

Astrocyte-shed extracellular vesicles regulate the peripheral leukocyte response to inflammatory brain lesions

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Abstract

Brain injury induces a peripheral acute cytokine response (ACR) that directs the transmigration of leukocytes into brain. This brain-to-peripheral immune communication affects patient recovery, thus understanding how it is regulated is important. Contrary to expectations it is not regulated by sympathetic innervation. Using a mouse model of an inflammatory brain injury, we set out to find a soluble mediator for this phenomenon. We found that extracellular vesicles (EVs) shed from astrocytes in response to intracerebral injection of interleukin-1 β (IL-1 β) rapidly entered into peripheral circulation and promoted the transmigration of leukocytes through modulation of the peripheral ACR. Bioinformatic interrogation of the protein and miRNA cargo of EVs identified XXXXXX Peroxisome proliferator-activated receptor- α (PPAR- α) as a primary molecular target of astrocyte-shed EVs. We confirmed in mice that astrocytic EVs promoted the transmigration of leukocytes into the brain by inhibiting PPAR α resulting in the increase of NF- κ B activity thus, triggering the production of cytokines in liver. These findings expand our basic understanding of the mechanisms regulating communication between the brain and peripheral immune system, and identify astrocytic EVs as a molecular regulator of the immunological response to inflammatory brain damage.

Introduction

The historic concept that brain responds to immune challenges independent of the peripheral system has been amended in recent years. The brain is now more accurately described as 'immunologically specialized', owing to the atypical nature of responses to injury that involve bidirectional communication between the brain and peripheral immune systems (1, 2). Brain injuries such as stroke, trauma, and inflammatory lesions are associated with both an acute injury and secondary damage that involves the infiltration of immune cells that can continue for weeks to months post-injury (3, 4). The infiltration of immune cells into brain involves the induction of a peripheral acute cytokine response

(ACR) that primes leukocytes to transmigrate to the site of brain damage. Although the peripheral ACR to brain damage occurs in multiple peripheral organs, it is strongest in liver, and is associated with induction of inflammatory cytokines, chemokines, and acute-phase proteins that play essential roles in priming leukocytes for transmigration to the site of brain injury (5-10). Despite decades of research the precise mechanism by which the injured brain instructs the peripheral immune system remains unknown. Resection of the Vagus nerve has been shown to block some elements of this communication in a protective fashion following stroke (11). However, data from focal inflammatory lesion studies in sympathectomised and parasympathectomised animals suggests that the communication of brain injury to the peripheral immune system does not require intact nerve fibers (12), but does involve unidentified blood-borne factors that initiate the liver ACR (13). Here we show that astrocyte-shed extracellular vesicles (EV) promote leukocyte transmigration through regulation of the ACR to interleukin-1 β (IL-1 β)-induced inflammatory brain lesion.

Results

Brain nSMase2 and ceramide regulate leukocyte trafficking in response to IL-1 β induced brain injury

The inflammatory cytokine IL-1 β is a potent regulator of the inflammatory response to brain injury (14-19). Based on evidence that ligation of the IL-1 β receptor rapidly activates a neutral sphingomyelin hydrolase (nSMase; 20), we first determined if this pathway contributed to the influx of leukocytes in response to a focal IL-1 β lesion. Intra-striatal injection of IL-1 β promoted an influx of Ly6b⁺ leukocytes to the lesion site that was reduced in mice expressing a mutated form of nSMase2 (*m-smpd3*; Fig. 1A,B) (21). As

the *m-smpd3*^{fro/fro} mouse expresses an inactive form of nSMase2 in all tissues, we determined whether inhibition of brain nSMase was sufficient to inhibit leukocyte influx in response to IL-1 β . Co-administration of IL-1 β with the non-competitive nSMase2 antagonist GW4869 reduced leukocyte influx, while co-administration of IL-1 β with a general nSMase antagonist altenusin (known to inhibit multiple isoforms of nSMase; 22) produced near complete blockade of leukocyte influx (Fig. 1A,C). As altenusin also inhibits the protein tyrosine kinase p60c-Src (23), we administered a selective inhibitor of p60^{c-src} (PP1; 24) with IL-1 β and found no reduction in leukocyte recruitment (fig. S1A,B). IL-1 β rapidly increased long chain ceramides (C16:0-C20:0; Fig. 1D, and fig. S1C) with corresponding decreases in long chain sphingomyelins (fig. S1D), but had no effect on brain ceramide in *m-smpd3* mice (Fig. 1D,S1C). Injection of IL-1 β , but not saline was associated with a robust microglia and astrocyte activation (Fig. 1E, S1E), and increased brain expression of IL-1 β , TNF α , CCL2, and IL17 (Fig. 1F). Microglial activation, and induction of cytokine expression were reduced to levels not different than control by simultaneous administration of IL-1 β + altenusin (Fig. 1E,F,S1E). As the Ly6b antibody may also detect macrophage precursors, we confirmed the identification of leukocytes using a second leukocyte specific antibody (MBS), and co-stained sections with IBA-1 to identify microglia. None of the MBS+ cells in striatum of mice administered IL-1 β were dual positive for IBA-1 (fig. 1SF). These data suggest that IL-1 β induction of ceramide, microglia activation, cytokine expression, and leukocyte influx are regulated by brain nSMase.

