Approximately 150 μg of exosomes and the encapsulated siRNA (150 μg of inflammasome protein psiRNA) was injected via femoral artery per animal at 30 min after SCI. Three groups of rats were used in these studies: (i) sham-operated; (ii) spinal cord injured injected with siRNA encapsulated exosomes; and (iii) spinal cord injured injected with unmodified exosomes. To characterize the tissue distribution, we performed immunoblot analysis of spinal cord lysates. Systemic administration of unmodified exosomes was run as a control. To assess whether exosome-mediated siRNA delivery reduces the innate immune response *in vivo*, we assessed the levels of innate immune proteins by immunoblot analysis as described (de Rivero Vaccari *et al.* 2008).

Statistical analysis

Statistical comparisons between groups were done using a one-tailed Student's *t*-test for experiments involving two groups: Exosome control versus exosome siRNA against ASC and siRNA versus scrambled. A *p*-value of significance was set at $p < 0.05$. When more than two groups were compared, a one-way ANOVA followed by Dunnett's multiple comparisons test was used.

Results

Immunohistochemical expression of NLRP1 inflammasome proteins in the spinal cord and brain after injury

Spinal cord sections were obtained from decedents who had SCI. Immunohistochemical analysis combined with light microscopy indicated that NLRP1 is expressed in neurons of the ventral horn (short arrows), myelinated axons (arrow heads), and oligodendrocytes (long arrows) (Fig. 1a). Hematoxylin-stained oligodendrocyte nuclei appear round, whereas microglial nuclei are irregular in shape. Moreover, NLRP1 immunoreactivity in the penumbra (C7) was higher than in areas far distant to the penumbra (L2). Therefore, it appears that inflammasome protein expression is altered in areas distant to the primary lesion site, suggesting that large areas of the spinal cord show an innate immune inflammatory response after injury.

In addition, DAB immunoreactivity for caspase 1 was detected in swollen axons (spheroids, arrow heads) (Fig. 1a), and arterioles (data not shown). At areas of the penumbra, caspase 1 staining was present in motor neurons (short arrows) of the ventral horn, and was present in the white matter in oligodendrocytes (long arrows). Caspase 1 immunoreactivity in oligodendrocytes (long arrows) was the same at all levels of the spinal cord examined, regardless of proximity to the epicenter. At areas distal to the epicenter (T12), caspase 1 was also present in motor neurons (short arrows) but with decreased immunoreactivity than the penumbra (C7). These findings indicate that caspase 1 immunoreactivity in neurons may decrease as function of the distance from the epicenter, similar to NLRP1.

