Full Title: Exacerbation of Acute Traumatic Brain Injury by Circulating Extracellular Vesicles

Running Title: Inflammatory EVs and TBI

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Abstract

Inflammatory lesions in the brain activate a systemic acute phase response (APR), which is dependent on the release of extracellular vesicles (EVs) into the circulation. The resulting APR is responsible for regulating leukocyte mobilization and subsequent recruitment to the brain. Factors that either exacerbate or inhibit the APR will also exacerbate or inhibit CNS inflammation as a consequence, and have the potential to influence ongoing secondary damage. Here, we were interested to discover how the circulating EV population changes after traumatic brain injury (TBI) and how manipulation of the circulating EV pool impacts on the outcome of TBI. We found the number of circulating EVs increased rapidly after TBI, and this was accompanied by an increase in CNS and hepatic leukocyte recruitment. In an adoptive transfer study, we then evaluated the outcomes of TBI after administering EVs derived from either *in vitro* macrophage or endothelial cell-lines stimulated with LPS, or from murine plasma from an LPS-challenge using the air-pouch model. By manipulating the circulating EV population, we were able to demonstrate that each population of transferred EVs increased the APR. However, the characteristics of the response were dependent on the nature of the EVs, specifically it was significantly increased when animals were challenged with macrophage-derived EVs, suggesting that the cellular origins of EVs may determine their function. Selectively targeting EVs from macrophage/monocyte populations is likely to be of value in reducing the impact of the systemic inflammatory response on the outcome of traumatic CNS injury.

Key words: traumatic brain injury, inflammation, blood brain barrier, extracellular vesicles, microglia

Introduction

Whilst the causes of traumatic brain injury are largely unavoidable, the ensuing inflammatory response to the injury may be susceptible to therapeutic intervention.[1] However, targeting inflammation within the brain is problematic owing to the presence of the blood-brain barrier. It has become clear that the magnitude of the inflammatory response within the brain is dependent on the degree of activation of the acute-phase response (APR) in the periphery and in the liver in particular.[2,3] The anatomical and molecular pathways that carry injury signals from the brain to the liver to activate the APR are poorly understood.[4] However, inhibition of the APR using neutralising agents directed against acute-phase proteins[5] or by the inhibition of NFkB[6] has shown that it is possible to ameliorate inflammation in the brain by suppressing the hepatic APR. Thus, targeting the afferent signalling pathways from the brain to the liver may provide some level of neuroprotection after acute traumatic brain injury (TBI).

Simple humoral or neural pathways were considered to be responsible for initializing the APR after TBI, but other studies have cast doubt on these claims; vagotomised animals still exhibit a peripheral response to CNS injury[7] and although the ratio of local and systemic cytokine/chemokine production after CNS injury has been found to be a critical factor dictating local leukocyte recruitment,[8] no cytokine/chemokine route between the CNS and the periphery has been established.[4] The emerging field of extracellular vesicles (EVs)[9] provides a potentially promising vehicle for brain-liver communication after TBI[10]. EVs are divided into three main categories depending on their biogenesis[11]: (1) exosomes (40-150nm) are small EVs that are released from endosomes; (2) microvesicles, also referred to as ectosomes and microparticles (150nm-1000nm), bud directly from the plasma membrane; and (3) apoptotic bodies (1000nm-5,000nm) are secreted after programmed cell death. EVs of types 1 and 2 are secreted by almost all cell types and are known to be released by neurons, astrocytes,[12] endothelial cells,[13,14] microglia[15,16] and oligodendrocytes in the CNS.[17] Indeed, EVs derived from the circulation of an animal following a CNS inflammatory challenge have been

shown to activate the hepatic APR, and are likely to be largely, although not necessarily exclusively, of endothelial cell origin.[18] Thus, EVs present a novel form of paracrine communication after CNS injury and may induce the hepatic APR responsible for secondary damage after TBI.

Here, we show that CNS trauma is directly correlated with an increase in circulating EV concentration. Furthermore, we have demonstrated that by manipulating the circulating EV pool by delivering EVs derived from an inflammatory background it is possible to augment the APR, and exacerbate TBI, in a cell-line-dependent manner. This data introduces, for the first time, a new level of functionality for circulating EV populations after traumatic CNS injury.