Following inflammatory brain injury peripheral leukocytes transmigrate to the site of brain injury by a mechanism that is dependent on a peripheral ACR (5, 10). Therefore, we determined if brain nSMase regulates the ACR in liver, lung and spleen, as these are the

three major organ systems implicated in the ACR response to brain injury (10, 25, 26). Intra-striatal administration of IL-1 β was associated with a rapid induction of the inflammatory cytokines IL-1 β , IL6, TNF α and the chemoattractant CCL2 in liver (Fig. 1G), with a similar cytokine induction in lung (+ IL-17) (Fig. 1H), with a less robust response in spleen (IL-1 β and CCL2 only) (Fig. 1I). Co-injection of IL-1 β + altenusin prevented the ACR in liver, and lung but not in spleen (Fig. 1, G-I). Twenty-four hours following striatal IL-1 β administration, liver and lung ACR diminished to control levels with the exception of liver CCL2 (fig. S2A,B). The spleen ACR response was sustained for at-least 24h following intra-striatal infusion of IL-1 β with a progressive increase in TNF α expression (fig. S2C). Thus, the ACR following inflammatory brain lesion is regulated in liver by mechanisms dependent on brain nSMase, while the ACR in lung is partially, and spleen entirely independent of brain nSMase.

Protein and miRNA cargo of astrocyte-shed EV regulate the peripheral acute cytokine response to inflammatory brain lesion

Based on data suggesting that ceramide and nSMase regulate the production and release of EV (27, 28) we reasoned that IL-1 β may induce the release of EV that enter into peripheral circulation to regulate the ACR. Striatal injection of IL-1 β resulted in a trend towards increased circulating EVs in plasma at 2h, with significant increases at 24h from $1.6 \times 10^{10} \pm 6.6 \times 10^9$ in control (not injected) to $5.8 \times 10^{10} \pm 1.0 \times 10^{10}$ in IL-1 β injected mice. Circulating EVs in mice injected with saline were not different from control ($2.3 \times 10^{10} \pm 7.7 \times 10^9$) (fig. S2, D,E). We isolated EV from the plasma of mice injected intra-striatal with either saline or IL-1 β , and infused 3.0×10^9 EV (fig. S3, A-D) into the tail veins of recipient mice with a central ACR block produced by administration of IL-1 β + altenusin

(Fig. 2A). The adoptive transfer of EV from mice injected with IL-1 β induced liver expression of IL-1 β , IL-6, TNF- α and CCL2 (Fig. 2B), and restored the transmigration of leukocytes to the site of IL-1 β + altenusin injection (Fig. 2C,D). Twenty-four hours after the adoptive transfer of EV, the liver ACR returned to basal levels with the exception of CCL2 (fig. S3F). This liver ACR, and the recruitment of leukocytes into striatum following the adoptive transfer of EV into mice with a central ACR block was indistinguishable from the response produced after a simple intra-striatal administration of IL-1 β (compare to Fig. 1, A and G). However, because the brain cytokine response to IL-1 β was blocked by altenusin (Fig. 1F) it was not clear how leukocytes targeted to striatum. We found that although the brain cytokine response was blocked by co-injection IL-1 β + altenusin, focal expression of the vascular adhesion protein ICAM-1 was not affected by central inhibition of nSMase (Fig. 2E). EV isolated from the plasma of mice receiving brain injections of saline, or EV isolated from plasma of mice administered IL-1 β but stripped of protein and miRNA content (fig. S3E) did not induce a liver ACR, and did not restore the transmigration of leukocytes into brain parenchyma (Fig. 2, B-D). These data suggest that the protein and miRNA cargo of EV regulates the peripheral ACR and leukocyte response to an inflammatory brain lesion. Leukocytes appear to target vascular adhesion molecules expressed in response to IL-1 β , and this expression is independent of nSMase2 activation.

While an assortment of CNS cells are capable of shedding EV (29-32) we focused our efforts on astroglia based on the intimate association of these cells with the blood brain barrier (BBB; 33), and observations that astrocytes shed far more EV in response to IL-1 β compared with microglia. IL-1 β induced a rapid release of EV from cultured primary astrocytes ($2.17 \times 10^9/\text{ml} \pm 2.89 \times 10^8/\text{ml}$ EVs were released within 2h in response to a

media change with serum removal, and $2.37 \times 10^9 \pm 2.08 \times 10^8/\text{ml}$ were released in response to IL-1 β), that we visualized using the styryl pyridinium dye FM1-43 (Fig. 3A, Movie S1). EV release occurred at sites where IL-1 β induced a rapid formation of membrane microdomains enriched for ceramide, the ganglioside GM1, and nSMase2 (Fig. 3, A-C). EV release was blocked by pre-treatment with an IL-1 receptor antagonist, or the nSMase antagonist altenusin (Fig. 3, A-E). IL-1 β stimulation in the presence of altenusin resulted in the formation of sub-cellular vacuoles indicative of multivesicular bodies, but these appeared to not fuse with the plasma membrane (Figs. 3A-C far right column). These data are consistent with our previous reports that plasma membrane fusion events involve a regulated production of ceramide by nSMase (34, 35), and with reports that nSMase and ceramide are required for EV biogenesis and release (36, 37). Rapid formations of membrane microdomains and EV release were also observed in astrocytes exposed to TNF α (IL-1 β and TNF α are both potent stimulators of nSMase activity), but not in response to IL-10, which has no known association with nSMase (Fig. S4A-C). These data suggest that IL-1 β induced a rapid formation of ceramide, GM1, and nSMase2 enriched membrane microdomains located to astrocyte lipid rafts that were the primary sites of EV release.