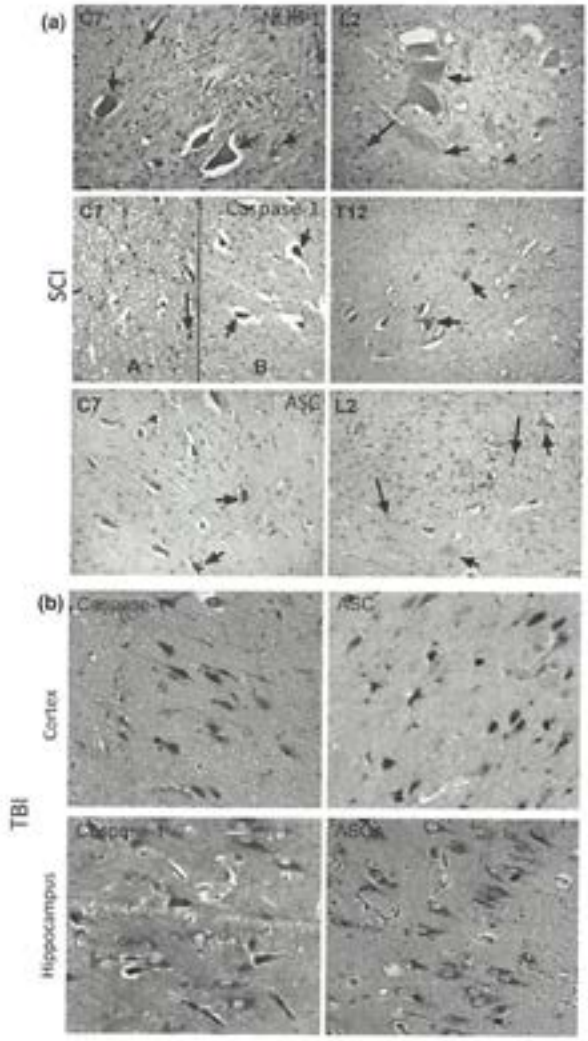

Fig. 1 NLRP1 inflammasome proteins are expressed in cells of the CNS. Spinal cord and brain sections were obtained from decedents that had injury to either the spinal cord (a) or the brain (b). (a) NLRP1 is expressed in neurons of the ventral horn (short arrows), myelinated axons (arrowheads), and oligodendrocytes (long arrows); moreover, NLRP1 immunoreactivity in areas of the penumbra (C7) was higher than in areas distant to the penumbra (L2). Caspase 1 was detected in swollen axons (spheroids, arrow heads), in motor neurons (short arrows) of the ventral horn, and in the white matter in oligodendrocytes (long arrows). At areas distal to the epicenter (T12), caspase 1 was also present in motor neurons (short arrows) but with decreased immunoreactivity than the penumbra (C7). ASC was present in the penumbra (C7) and distal to the epicenter (L2) in neurons in the ventral horn (black arrows), white matter oligodendrocytes (long arrow) and in macrophages/microglia at the epicenter (short arrow). (b) Immunohistochemical analysis of brain sections indicates that caspase 1 and ASC immunoreactivity is present in the cortex and hippocampus of humans.