Materials and Methods

Animals: Male C57BL/6 mice, 8-10 weeks of age, were housed under standard diurnal lighting conditions (12 hours) with *ad libitum* access to food and water. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 and licenced protocols were approved by local committees (LERP and ACER, University of Oxford) and carried out under licence number 30/3076. For surgery, animals were anaesthetized in a 2% isoflurane/oxygen mix (2 L/min) and placed in a stereotactic frame (Stoetling Co., USA) under maintenance anaesthesia (1.5%). All efforts were made to adhere to the ARRIVE guidelines at all times.

Traumatic Brain Injury (TBI) model: A well characterised Controlled Cortical Impact (CCI) model was employed to simulate a traumatic brain injury (TBI).[19] The instrument was composed of a linear unit, consisting of an interconnected motor and ram, as well as a microprocessor (Hatteras). The size and shape of the impactor tip, velocity of the impact, contusion depth and dwell time could be adjusted to vary injury severity. For this study, a 1.1mm stainless steel flat tip with bevelled edge was used with a speed of 1.5m/s and dwell time of 85ms to a depth of 0.5mm.

Surgery: Following excision of the scalp, a circular craniotomy of 2mm diameter was performed at Bregma A/P -2mm, M/L -2mm. Impactor tip was then centered at the craniotomy site, perpendicular

to the exposed brain. The tip was then lowered until it made contact with the dural surface without tissue deformation. After an upwards retraction of 20mm, the tip advanced a total of 20.5mm into the somatosensory cortex to induce the TBI. Post-TBI, bleeding was allowed to resolve before the skull section was replaced and the scalp was closed using glue. Animals were then allowed to survive for 2 hours, 6 hours, 12 hours and 24 hours before culling and tissue collection. Sham operated mice underwent craniotomy, but not cranial trauma. All animals received sub-cutaneous saline in the immediate post-operative period and wound sites were treated with Marcaine.

Tissue collection: Blood was collected via cardiac puncture into heparinised needles. Animals underwent intracardiac perfusion with heparinised saline and fresh spleen and liver tissue were snap frozen at -80^{o}C for PCR. Tissue was then fixed by intracardiac perfusion with 4% paraformaldehyde (PFA), and brain, liver and spleen were collected and cryoprotected using 30% sucrose. Tissue was cut at 10-12µm with Leica cryostat.

Immunohistochemistry: Immunostaining was performed as previously described using an avidin-biotin-peroxidase method using 3,3'-Diaminobenzidine (DAB, Abcam) to visualize the stain.[20] To assess cellular changes, brain sections were stained for neutrophils (MBS; made in house (rabbit antibody harvested from a rabbit immunized with whole rat neutrophils and stimulant *Megathura crenulata* haemocyanin); 1:1000 as previously described[21,22]) and Iba1+ microglia (Iba-1; AbCam ab178847, Cambridge, UK; 1:500) with a cresyl violet counterstain. BBB breakdown was assessed by quantifying the presence of serum in the parenchyma (IgG; VectorLabs, UK BA-9200; 1:200) and lesion volume was established using a cresyl violet stain. Liver and splenic tissues were stained with MBS and Iba-1 with haemotoxylin counter stain.

Quantification of macrophage and neutrophil number in liver and spleen: Quantification of splenic and hepatic macrophages and neutrophils, positive for Iba-1 and MBS respectively, was performed in five representative fields at 40x where the average number of positive cells was calculated and

expressed as number of cells per mm^2. The five fields were selected from three 10µm sections chosen at random from the liver or spleen.

Quantification of blood brain barrier (BBB) breakdown: IgG staining density was used as a surrogate marker for BBB breakdown and was analysed using imageJ (version 1.8.0_101) software on images of whole coronal sections. The threshold of the images was adjusted after subtracting an appropriate background to ensure that only specific staining was quantified. Total area of IgG stain was measured, as well as stain intensity to quantify the amount of tissue IgG.