To determine whether astrocyte-shed EV were sufficient to promote leukocyte influx *in vivo*, we injected an adenovirus vector to express a GFAP driven shRNA to selectively reduce nSMase2 in astrocytes (Fig. 3F). Protein expression of nSMase2 was reduced 3 days after injection of adenovirus (Fig. 3, G and H), and leukocyte influx in response to injection of IL-1 β was blocked (Fig 3I, fig. S4D). To further demonstrate that astrocyte EV were sufficient to promote the transmigration of leukocytes into brain, we next purified GFP+ EV from cultured GFAP-GFP astrocytes (shed in response to IL-1 β), and infused

3.0 X 10⁹ EV, or protein and RNA depleted EV (figs. 3J, and fig. S4, E and F) into the tail vein of mice with an ACR block produced by co-administration of IL-1 β + altenusin into striatum. Venous infused GFP+ EV were observed in liver (fig. S4, G and H), were associated with a liver ACR (fig. 3K), and promoted leukocyte influx into brain (fig. 3, L and M) in a manner indistinguishable from animals administered IL-1 β alone (compare with fig. 1, A-C and G). Infusion of protein and RNA depleted astrocyte EV did not induce a liver ACR, and did not promote leukocyte influx into brain parenchyma (fig. 3, K-M). These data suggest that EV shed from astrocytes in response to IL-1 β are sufficient to promote a robust transmigration of leukocytes into brain.

Astrocyte-shed EV rapidly cross the blood-brain-barrier and are targeted to peripheral organs

If astrocyte-shed EV facilitate the recruitment of leukocytes to the site of inflammatory brain lesion they must efficiently cross the BBB. To address this question we first used an in vitro transwell model of the BBB cultured with brain vascular endothelial cells and astrocytes from GFAP-GFP mice. Exposure of the astrocyte side of the bilayer to IL-1 β induced a rapid flux of GFP+ EV into the luminal side of the model, without any change in barrier permeability (fig. 4A; fig. S5A and B). Inhibition of nSMase with altenusin, IL-1 receptor blockade, or physical disruption of membrane microdomains with the cholesterol chelating agent β -cyclodextran (38) prevented the luminal flux of EV (Fig. 4A). Using transmission electron microscopy (TEM) we observed the formation and fusion of multivesicular bodies in astrocytes, endothelial uptake, and release of EV in IL-1 β stimulated BBB cultures (fig. S5, C-H). Immunogold TEM of GFP in the BBB model (Fig. 4B), and in brain tissues from GFAP-GFP mice injected with IL-1 β (Fig. 4C) confirmed *in*

vivo that EV observed in the cytosol of endothelial cells originated from astrocytes. Immunogold labelling of GFP was not detected in Wt mice (Fig. 4D). Although IL-1 β has previously been shown to not perturb the BBB in adult mice (39), we confirmed the absence of IgG in brain parenchyma of adult mice injected with IL-1 β (fig. S5, I and K). IgG was readily apparent brain parenchyma of 2 week old mice injected in striatum with IL-1 β (fig. S5, J and K), consistent with a more permeable BBB in immature mice. These data demonstrate that EV released from astrocytes can cross endothelial cells, and are released into circulation.

We next used GFAP-GFP mice to identify the peripheral targets of astrocyte-shed EV (Fig. 4, E-L). We observed a basal level of GFP+ EV in the liver, lung and spleen that was not apparent in non-transgenic mice (Fig. 4, E,F,H,I, K, and L), suggesting there is a constitutive low-level trafficking of EV from astrocytes into circulation. Two hours after injection of IL-1 β into the brain we observed a 3-4 fold increase in the number of GFP+ EV in liver, lung, and spleen that was not apparent when IL-1 β was injected along with alenudin (Fig. 4, E,F,H,I, K, and L). Twenty four hours after IL-1 β injection there were sharp reductions in the number of GFP+ EV in each of these organs compared to the 2-hour peak response (Fig. 4, G,J, and M), presumably reflecting a reduction in release, and the processing of incorporated EV. To confirm that GFP+ EV observed in liver originated from brain astrocytes we co-infused FM-143 (to label brain cells), and IL-1 β (to simulate EV release) into striatum of GFAP/EGFP mice, and observed dual labelled EV in liver (Fig. 4N), demonstrating that GFP+ EV in liver originated from brain astrocytes.

Analysis of EV cargo identified PPAR α as a subcellular target in liver that regulates the peripheral immune response to inflammatory brain lesion

To determine the molecular mechanisms by which EV regulate the peripheral ACR to brain injury we characterized the protein, lipid, and miRNA content of EV shed from IL-1 β stimulated astrocytes. EV released during a 2h time-frame following IL-1 β stimulation were 66.28 ± 23.46 nm in diameter (Fig. 5A,B), CD63, TSG101 and Flotillin immunopositive, Actinin-4, Microfilin and β -actin immunonegative (Fig. 5C). Proteomic analysis of EV shed from astrocytes in response to IL-1 β identified 212 distinct proteins that were largely related to molecular function, catalytic activity, and binding (tables S1-2). There were 10 proteins unique to EV shed in response to IL-1 β , 127 proteins unique to constitutively released EV, and 75 proteins common to both groups. None of the 124 different miRNAs detected were unique to any one group, but 23 were enriched 2-fold or more in EV shed from IL-1 β stimulated astrocytes (fig. S6B, table S3). Some of the miRNA identified in EVs from other studies including were not present in our analyses including miR-155, miR-146a and miR-124 (40-42). There are several possible explanations for differences in the EV microRNA content. There are likely differences in the miRNA content of exosomes released from different cell types in response to the same stimulus. There are likely differences in the miRNA content depending on when the EVs are collected following stimulation and different stimuli may produce slightly different compositions of miRNA cargo. As the cargo of EV is complex, we reasoned that a focus on any one protein or miRNA would not likely provide an accurate representation of the function for these signaling complexes. Therefore, we examined the protein and miRNA cargo of EV, and identified PPAR α as a likely molecular target of astrocyte EV shed in response to IL-1 β . This pathway was suggested by a protein mediated positive regulation of HSP90, (a repressor of PPAR- α) (43), negative regulation in the enzymatic production of cAMP and