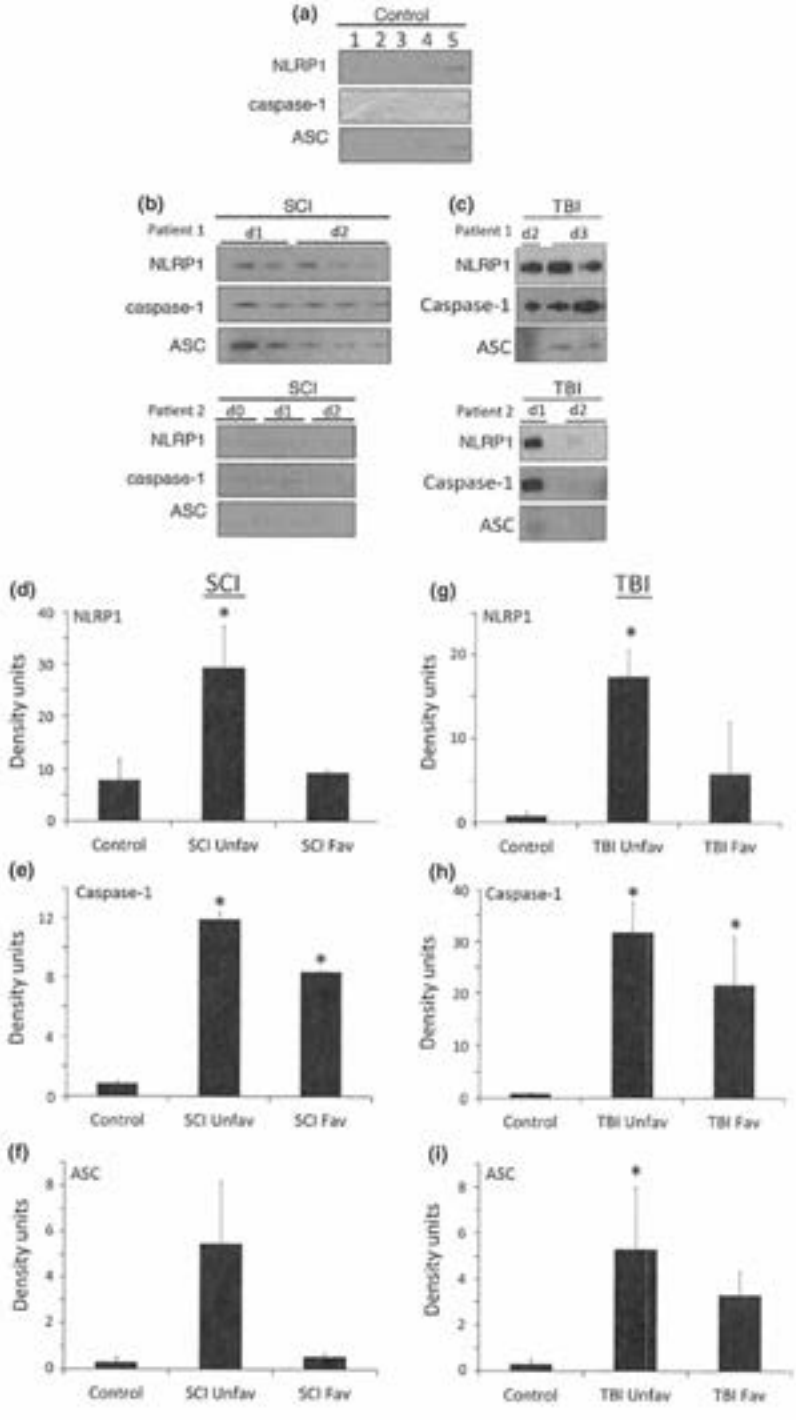

Fig. 2 NLRP1, caspase 1, and ASC are elevated after SCI and traumatic brain injury (TBI). (a) CSF samples from uninjured patients were used as controls (1–5). (b) CSF samples were immunoblotted with antibodies against NLRP1, caspase 1 and ASC. d0 = day of injury and d1, d2 = day 1 and day 2 after injury. Immunoblot analysis of two different cases of patients with SCI indicates an increase in the levels of NLRP1 (d), caspase 1 (e) and ASC (f) in the CSF of patients with SCI when compared to CSF from control subjects (patient 1). Patient 2 presented lower levels of inflammasome proteins in the CSF and had a better AIS score at discharge than patient 1 (d vs. c). (c) Immunoblot analysis of two different cases of patients with TBI indicates an increase in the levels of NLRP1 (g), caspase 1 (h) and ASC (i) in the CSF of patients with TBI when compared to CSF from control subjects (patient 1). Patient 2 presented lower levels of inflammasome proteins in the CSF and had a better GCS than patient 1 (7 vs. 5). Data are presented as mean ± SEM $*p < 0.05$, $n = 3$–6 per group. UnFav = unfavorable outcome after injury; Fav = favorable outcome after injury.

In the penumbra (C7) and distal to the epicenter (L2), neurons in the ventral horn (black arrows) and white matter oligodendrocytes (long arrow) showed ASC immunoreactivity. In addition, ASC was also present in macrophages/microglia at the epicenter (short arrow). Moreover, ASC immunoreactivity was also detected in the substantia gelatinosa (dorsal horn) at C7 and L2 (data not shown).

Caspase 1 and ASC were also noted in cortical and hippocampal neurons following TBI (Fig. 1b) indicating that neurons predominantly show increased inflammasome expression following CNS injury. Thus, it appears that

CNS trauma induces increased NLRP1 inflammasome protein immunoreactivity in CNS neurons, and these findings are consistent with previous work demonstrating inflammasome protein expression in neurons, oligodendrocytes, and microglia after SCI in rats (de Rivero Vaccari *et al.* 2008).