Quantification of CNS microglia and neutrophil numbers: Neutrophil and microglial numbers were quantified from sections -1.6mm to -2.8mm relative to Bregma in 10µm sections at 100µm intervals. The region of interest in each slice was specified by the area of neuronal loss (as defined by absence of cresyl violet staining) and area of infiltration of MBS+ neutrophils or Iba-1+ microglia using a Leica microscope. Total number of cells across 12 sections was quantified and expressed as average number of cells per mm^2.

Quantification of lesion size: Lesion size was determined by measuring the area of dead or dying cells in the cortex following TBI. Sections were stained with cresyl violet and the lesion was traced with the camera lucida (Leitz Laborlux). Tissue was considered dead or dying when cresyl violet staining was reduced and cells were small and deformed with no visible nucleolus. The average area of cell death across 3 sections per animal was calculated with ImageJ.

Cell culture: Murine brain vascular endothelial cells (bEND.3; ATCC® CRL-2299™) and murine macrophages (RAW 264.7; ATCC® TIB-71™) were cultured under standard cell culture conditions (5% CO_2 in air, 37°C humidified) using readily available cell culture medium (DMEM, 10% EV-free foetal calf serum and 1% antibiotics). Cells were stimulated with 10ng/mL LPS for 6 hours prior to harvesting of EVs from supernatant as above. EV-free serum was generated in house by ultracentrifuging standard heat-inactivated FCS for 18 hours at 120,000*g*.

Air pouch model: The air pouch model of systemic inflammation was performed using C57BL/6 mice as previously described[23]. Mice were injected with 2.5mL of sterile air twice under general anaesthesia; 48 hours apart. 24 hours after the second sterile air injection, the mice were injected with 1mL of 1.5mg/kg LPS. 6 hours post-injection, animals were culled and blood was collected via heparinised needle.

EV isolation: Blood from animals was spun at 2,700*g* for 10 minutes at 4ºC to separate plasma and remove platelets. Platelet-free plasma was then ultracentrifuged at 120,000*g* for 120 minutes to pellet the EVs, which were then resuspended in phosphate buffered saline (PBS, Thermo Fisher Scientific). For cell culture supernatant, dead cells and debris were spun at 3,000*g* for 10 minutes at 4^{o}C prior to ultracentrifugation at 120,000*g* for 120 minutes to pellet EVs, which were resuspended as above. All ultracentrifugation steps were performed using the Beckman Coulter (Brea) Type 70 Ti rotor at 4°C.

Tuneable resistive pulse sensing (TRPS): TRPS measurements were performed with the qNano (Izon) on plasma isolated EVs. A NP150 nanopore was used to accurately measure particles between 80nm and 200nm. CPC100B calibration particles at dilutions of 1:500, 1:1000, 1:3000 and 1:5000, at pressures 5, 7 or 10 were used to calibrate the pore. Calibration curves were run until 1000 particles had been counted. Samples were diluted 1:50 and the particle rate curve was run at corresponding calibration pressures until 500-1000 particles had been counted. Samples were calibrated and evaluated at 5nm intervals.

Transmission Electron Microscopy (TEM): TEM imaging was used to validate the presence of EVs in our pellet. Plasma-derived EVs were fixed in 2% PFA and diluted 1:20 in PBS for optimum imaging. 10µl of isolated EVs were applied to freshly glow discharged carbon formvar 200 mesh copper grids for 2 minutes before being blotted with filter paper. The grids were then stained with 2% uranyl acetate for 10 seconds, blotted and air dried. Grids were imaged in a FEI TEcnai 12 TEM at 12kV (GAtan OneView CMOS camera).

Western blotting: Total protein was extracted from plasma-derived vesicle fractions using RIPA buffer and quantified using a micro BCA kit (Thermo Scientific, UK). 25µg total protein extracted from EVs was loaded for electrophoresis, in parallel with protein extracted from whole brain lysates as a control, and transferred onto a PVDF membrane. Blotting was performed overnight using antibodies against vesicle markers Tsg101 (4A10 Ab83; AbCam, Cambridge, UK; 1:250 in 5% BSA), Alix (3A9 ab117600; AbCam; 1:500 in 5% milk), CD31 (AbCam, UK; 1:2000 in 5% milk), CD68 (AbCam, UK; 1:2000 in 5% milk) and histone-H3 (DIH2; New England Biolabs, UK; 1:2000 in 5% milk). Detection was performed using horse-radish peroxidase conjugated secondary antibodies (1:10,000) and blots were visualized using chemiluminescence and a ChemiDoc (Bio-Rad, UK).