diacylglycerides (promotes PPAR- α activity through PKA, or PKC) (44, 45), and inhibition of Ras by several miRNAs in the let-7 family (Fig. 5E, Table S3). Because PPAR actively suppress cytokine expression through inhibitory effects on NF- κ B (46), and is enriched in tissues that oxidize fatty acids, with highest expression in liver (47, 48), we conducted immunoprecipitation of chromatin isolated from liver samples of mice following striatal injection of IL-1 β and found increased binding of the NF- κ B subunit c-Rel to promoter regions of *CCL2*, *IL-1 β* , and *TNF α* , but not to *IL-17*, consistent with the observed liver ACR response (Fig. 5F-I, S6C,D). Together, these data suggest that EV shed from astrocytes in response to IL-1 β may induce a liver ACR through suppression of PPAR α . To test this possibility we administered the PPAR α agonist fenofibrate intraperitoneal (known to have low CNS bioavailability; 49), just prior to striatal IL-1 β infusion, and showed that fenofibrate blocked NF- κ B binding to *CCL2*, *IL-1 β* , and *TNF α* promoter regions (Fig. 5F-I), and prevented leukocyte transmigration into brain parenchyma (Figs. 5J,K). In mice with an ACR block produced by co-administration of IL-1 β + altenusin we found that IP administration of the PPAR- α antagonist GW6471 re-established leukocyte recruitment to the site of IL-1 β injection (Fig. 5L,M). These data suggest that EV shed from astrocytes in response to IL-1 β regulate the expression of inflammatory cytokines in liver through suppression of PPAR α .

Discussion

Despite considerable evidence that inflammatory brain lesions promote a peripheral leucocytosis through the release of soluble mediators from brain, attempts to identify these factors have been unsuccessful. Here we provide evidence that the molecular cargo of EV released from brain astrocytes is sufficient to promote leukocyte recruitment by regulating the peripheral ACR to a focal IL-1 β inflammatory lesion. This identification of a

soluble mediator fulfills a critical gap in our understanding of how the brain communicates with the peripheral immune system. While our data show that EV released from astrocytes are sufficient to induce a peripheral ACR and leukocyte transmigration, it is likely that EV released from other brain resident cells, and/or vascular endothelium play important roles in fine tuning the immunological response to brain damage. Indeed, several recent studies have demonstrated that EV are released into circulation in the setting of glioblastoma multiforme (50), focal cerebral ischemia, traumatic brain injury (51), Parkinson's disease (52), and Alzheimer's (53, 54) carry disease specific cargo. In addition to trafficking from brain to peripheral circulation, it has been recently shown that hematopoietic cells release EV that traffic into brain to deliver functional RNA to neurons (55), and recent data have shown that choroid plexus epithelium cells sense and transmit information about the peripheral inflammatory status to brain through the release of EVs that are taken up by astrocytes and microglia (41). These findings combined data presented in this paper suggest that EV regulate bi-directional communication between the brain and immune system, and differences in cargo may regulate disease specific biological responses. Unraveling the molecular mechanisms that regulate this long distance communication will extend our understanding of how the brain and peripheral systems communicate, and may open new therapeutic possibilities for the treatment of neuroimmunological disease.

Materials and Methods

Animals

Adult (2-3 month) male C57BL/6J (Jackson Laboratories), *Smpd3^{fro/fro}* (nSmase2 inactive, kindly provided by Dr. Christophe Poirier)(56), and GFAP-EGFP mice (Jackson Laboratories) mice were used for these studies. All animal procedures were performed in accordance with the NIH guidelines on animal care, and were approved by the JHU Institutional Animal Care and Use Committee.

Focal brain injury model

Striatal injections were performed as previously described (15). The striatum was chosen as the injection site since it is an area of brain parenchyma, distant from the meninges, in which the inflammatory responses to the cytokines, endotoxin have been well characterized previously, and to avoid involvement of the ventricles or meninges. We have been unable to detect increased levels of exogenously administered IL-1 in the blood after focal microinjection of IL-1 β into the striatum. While the striatum is affected in Parkinson's disease and Huntington's disease, which makes it a clinically relevant site, the focal unilateral inflammation induced by IL-1 β does not produce any overt behavioral changes that affects an animal's ability to access to food or water or induce heightened sensitivity to pain, which can be a feature if the IL-1 β is injected more caudally (in the DRN for example). Mice were anesthetized with 3% Isoflourane (Baxter) in oxygen (Airgas), and placed in a stereotaxic frame (Stoelting Co.). A small burr hole was drilled in the skull over the left striatum using a dental drill (Fine Scientific Tools). IL1 β (0.1ng/3 μ l) alone, with altenusin (50 μ M), or GW4869 (10 μ M) was injected (total volume of 3 μ l) at the rate 0.5 μ l/min *via* a pulled glass capillary (tip diameter <50 μ m; 57) using the stereotaxic coordinates: A/P +1; M/L -2; -3 D/V. Saline containing the same amount of DMSO (0.67%/volume) was used as a control. Following infusion, the capillary was held in place for 5 min to allow for solution to diffuse into the tissue. Animals were sacrificed at 2 and 24h by an overdose of anesthetic, and transcardically perfused with ice-cold saline containing heparin (20 μ l per 100ml, Sigma). Liver, lung and spleen were dissected and flash frozen. Brains were rapidly extracted and flash frozen or post fixed in 4% PFA followed by cryoprotection in a 30% sucrose solution and frozen at -80°C.