NLRP1 inflammasome proteins are present in the CSF of patients with TBI and SCI

To determine whether inflammasome proteins were present in CSF after TBI and SCI, we analyzed CSF samples by immunoblotting from uninjured controls (Fig. 2a) and SCI (Fig. 2b) and TBI (Fig. 2c) individuals with antibodies against NLRP1, caspase 1, and ASC. Control samples contained very low levels of NLRP1 inflammasome proteins. In contrast, immunoblot analysis of two different SCI patients (Fig. 2b, d–f) and two different TBI patients (Fig. 2c, g–i) showed an increase in the levels of NLRP1, caspase 1, and ASC in the CSF when compared to control subjects. It should be noted that SCI patient 1 showed high levels of inflammasome proteins at days 1 and 2 (d1, d2) after SCI and had a poor prognosis, whereas patient 2 had low levels of inflammasome proteins acutely after SCI and had a good prognosis. Similarly, after TBI, patient 1 expressed higher levels of inflammasome proteins than patient 2, consistent with patient 2 having a less severe injury (GCS 7 vs. 5, Table 2). Thus, these findings are consistent with our previous study after TBI showing that individuals that present with low levels of caspase 1 in the CSF early after CNS injury may have a better prognosis than those individuals who show increased levels of these proteins. Further studies that include more patients are needed to accurately determine the predictive value of inflammasome proteins in the CSF on outcomes after CNS injury (Adamczak *et al.* 2012).

Inflammasome proteins are secreted in CSF exosomes after SCI and TBI

To determine whether inflammasome proteins are secreted in association with exosomes in CSF after CNS trauma, we isolated exosomes from TBI and SCI patients and analyzed inflammasome protein expression using immunoblot analysis (Fig. 3). ASC, NLRP1, and caspase 1 (C1) were present in CSF of SCI and TBI patients and non-trauma controls. NLRP1, ASC, and caspase 1 (p20) were released into CSF in association with exosomes (Fig. 3). However, in each case, the inflammasome proteins in exosomes ran slightly faster on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, indicating that these proteins in exosomes are lower in molecular weights than those in CSF alone. GOS for each TBI patient and AIS scale for SCI patients show that caspase 1 and NLRP1 are enriched in the exosomal fraction from two of the three patients with poor outcome.

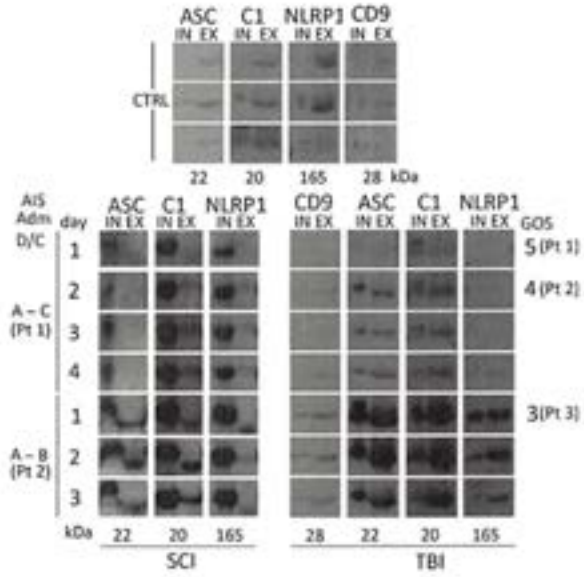

Fig. 3 ASC, Caspase 1, and NLRP1 are released into CSF in association with exosomes. Two micrograms of protein from CSF from spinal cord- and brain-injured samples (IN: input) and isolated exosome fractions (EX) were analyzed by immunoblotting for exosome-enriched protein expression. AIS and Glasgow Outcome Scores (GOS) for each SCI and traumatic brain injury (TBI) patient, respectively, are shown to the left and right of the immunoblots. CD9, confirms exosome-positive fractions. C1 and NLRP1 are enriched in the exosomal fractions from patients with poor outcome (AIS B and GOS-3). ASC is enriched in the exosomal fraction from two of the three patients (Pt.) with poor outcome after TBI. Molecular weights in kDa are shown.