EV adoptive transfer: C57BL/6 mice received an i.v. injection of 150uL of either sterile saline, plasma-derived EVs isolated from the air pouch model, EVs isolated from murine macrophages (RAW264.7) or EVs isolated from brain vascular endothelial cells (b.END3) immediately after TBI. Animals were allowed to survive for 6 hours before fresh and fixed liver, and fixed brain were collected as previously described. Before the injection of EVs derived from plasma, macrophages or endothelial cells into TBI animals, their concentration and size were measured using TRPS. A ~10x higher concentration of plasma-derived EVs was injected in comparison to RAW 264.7 macrophage-cell-line-derived EVs due to the lower specificity of plasma-derived EVs as macrophage-derived and endothelial cell-derived EVs will originate from one cell-line only, whereas plasma-derived EVs are originating from a multitude of cell types and represent only a small proportion (c.10%) of the EVs in the circulation.[23] Macrophage-derived and endothelial cell-derived EV injections were adjusted to the same concentration of 9.81×10^8 particles/mL before injection.

RNA extraction and cDNA conversion: RNA extraction was performed on snap-frozen liver tissue using the Qiagen® RNEasy Mini Kit (Qiagen®, UK) as per manufacturer's instructions. Eluted RNA was quantified using a Nanodrop and considered acceptable for cDNA conversion if 260/280 and

260/230 ratios were >2. 1000ng whole RNA was converted to cDNA using the high capacity cDNA kit (Applied Biosystems, UK), as per manufacturer's instructions.

Quantitative PCR: Real-time quantitative PCR was performed on 25ng cDNA using SYBR green and the relative standard curve method, as described previously.[24] Forward and reverse primers for *tnf* (F: GCCTCCCTCTCATCAGTTCTAT, R: TTTGCTACGACGTGGGCTA), *il1β* (F: CAACCAACAAGTGATATTCTCCAT, R: GGGTGTGCCGTCTTTCATTA), *il6* (F: TCCATCCAGTTGCCTTCTTG, R: GGTCTGTTGGGAGTGGTATC), *cxcl1* (F: GCTGGGATTCACCTCAAGAAC, R: TGTGGCTATGACTTCGGTTTG), *cxcl10* (F: CATCCCGAGCCAACCTTCC, R: CACTCAGACCCAGCAGGAT) and *saa2* (F:, R:) were used.

Statistical analysis: All data was analysed using Graphpad Prism 6 software. Analysis of data was performed using two-way analysis of variance (ANOVA) or one-way ANOVA as appropriate. Data was considered significant at $p<0.05$. Tukey or Holm-Sidak's multiple comparisons post-hoc testing was applied as appropriate. All data sets are presented as mean ± SEM, n are included in figure legends.

Results

Cortical impact results in blood brain barrier breakdown and localized inflammation

Here we wished to perform a basic characterization of a model of cortical impact where the centre of the lesion was positioned posterior to Bregma, at the level of the posterior parietal association area. This position was chosen to be clinically silent, with minimal behavioural effects, in order to focus study on the molecular pathology (supplementary Fig. S1). To assess BBB breakdown, infiltration of serum IgG was used, quantifying both the volume of tissue positive for IgG immunoreactivity (Fig. 1A) as well as the density of staining (Fig. 1B). Sham animals showed no BBB breakdown at any time after surgery, as such only one sham column has been included in the graph but shams for each time point were included in the analysis, at no time point were shams any different from one another. The volume of IgG infiltration increased with time after TBI (Fig.1C) with