Astrocyte Culture

Primary cortical astrocyte cell cultures were established and maintained using methods similar to those described previously (58). Briefly, Primary astrocytes were isolated from the cerebral cortex of postnatal day 1 C57B6J mice and GFAP-EGFP mice. Cells were mechanically dissociated in HBSS, and plated in poly-D-lysine culture flasks containing DMEM/F12 MEDIA (Gibco BRL) AND 10% fetal bovine serum (Gibco BRL). Type 1 astrocytes were purified by the mechanical removal of less adherent cells. Cultures were 98% GFAP+ astrocytes with type I morphology and were used for experiments between 3-10 passages.

EV isolation and quantitation

Plasma derived EV: Blood was collected *via* cardiac puncture using a heparin (Sigma Aldrich) coated syringe and EDTA tubes (BD) 2h following striatal injections. Blood was immediately centrifuged at 2700g for 15min (20°C) to obtain plasma. Plasma was further centrifuged at 10,000g for 15min (4°C) to generate platelet free plasma. Plasma-derived EV were isolated *via* ultracentrifugation at 100,000 g for 3h (4°C). Pellets containing EV were washed twice with 5 ml saline and the final pellet resuspended in saline. EV size and quantity were determined using Nanosight as described below. EV were used for experiments the same day as isolation. Astrocyte-shed EV: Eight-ten T150 culture flasks of GFAP-EGFP primary astrocytes (~80% confluent) were gently washed 3X with warm PBS to remove endogenous EV. Astrocyte EV release was stimulated by the addition of fresh EV-free media containing IL-1 β (200ng per ml, R&D systems). Media was collected 2h after stimulation, and EVs isolated by a multistep ultracentrifugation. Media was centrifuged at 10,000g for 15min (4°C). Supernatant was removed and ultracentrifuged at

100,000 g for 3h (4°C) to isolate EVs. This short stimulation procedure resulted in the release of EVs with a narrow size range and protein markers consistent with exosomes. EV size, distribution, and concentrations were determined using a NS500 Nanosight™ nanoparticle tracking analysis system (Malvern Instruments). EV were visualized with scattered light from a 488nm laser beam onto an optical microscope containing a CCD video camera. Five exposures of 20sec each were randomly chosen by the software to measure EV size and concentration based on the Stokes-Einstein equation, and linear fitting using standard particles with a known diameter. Data was binned and plotted as a continuous histogram.

Depletion of proteins and RNA from EV

The lipid components from plasma and astrocyte derived EV were isolated using a modified Bligh and Dyer procedure as previously described (59). Using this procedure, RNA localizes to the upper aqueous fraction, lipids to the lower chloroform layer, and proteins are precipitated at the interface of the aqueous and organic layer. The chloroform fraction containing a crude lipid extract was dried under a stream of nitrogen, resuspended in saline, and uniform sized vesicles were created using a mini-extruder with 80nm pore-sized membrane (Avanti). Liposomes were used on the same day of isolation for experiments.

Adoptive transfer of EV

EV were isolated from the plasma of “*donor*” mice 2 h following striatal injection of IL-1 β (0.1ng in 3 μ l) or saline (3 μ l). Plasma EV (3.0×10^9) were infused into the tail veins of “*recipient*” mice 2h with an ACR block produced by striatal co-injection of IL-1 β + neutral

sphingomyelinase antagonist altenusin (50 μ M). Mice were sacrificed 2, or 24 h following EV infusions. Organs were isolated for biochemical and histological analyses.

Transwell model of the blood-brain-barrier

An *in-vitro* model of the blood-brain-barrier was generated using a previously published method (60) with minor modifications. GFAP-EGFP primary astrocytes were used in order to visualize EV (EV shed from these cells carry EGFP). BBB barrier function was confirmed by the presence of high transendothelial electrical resistance, and by a lack of permeability to fluorogenic labeled albumin, and insulin.

Cytokine expression

RNA was isolated from fresh frozen tissues (10-50mg) using the RNeasy Mini Kit (Qiagen). Total RNA was reverse transcribed and quantified using previously published methods (61). For quantitative real time PCR (qRT-PCR), each reaction contained SYBR Green Master Mix (12.5 μ l; Life Technology), DEPC H₂O (10.5 μ l), forward and reverse primers to CCL2, TNF α , IL-6, IL-1 β , IL-17, IL-10, IGFR1, and CXCL1 (0.5 μ l of each, Sigma), and cDNA (1 μ l). Each 96 well plate included a non-template control and samples were analyzed in triplicate on an Applied Biosystem 7300 (Life Technology, Carlsbad, CA). Cycling parameters were as follows: 1 cycle at 50°C for 2min, 1 cycle at 95°C for 10min, 40 cycles of 95°C for 15sec, and 60°C for 1min. The change in threshold cycle (Δ Ct) for each sample was normalized to β -actin, and $\Delta\Delta$ Ct's were calculated by comparing Δ Ct for the treatment group to the average Δ Ct of the control group (62).