In vitro and *in vivo* delivery of siRNA by neuronal-derived exosomes

To explore whether exosomes may be used as a therapeutic treatment to block inflammasome activation after SCI, we first assessed whether neuronal-derived exosomes loaded with siRNA against ASC would be able to deliver their cargoes *in vitro* (Fig. 4a). Rat cortical neurons were grown for 7 days, the culture medium was collected and exosomes were extracted. Exosomes were loaded with either siRNA-GFP against ASC or scrambled siRNA-GFP and added to cortical neurons for 72 h. After 72 h of exosome treatment, the knockdown of ASC protein was specific with a knockdown efficiency of approximately 32% (Fig. 4a).

Next, we investigated the potential for exosome-mediated systemic siRNA delivery *in vivo*. To establish whether exosomes crossed the blood–spinal cord barrier penetrated into the spinal cord parenchyma and delivered their cargo, exosomes containing si-RNA-GFP were injected into the femoral vein at 30 min after SCI and after 24 h. The spinal cord was harvested, sectioned, and examined by immunofluorescence microscopy. As shown in Fig. 4(b), cells in the injured spinal cord were GFP positive in the lesion epicenter (C5) but not in areas distant to the epicenter,

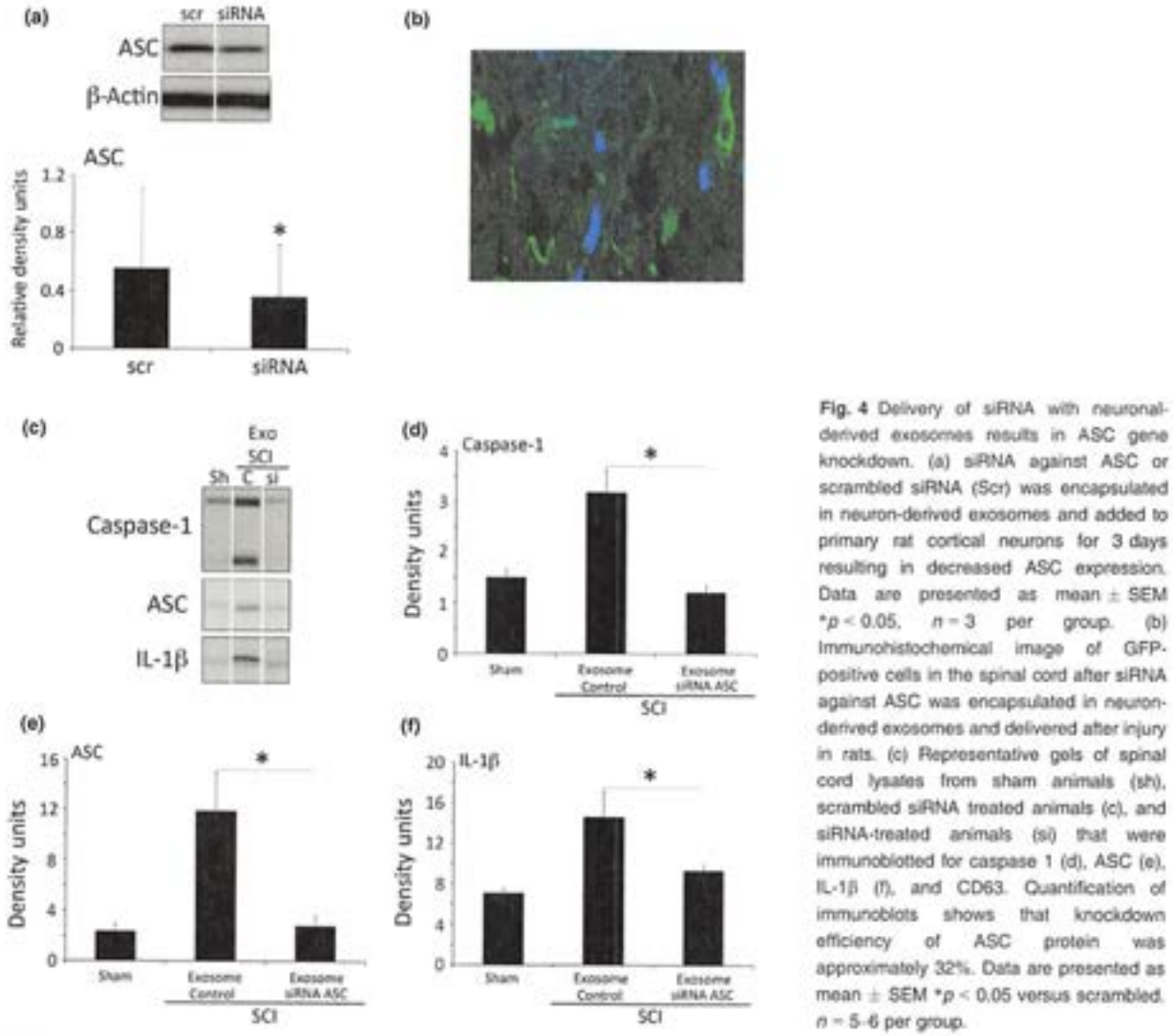

Fig. 4 Delivery of siRNA with neuronal-derived exosomes results in ASC gene knockdown. (a) siRNA against ASC or scrambled siRNA (Scr) was encapsulated in neuron-derived exosomes and added to primary rat cortical neurons for 3 days resulting in decreased ASC expression. Data are presented as mean ± SEM $*p < 0.05$, $n = 3$ per group. (b) Immunohistochemical image of GFP-positive cells in the spinal cord after siRNA against ASC was encapsulated in neuron-derived