significant differences found between the volume of tissue containing IgG after 2 hours and 6 hours compared to 24 hours (Fig. 1A; two-way ANOVA; injury $p<0.0001$; time $p<0.01$; injury:time $p<0.01$). The volumes after 2 hours, ($0.7725\pm0.4253mm^3$), 6 hours ($1.37\pm0.4981mm^3$) but not 12 hours ($4.332\pm1.575mm^3$) were found to be significantly smaller than 24h post-TBI ($6.965\pm1.553mm^3$; Tukey post-hoc vs 2 hours $p<0.01$, vs 6 hours $p<0.05$). IgG density is correlated with the amount of IgG that has 'leaked' into the tissue. It is accepted that this is a semi-quantitative measure. IgG density increased over time (Fig. 1B; two-way ANOVA; injury $p<0.0001$; time $p<0.01$; injury:time $p<0.01$). The most intense IgG stain was observed at 24 hours post-TBI which gave an intensity of 138.4 ± 5.918 (arbitrary units), which was significantly greater than the densities at 12 hours (57.31 ± 18.10; Tukey post-hoc $p<0.01$), 6 hours (27.74 ± 19.24; $p<0.01$) and 2 hours (8.6 ± 4.397; $p<0.0001$) post-TBI (Fig. 1B).

Lesion size was measured by quantifying the area of dead and dying neurons on a Nissl stain (Fig. 1D & E; two-way ANOVA; injury $p<0.0001$; time $p<0.01$; injury:time ns). TBI animals had significantly larger lesions at 6 hours ($0.647\pm0.1310mm^3$; Tukey post-hoc; $p<0.05$), 12 ($1.092\pm0.2588mm^3$; $p<0.01$) and 24 hours ($1.307\pm0.1875mm^3$; $p<0.001$) after TBI when compared to sham animals. Lesion size increased with time, with area of cell death significantly greater at 24 hours compared to 2 (Tukey post-hoc test; $p<0.05$) and 6 hours ($p<0.05$), but not 12 hours.

Any traumatic CNS injury is associated with some degree of CNS inflammation. Here, we investigated infiltrating polymorphonuclear cells, as well as Iba-1+ microglia. Total brain neutrophil infiltration into the lesion was found to be significantly higher at later time points (Fig. 1F & G; two-way ANOVA; injury $p<0.0001$; time $p<0.0001$; injury:time $p<0.0001$). Post-hoc testing (Tukey) revealed significant differences between 2 hours and 6 hours post-TBI (4.33 ± 2.333 and 4.5 ± 1.936, respectively) when compared to 12 hours, 59.25 ± 5.105 (2 hours $p<0.01$ and 6 hours $p<0.05$). Animals surviving 24 hours post-TBI (80.33 ± 13.91) also had significantly higher CNS neutrophils compared to the 2 or 6 hour time points post-TBI (2 hours $p<0.001$; 6 hours $p<0.01$).

As well as an increase in neutrophil number, there was also a significant difference in the number of Iba-1+ microglia found after TBI (43.96±3.11/mm^2) when compared to sham surgery (23.14±1.215/mm^2; Fig. 1H & I; two-way ANOVA; injury $p<0.001$; time ns; injury:time ns). Whilst this general increase in Iba-1+ cells and did not change over time, it is interesting to note that when Iba-1-positivity was assessed at regular intervals laterally from the core of the lesion (similar to analysis and digitization performed previously[21]), the spread of Iba-1+ microglia increased with time, with Iba-1+ cells being found as far as 1.2mm away from the epicentre of the lesion by 24 hours (supplementary Fig. S2).

It should be noted that this lesion generates a significant systemic inflammatory response. Peripheral macrophage and neutrophil recruitment were examined in the liver and spleen (supplementary Fig. S3). Hepatic macrophage numbers were not affected by TBI whereas splenic macrophages were (two-way ANOVA; injury $p<0.01$; time ns; injury:time ns), and neutrophil infiltration was increased at all times after injury in both liver (two-way ANOVA; injury $p<0.0001$; time $p<0.05$; injury:time ns) and spleen (two-way ANOVA; injury $p<0.0001$; time $p<0.5$; injury:time ns).