miRNA expression analysis

RNA was isolated from EV using the miRNeasy Micro Kit (Qiagen). Small RNAs were tagged on their 3' end to normalize T_m 's according to the manufacturers instructions (Nanostring Technologies). Briefly, small RNA's were bridged with tagged DNA in annealing buffer (94°C for 1min, 65°C for 2min, 45°C for 10min, hold at 48°C), PEG and ligation buffer were added at 48°C for 5min, then Ligase was added (48°C for 3min, 47°C for 3min, 46°C for 3min, 45°C for 5min, 65°C for 10min, hold at 4°C). To remove excess tags and bridges, samples were purified using a ligation clean-up enzyme (37°C for 120min, 70°C for 10min, hold at 4°C). RNA was further prepped for hybridization by adding water (40µl), heating the samples to 85°C for 10min, then immediately placing them on ice. RNA (5µl) was hybridized to miRNA Reporter CodeSet master mix (20µl) at 65°C for 18h. Hybridized RNAs were bound to nCounter cartridges, and analyzed using an nCounter Digital Analyzer (Nanostring).

Chromatin immunoprecipitation (ChIP)

ChiP assays were conducted as previously described (63). Frozen tissue samples (40mg per IP) were cut into small pieces and incubated with PBS containing 1% formaldehyde for 8 min at room temperature and quenched with 0.125 M glycine. After rinsing twice with ice-cold PBS containing complete protease inhibitors (PIC, Roche), tissues were collected by centrifugation, and disaggregated using a homogenizer (Dounce) in 1ml of ice-chilled PBS (+PIC). Crosslinked cells were collected and resuspended in lysis byffer (5mM PIPES, pH8, 85mM KCl, 0.5% NP40 vol/vol) with PIC. After 30 min incubation with lysis buffer nuclei were pelleted by centrifugation (10min at 3000rpm) and incubated with 300ml of nuclei lysis buffer (50mM Tris-HCl, pH8.1, 10mM EDTA, 1%SDS) with PIC for 15min on ice and sonicated using a Bioruptor® Sonication System (Diagenode). Lysates were clarified by centrifugation at 10,000rpm for 10min at 4°C. The size of DNA in the cleared chromatin fragments was tested before precipitation to ensure that the majority of

fragment size was 200–400 bp. Immunoprecipitation was performed with 1µg of cRel antibody (Santa Cruz, sc-70x), and 1µg normal rabbit IgG/1 unit OD260 readings. Two units of chromatin were used per condition. Chromatin was immunoprecipitated using protein A-Dynabeads (Invitrogen) overnight at 4°C. Protein-DNA complexes were washed two times with Low Salt ChIP Buffer (50mM Tris-HCl, pH8, 150mM NaCl, 0.5% Sodium deoxycholate, 0.1% SDS, 1%NP40, 1mM EDTA), two times with High Salt ChIP Buffer (50mM Tris-HCl, pH8, 500mM NaCl, 0.5% Sodium deoxycholate, 0.1%SDS, 1%NP40, 1mM EDTA), twice with LiCl Buffer (10mM Tris-HCl, pH8, 250mM LiCl, 0.5% Sodium deoxycholate, 0.1%SDS, 1%NP40, 1mM EDTA), and one final wash with TE Buffer (10mM Tris-HCl (pH8), mM EDTA). Chromatin was eluted from the beads twice by incubation with 50µl of elution buffer (50mM NaHCO₃, SDS1% vol/vol) and heating at 65°C with occasional vortexing for 15min, and decrosslinked over night at 65°C. Input sample underwent a crosslink reversal. Samples were treated with RNase A and proteinase K according to the manufacture's specifications. DNA was purified by phenol–chloroform extraction, followed by ethanol precipitation. Fold enrichment was calculated using the 2 Δ Ct method. The primer sequences of qPCR for ChIP are provided in Table S4.

Immunohistochemistry

Coronal brain sections (20-40µm) were prepared using a cryostat microtome (Leica). Endogenous peroxidase activity was quenched using a 1% solution of H₂O₂ in MeOH, and primary antibodies Ly6b (1:1000 AbD Serotec), IBA-1 (1:500, Abcam), or ICAM-1 (1:100, Abcam) were incubated overnight at 4°C. Sections were washed (3xPBS), and the appropriate biotinylated secondary antibody (1:100, Vector labs) was added for 2h at room temperature. Staining was visualized using an avadin-biotin complex (1:100 of A and B, Vector labs) and DAB-HCl using a microscope to monitor staining progression.

Stereological quantitation was performed using a one-in-five series (200 μ m spacing), from the rostral point of bregma -1.22 mm to the caudal point of bregma -2.80 mm) as previously described (64). A similar protocol was used to determine BBB integrity, except no primary antibody was used and the secondary antibody (targeted towards mouse IgG) was visualized (IgG is not present in brain parenchyma when the BBB is intact).