exosomes and delivered after injury in rats. (c) Representative gels of spinal cord lysates from sham animals (sh), scrambled siRNA treated animals (c), and siRNA-treated animals (si) that were immunoblotted for caspase 1 (d), ASC (e), IL-1β (f), and CD63. Quantification of immunoblots shows that knockdown efficiency of ASC protein was approximately 32%. Data are presented as mean ± SEM $*p < 0.05$ versus scrambled. $n = 5$–6 per group.

indicating that neuronal-derived exosomes effectively delivered their cargo *in vivo*.

To confirm the therapeutic potential of neuronal exosomes *in vivo*, we next investigated delivery of siRNAs that silenced ASC protein expression (Fig. 4c–f). Exosomes loaded with siRNA against ASC knockdown ASC protein expression approximately 76% when compared to SCI rats treated with scrambled siRNA (Fig. 4c). ASC knockdown also leads to a significant decrease in caspase 1 activation (Fig. 4d) and processing of IL-1β (Fig. 4f) after SCI, indicating that exosome-mediated siRNA delivery may be a strong candidate to block inflammasome activation following SCI.

Discussion

Despite recent advances in uncovering pathomechanisms of secondary injury, there are no effective therapeutic targets to treat SCI or TBI. Our results show that in humans, NLRP1 inflammasome proteins are expressed in neurons following trauma. These inflammasome proteins are released into the CSF in exosomes in patients following CNS trauma. Moreover, exosome-mediated systemic siRNA delivery against ASC significantly decreased inflammasome activation following SCI and decreased caspase 1 activation and processing of IL-1β in rodents. Thus, exosomes offer a new therapeutic alternative to deliver RNA drugs to block inflammation after CNS injury.