TBI causes an increase in circulating EV numbers

To assess the concentration of EVs circulating in the plasma, pelleted EVs were analysed using TRPS. Compared to sham, TBI caused a significant increase in EV number in the circulation independent of time (Fig. 2A; two-way ANOVA; injury $p<0.05$; time ns; injury:time ns). A trend was observed between sham and TBI animals where increasing survival time appeared to be associated with an increase in EV number. 24 hours gave the highest concentration of circulating EVs ($4.27x10^{11}$ particles/mL). However, this did not reach significance. When the mean diameters of the particles were compared, we observed that the vesicles decreased in size after injury (Fig. 2B; two-way ANOVA; injury $p<0.01$; time $p<0.01$; injury:time ns). Post-hoc testing (Tukey) revealed that the mean diameter at 24 hours (94.33±2.404nm) was significantly different from both 6 hours

(101.67±7.12nm; $p<0.05$) and 12 hours (102.66±2.88nm; $p<0.01$), indicating that smaller EVs get released with longer survival times. All EV fractions isolated from plasma showed a standard distribution when analysed with TRPS (Fig. 2C-F). EVs were confirmed via TEM and Western blotting as per ISEV guidelines and our previous work.[22,25,26] TEM revealed the presence of EVs of both microvesicle and exosome biogenesis, based on size (Fig. 3A). Western blotting showed plasma derived EV samples were positive for EV markers TSG101, Alix and CD31, and negative for cell lysate controls CD68 and H3 (Fig. 3B). These results indicate the presence of a mixed population of EVs (exosomes and microvesicles) after isolation without cellular contamination.

Exogenous inflammatory EVs exacerbate acute injury following TBI

To determine the degree to which the composition of circulating inflammatory EVs contributes to the outcomes of TBI, we established a model of systemic inflammation using an LPS air-pouch model (a standard method for pyrogen testing; *see* materials and methods), as well as using *in vitro* models of inflammation using LPS-challenged mouse macrophages (RAW 264.7) or brain vascular endothelial cells (b.END3). EVs are released into the circulation after injury in a rapid manner, thus we aimed to look at an acute time point after injury (6 hours) in order to determine what effects early manipulation of the circulating EV pool had.

The volume of IgG extravasation increased when EVs were injected into the bloodstream (Fig. 4A, B & D; one-way ANOVA; $p<0.01$). In post-hoc testing (Holm-Sidak), significant differences were found between IgG volume after plasma-derived EV injection (5.442±0.4987mm^3; $p<0.01$), macrophage-derived EV injection (5.411±0.5882mm^3; $p<0.01$) and endothelial cell-derived EV injection (5.398±0.3043mm^3; $p<0.01$) when compared to a sterile saline injection (2.636±0.5018mm^3). The most intense IgG stain was observed in the macrophage-derived EV injected group (Fig. 4B; 56.11±6.606). This was the only treatment found to be significantly different when compared to the sterile saline injected group in post-hoc tests (36.84±14.30; $p<0.05$). The area of cell death in the brain was also exacerbated by exogenous EVs (Fig. 4C & E; one-way ANOVA; $p<0.05$). Animals

injected with endothelial-derived EVs (2.689±0.3315mm^2) had significantly larger lesions than saline injected animals (1.164±0.2445mm^2) as determined by Holm-Sidak post-hoc test ($p<0.01$). Animals injected with plasma- and macrophage-derived EVs also exhibited greater cell death, with lesion sizes of 2.066±0.2262mm^2 and 1.816±0.3138mm^2 respectively, however these data did not reach significance. In addition to the investigation of structural changes in animals challenged with exogenous inflammatory EVs, we also investigated the degree to which CNS microglia and recruited neutrophil populations were affected by the manipulation of the circulating EV pool. Significant differences in microglial numbers/mm^2 were found in animals injected with EVs derived from plasma and cell culture (Fig.4F; one-way ANOVA; $p<0.01$). Post-hoc testing (Holm-Sidak) showed that significant increases in microglial number in plasma-derived EV injected animals (80.87±6.502/mm^2; $p<0.01$), macrophage-derived EV injected animals (79.49±2.95/mm^2; $p<0.01$) and endothelial cell-derived EV injected animals (71.1±1.988/mm^2 $p<0.05$) when compared to controls (52.65±5.511/mm^2). In animals receiving a TBI with a saline challenge, there was an average of 49.8±6.367 neutrophils in the brain which increased upon challenge with exogenous inflammatory EVs (Fig. 4G; one-way ANOVA; $p<0.05$). Animals receiving EVs from LPS-challenged macrophages displayed an average of 101.3±12.7 neutrophils, which was significantly elevated when compared to animals receiving a saline injection ($p<0.05$).