Immunofluorescence

Astrocytes isolated from C57B6J mice were cultured on glass coverslips, and treated with IL-1 β (100ng/ml; Millipore), IL-1 β + altenusin (50 μ M; Enzo Life Sciences), IL-1 β + GW4869 (20 μ M; Calbiochem), IL-1 β + IL-1RA (100ng/ml), TNF- α (100ng/ml), or IL-10 (100ng/ml) for 2-60 min. Cells were fixed with 4% paraformaldehyde, and lipid raft membrane microdomains were identified using a cholera toxin subunit B conjugated to Alexa Fluor 555 which binds the ganglioside GM1 (CTB-555; Invitrogen/Molecular Probes Inc.) (34, 64). CTB-555 labeled cells were incubated with primary antibodies to ceramide (1:200; SantaCruz), or nSMase (1:200; SantaCruz), and the corresponding secondary antibodies conjugated to AlexaFluor 488, or AlexaFluor 546 (1:1000; Invitrogen/Molecular Probes). Fluorescence was imaged with a 100X objective by optical sectioning using structured illumination (Carl Zeiss Inc., Thornwood, NY, USA). All images for quantification were taken with identical settings, and performed on a single plane of focus through the brightest point. Colocalization was confirmed by three-dimensional reconfiguration of z-stack images using orthogonal views as previously described (35).

Localization of astrocyte-shed exosomes in peripheral tissues

EV released from astrocytes of GFAP-EGFP mice were visualized by fluorescent imaging of EGFP+ puncta in liver, spleen and lung tissue sections. Tissues were post fixed in 4%

PFA, cryoprotected in 30% sucrose, and 20µm sections were cut using a cryostat microtome (Leica). Non-specific binding was blocked with 5% normal goat serum plus 5% normal horse serum in Tris-buffered saline (TBS-T) containing 0.1% Triton X100 (Fisher Scientific). EGFP was enhanced by incubating sections with anti-GFP rabbit sera (1:500; Invitrogen) overnight at 4°C, followed by incubation with a secondary antibody conjugated to Alexa 488 (1:1000, Invitrogen). Cells were visualized with F-actin (1:100; Abcam), and nuclei were stained with DAPI as previously described (65). Imaging was performed with 40 and 100X objectives using optical sectioning by structured illumination (Carl Zeiss Inc., Thornwood, NY, USA).

Western blotting

Proteins were resolved by 10% SDS-PAGE, and transferred to PVDF membranes (BioRad, Hercules, CA, USA). Non-specific binding sites were blocked with 5% (w/v) milk in TBS containing 0.1% Tween 20 (TBS-T). After blocking, blots were incubated overnight with the primary polyclonal antibody Flotillin 1 (1:1000, Abcam), CD63 (1:200; Santa Cruz), TSG101 (1:1000; BD), Actinin-4 (1:1000; GenTex), Microfilin 1:5000; Thermo), nSmase2 (1:500; Santa Cruz), transferrin receptor (1:1000, Invitrogen), β -actin (1:5000; Sigma). Following washes with TBS-T, blots were incubated for 2 h with the appropriate IgG HRP-linked secondary antibody (1:1000; Cell signaling technology, Danvers, MA, USA), and developed by enhanced chemiluminescence. Image analysis was performed using a G:BOX Imaging system (Syngene, Frederick, MD, USA).

Electron microscopy

For the negative stain, glow discharged 400 mesh copper carbon coated grids (Electron Microscopy Sciences) were placed on 5 to 10µL of liquid sample for 1 min, then briefly

washed with drops of distilled water. The grids were stained in 2% uranyl acetate (EMS) for 1min and blotted dry on Whatman filter paper (Sigma Aldrich Aldrich). Imaging was performed on a Libra 120 electron microscope (Zeiss) at an acceleration voltage of 120 Kv using a Veleta camera (Olympus).

For the immunogold electron microscopy, thin sections of Epon (EMS) embedded tissue (66) were placed on nickel grids (EMS) and etched in 3% sodium meta-periodate (EMS) two times for 30min each and then washed with ddH₂O. Sections were incubated in PBS containing 10% FBS for 30min, and incubated overnight in GFP antisera (Invitrogen; 1:50 at 4°C). After several washes in PBS grids were incubated at room temperature in goat anti-rabbit secondary (Jackson ImmunoResearch; 1:20). Grids were washed in PBS, and fixed briefly in 2% glutaraldehyde (Sigma Aldrich Aldrich). Staining was done using uranyl acetate, followed by lead citrate. Grids were viewed on a Libra 120 electron microscope (Zeiss) using a Veleta camera (Olympus).

Ceramide analysis

Ceramides were quantified by mass spectrometry as previously described (67), with slight modifications. A crude lipid extraction was prepared from tissues using a variation of the Bligh and Dyer procedure (68) with ceramide d18:0/12:0 (Avanti Polar Lipids) included as an internal standard. The organic layer containing a crude lipid extract was dried in a nitrogen evaporator (Organomation, Berlin, MA, USA) and suspended in MeOH prior to analysis. Chromatographic separations were conducted using a Shimadzu ultra fast liquid chromatography system (Shimadzu) coupled to a C18 reverse-phase column (Phenomenex). Eluted samples were injected into an API3000 triple quadrupole mass spectrometer (AB/Sciex) where individual ceramide species (C16:0-C26:1) were detected by multiple reaction monitoring. Eight-point calibration curves (0.1–1000ng/mL) were constructed by plotting area under the curve (AUC) for each calibration standard

d18:1/C16:0, d18:1/C18:0, d18:1/C20:0, d18:1/C22:0, d18:1/C24:0 normalized to the internal standard. Correlation coefficients (R²) obtained were >0.999. Ceramide concentrations were calculated by fitting the identified ceramide species to these standard curves based on acyl chain length. Instrument control and quantitation of spectral data were performed using Analyst 1.4.2 and MultiQuant software (AB Sciex).