Exosomes are bioactive vesicles derived from the cell's endosomal membrane system and are secreted into surrounding body fluids. Exosome formation, cargo content, and delivery to surrounding cells are of immense biological importance considering the role exosomes play in various pathological conditions (Taylor and Gercel-Taylor 2011; Pant *et al.* 2012). Exosomes exhibit anti-inflammatory or pro-inflammatory properties depending on the parent antigen-presenting cell's conditioning. For example, exosomes

are enriched in MHC class I and II antigens and play a role in stimulation of immune responses (Filipazzi *et al.* 2012; Pant *et al.* 2012). A recent report suggests that exosomes carry bioactive cytokines such as IL-1β and inflammasome components (Qu *et al.* 2007). Through this mechanism, exosomes trigger an innate immune response and amplify such response *via* the cargo of protein, RNA or, miRNA that transfers immune responsiveness to neighboring cells.

Our results showing that exosomes are increased in CSF after SCI and TBI indicate a new unexplored role of exosomes in immune function after CNS trauma. The fact that both TBI and SCI are associated with altered vascular permeability possibly enhances the movement of exosome trafficking into CSF pools (Lotocki *et al.* 2009; Bramlett and Dietrich 2014; DeFazio *et al.* 2014; Figley *et al.* 2014). However, it will be important to trace the cell lineage producing these exosomes. In the CNS, neurons, astrocytes, microglia, and oligodendrocytes have been reported to secrete exosomes into the extracellular environment (Chivet *et al.* 2012; Fruhbeis *et al.* 2013). Exosomes contain a distinct set of proteins that are conserved across different cell types and species. Examples of these include cytoskeletal proteins such as tubulin and actin, heat-shock proteins (Hsp 70 and 90), metabolic enzymes of glucose metabolism, Flotillin-1, signal transduction proteins (kinases, heterotrimeric G proteins), MHC molecules, clathrin, proteins involved in transport and fusion (annexins, Rab proteins), and translation elongation factors. Strikingly abundant are proteins of the tetraspanin family (CD9, CD63, CD81, CD82) (van Niel *et al.* 2006; Thery *et al.* 2009). Our observation that cultured neurons secrete innate immune proteins in exosomes indicates a new unexplored role of exosomes in immune function regulation. Moreover, since exosomes reflect the cell's content, they provide a means for 'liquid biopsy', and the cell- and condition-specific cargos may be used as potential biomarkers (Clayton 2012; Yokobori *et al.* 2013; Diaz-Arrastia *et al.* 2014; Forde *et al.* 2014; Papa *et al.* 2014; Pouw *et al.* 2014). As shown in our recent publication, CSF from TBI patients contains innate immune proteins that may predict functional outcomes (Adamczak *et al.* 2014).

These data suggest a role for exosomes in the innate immune response after CNS injury in that they shuttle functional innate immune molecules, thus influencing the inflammasome signaling properties within the CNS. These findings show that exosomes loaded with siRNA specifically deliver their cargo and silence ASC, a key component of inflammasome signaling after SCI and TBI (de Rivero Vaccari *et al.* 2008, 2009) in cells of the CNS. Thus, the neuronal-derived exosomes loaded with exogenous genetic cargoes may offer a novel therapeutic strategy to block inflammation after CNS trauma. In support of this idea is the recent report that demonstrates the therapeutic potential of exosome-mediated siRNA delivery to knock down mRNA and protein of the beta-site APP-cleaving enzyme-1 (BACE-1) protein, a therapeutic target in Alzheimer's disease (Alvarez-Erviti *et al.* 2011).

What is clear is that an improved knowledge of the components of inflammasomes and the interactions that govern their function will enhance our understanding of the fundamental mechanisms of inflammatory cytokine production. Such information will be applicable to diverse pathologies, including CNS trauma, mental retardation, seizure, multiple sclerosis, Alzheimer's disease, HIV encephalitis, dementia, and ischemic injury (Dirnagl *et al.* 1999; Block and Hong 2005; Lucas *et al.* 2006). This understanding may give rise to new insights into biologically relevant targets to control inflammatory diseases, altered blood–spinal cord barrier or infections, complementing or replacing existing therapies that are hindered by limited clinical efficacy or excessive adverse complications.

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