EV origin determines the degree of hepatic inflammation

By manipulating the circulating pool of EVs through delivery of 'primed' exogenous vesicles, we set out to find how this may affect both TBI, and its associated APR. Here, we examined both the recruitment of immune cells to the liver and spleen, as well as cytokine and chemokine mRNA expression in the liver in animals whose circulating pool of EVs had been manipulated with exogenous EVs from inflammatory sources. Plasma-derived EVs from the LPS air-pouch model and macrophage-derived EVs from LPS-stimulated RAW cells had the most significant effects on neutrophil recruitment, but little effect on macrophage recruitment, to both the liver and spleen

(supplementary Fig. S4). In the liver, the macrophage-derived EV injected group was found to be significantly different compared to the sterile saline group for all of the transcripts examined, with fold changes as high as 66.9 for the relative expression of *cxcl10* (Fig. 5E). In the animals injected with endothelial cell-derived EVs the gene expression varied significantly for *cxcl10* ($p<0.05$), and *il1β* ($p<0.01$) when compared to the relative gene expression of the saline injected animals (Fig.5; one-way ANOVA; Holm-Sidak post-hoc test). For the plasma-derived EV group, the relative gene expression levels did not vary significantly from the saline-injected group across for all transcripts except *saa2* (Fig. 5F). The plasma-derived EV injected animals displayed a significantly higher fold change for *saa2* (8.713±1.237) compared to the saline injected animals (Fig. 5F; one-way ANOVA; 2.060±1.097; Holm-Sidak post-hoc test; $p<0.05$).

Discussion

In this study we have shown, for the first time, that manipulating the circulating pool of EVs immediately after traumatic CNS injury significantly worsens lesion area, blood brain barrier breakdown and systemic inflammation. We have shown that traumatic injury to the CNS in the mouse results in an elevation of circulating EVs and that by enriching this population, particularly with LPS-stimulated macrophage-derived EVs, we can exacerbate the inflammatory response to TBI. To our knowledge this is the first time that circulating EVs have been shown to influence the outcome of traumatic CNS injury and significantly opens the field of EV research in trauma.

The model of TBI used in this study was focused on the posterior parietal association area, rather than either the sensory or motor cortices. The lack of significant behavioural effects resulted in a clinically silent lesion where we were able to study the CNS pathology and its effects on the systemic immune system in relative isolation. The model used here generated a typical lesion which increased in size over time, and showed classic signs of CNS inflammation including microglial activation, IgG infiltration and neutrophil recruitment. BBB breakdown was observed at all time points in this model. As increased barrier permeability and higher numbers of CNS neutrophils play key roles in

the progression of CNS inflammation,[27] understanding more about the mechanisms which may lead to this recruitment is fundamental to the development of acute therapies for traumatic injury. Leukocyte infiltration has been shown to rely on systemic events that follow CNS injury.[28] For example, the hepatic APR brings about mobilisation of leukocytes from reservoirs within the bone marrow and the cells then travel to the site of injury, as well as, surprisingly, to the liver itself.[29 30] These events occur very rapidly with reports of hepatic activation even prior to CNS immune activation in injury models.[31] As such we chose to investigate the mechanisms of this communication at a very early time point, 6 hours, where the CNS injury has yet to establish itself, but where systemic inflammation and CNS inflammation have begun to be observed. By studying and manipulating the pool of circulating EVs at this acute time point we may begin to understand the means by which the populations of peripheral immune cells are mobilised after the initial injury.

The BBB is uniquely placed to signal CNS injury to the systemic immune system, and as a consequence recruit immune cells to the parenchyma. There are two major routes which may mediate this communication: neural and humoral. Vagotomised animals still demonstrate a significant APR,[7] and the transport of centrally induced cytokines and chemokines is incredibly complex, with little egress of pro-inflammatory mediators from the inflamed brain.[32] Recent reports using choroid plexus explants and cells suggest that epithelial cells in this scenario are capable of secreting EVs as part of this communication system.[33] We were therefore interested to discover whether circulating EVs might be the means of communication between the CNS and the liver in TBI.

Previous reports, in line with the data herein, have shown that TBI, in both mouse and human, results in an increase in the number of circulating EVs.[34-36] In addition, in this study we have shown that the size of the EVs was different after TBI; showing a slight trend towards decreased mean diameter. This suggests a shift from microvesicles to exosomes in the acute phase after injury. It is possible that the level of barrier breakdown, or internal CNS inflammatory mechanisms, might play a role in this switch from larger EVs to smaller exosomes but the reasons for, and the functional

results of this change remain speculative. Recent studies have suggested that exosomes retain the cargo necessary for functional transcription of miRNA species, whereas microvesicles do not[37]. Taken with reports showing that EVs carry specific miRNA transcripts after TBI,[38] and data from this study showing EVs of different origins express different miRNAs (supplementary Fig. S5), this data could suggest a shift towards exosomes for the purposes of increasing miRNA regulation of gene transcription post-injury. However, experiments beyond the scope of this study are required to confirm this theory.

Given the increases in EVs after injury, we were interested to discover what impact manipulating the circulating EV pool had on the outcomes of CNS trauma. The leading cause of mortality after TBI is systemic infection and consequent multisystem organ failure.[39] However, the processes underlying this are very poorly understood. We aimed to shed some light on the issue by investigating whether EVs from primed sources were able to bring about inflammation in the periphery, and how that influences the outcomes of TBI. We chose to use EVs derived from cells from the CNS barrier system, as they are uniquely placed to respond to CNS trauma and have been shown to exert effects on the liver in inflammatory brain injury models[22], EVs derived from cells of the systemic immune system, which are known to mobilize after CNS injury, and EVs derived from an *in vivo* inflammation model to demonstrate the effects of a more heterogenous EV population.

We were able to successfully activate the systemic inflammatory response via adoptive transfer of EVs from animals with systemic inflammation (LPS air-pouch model) and from cell lines activated with LPS. This fills in some of the gaps in the literature as, to our knowledge, no existing studies have evaluated the effect of exogenous, activated EVs on CNS inflammation in an injury model. Our results demonstrate that EVs from different cell populations are capable of uniquely exacerbating TBI, when exogenously administered immediately post-injury. Strikingly, activated macrophages in particular induce a marked systemic APR and increase in neutrophil recruitment to the injured brain, suggesting some degree of cellular specificity when it comes to EV-related injury signals. Plasma-

derived EVs from our LPS air-pouch model had less of a pro-inflammatory effect on our TBI model. This could be due to a number of reasons. The population of EVs present in the plasma will be extremely heterogenous, with large numbers yet to be identified with common cellular markers.[23] As such, it could be hypothesized that LPS *in vivo* results in the generation of pro- and anti-inflammatory EVs from different cell populations. Unfortunately, as yet there are few reliable techniques for identifying the origins of EVs in a mixed population although specialized flow cytometry is beginning to advance the field.[40]

Previous work from our laboratory has demonstrated that the number of leukocytes recruited to the injured CNS could be increased by priming the animal with systemic injections of hepatic chemokines.[30] Based on the data presented here it could be hypothesized that our EVs might contain similar chemokines, or that they were able to inherently activate the liver, which in turn secretes chemokines activating leukocyte mobilisation. Our previous studies have demonstrated that EVs from an inflammatory brain model do not contain significant amounts of IL-1β,[22] however, primed EVs from cell culture have been reported to contain both IL-1β and TNF-α[9], both of which are capable of initiating an APR.[41] As a consequence of our findings, efforts directed towards preventing the EV population from reaching the liver might be of therapeutic importance.

Conclusion

To our knowledge this study is one of the first to investigate and differentiate inflammatory changes in both the periphery and CNS, as well as quantify EV populations in the bloodstream, during the first 24 hours following traumatic injury to the CNS. Furthermore, this is the first evidence showing that peripheral inflammation can be brought by manipulating the circulating EV population with 'primed' EVs in order to exacerbate CNS injury. This study highlights the need for some important further work to understand the both the differences in the molecular profile of EVs produced in response to injury, and to determine the exact routes of their communication, from biogenesis to uptake. Such experiments will lead to an improved mechanistic understanding of the physiological

events that are set in motion in the acute stages following a traumatic CNS injury, and place us in a better position to develop novel therapeutic strategies.

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Author Disclosure Statement

No competing financial interests exist.

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