Proteomics

EV isolated from astrocytes were digested with trypsin overnight and analyzed by liquid chromatography/tandem mass spectrometry (LCMS/MS) using LTQ Orbitrap Velos MS (Thermo Fisher Scientific) interfaced with the NanoAquity (Waters) as previously described (69) with precursor and the fragment ions analyzed at resolution 30,000 and 15,000, respectively. Proteins were identified from the MS/MS spectra extracted in Proteome Discoverer (v1.4, Thermo Fisher Scientific) interfaced with Mascot (v 2.5, Matrix science, London) to search the SwissProt_2014_07 database with the following criteria: *Mus musculus* species; precursor and the fragment ions mass tolerance of 0.050 Da and 15 ppm, respectively; fixed modification cysteine carbamidomethylation; and variable modifications of asparagine and glutamine deamination, methionine oxidation, and serine, threonine, and tyrosine phosphorylation. Protein identifications were confirmed using Scaffold v4.4.1 (Proteome Software Inc) at > 91% probability with < 1% false discovery rate (FDR) with at least two peptides sequences per protein. The gene ontology classifications were carried out in Scaffold v4.4.1 (Proteome Software Inc.). Proteins were classified using the Protein Analysis Through Evolutionary Relationship (PANTHER) tool(70)

***In vivo* gene silencing of nSMase2 in astrocytes**

A GFAP promoted shRNA targeting nSMase2 and co-expressing mCherry in an adeno-associated viral vector (serotype2) was designed using the previously published siRNA sequence (71), and manufactured commercially (SignaGen). A scrambled shRNA nSMase2 co-expressing mCherry in an adeno-associated viral vector (SignaGen) was used as a negative control. Viral vectors (5×10^9 particles in 0.5 μ l) were injected into striatum using a pulled glass capillary as described above. Intra-striatal injections were performed 72h following administration of viral vectors. Vector expression was confirmed by mCherry staining of brain slices, and knock-down of nSMase2 was confirmed by Western Blot. The animals were sacrificed 24h following injection of IL-1 β . Organs were isolated for biochemical, and histological analysis.

Pathway discovery and biological validation

Putative molecular pathways regulated by EV cargo were identified using Ingenuity® Pathway Analysis (IPA; Qiagen). Proteomic and miRNA data were converted to fold change and uploaded to the IPA server (Qiagen). A core analysis (IPAv23814503) was performed using all data sources available in the software. Resultant canonical pathways and interaction networks were examined, and pathways with relevance to the regulation of inflammation were considered for biological validation. The involvement of a central regulator in the identified pathway (PPAR- α) was confirmed *in vivo* by intraperitoneal injection of small molecule drugs to target PPAR- α that included the agonist fenofibrate (10mg/kg; Sigma), and the antagonist GW6471 (20mg/kg; Tocris Bioscience) following striatal administrations of IL-1 β , or IL-1 β + alenusa as described. Mice were sacrificed at 2h for biochemical and histological analyses, and at 24h for leukocyte transmigration.

Statistical analyses

Group differences were determined by ANOVA with Tukey post hoc comparisons. Hierarchical cluster analysis was conducted using log2 values of normalized median counts. Heatmaps and clustering were generated using “pheatmap” (v0.7.7; R package version 3.1.1) (72).

Supplemental information

Figure S1: Additional leukocyte, ceramide and sphingomyelin measurements.

Figure S2: Additional cytokine measurements following intra-striatal injection of IL-1 β .

Figure S3. EV isolation, quantitation and liver cytokine response 24 h following EV infusion in mice.

Figure S4. IL-1 β and TNF α promote formation of ceramide rich membrane microdomains and EV release from astrocytes.

Figure S5. EV rapidly transverse endothelial cells in a BBB model system.

Figure S6: Additional proteomic and chromatin immunoprecipitation (ChIP) results.

Table S1: List of all proteins identified in exosomes collected from control GFAP-GFP astrocytes.

Table S2: List of all proteins identified in exosomes collected from IL-1 β stimulated GFAP-GFP astrocytes.

Table S3: List of miRNA identified in exosomes and relative fold change

Table S4: List of ChIP primers

Movie S1: IL-1 β induced a rapid release of EV from cultured primary astrocytes that we visualized using the styryl pyridinium dye FM1-43.

References

1. R. M. Ransohoff, P. Kivisäkk, G. Kidd, Three or more routes for leukocyte migration into the central nervous system. *Nature Reviews Immunology* **3**, 569-581 (2003).
2. D. C. Anthony, Y. Couch, P. Losey, M. C. Evans, The systemic response to brain injury and disease. *Brain, Behavior, and Immunity* **26**, 534-540 (2012).
3. D. H. Smith, X. H. Chen, J. E. Pierce, J. A. Wolf, J. Q. Trojanowski, D. I. Graham, T. K. McIntosh, Progressive atrophy and neuron death for one year following brain trauma in the rat. *Journal of neurotrauma* **14**, 715-727 (1997).
4. D. C. Wilcockson, S. J. Campbell, D. C. Anthony, V. H. Perry, The systemic and local acute phase response following acute brain injury. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* **22**, 318-326 (2002).
5. S. J. Campbell, I. Zahid, P. Losey, S. Law, Y. Jiang, M. Bilgen, N. van Rooijen, D. Morsali, A. E. Davis, D. C. Anthony, Liver Kupffer cells control the magnitude of the inflammatory response in the injured brain and spinal cord. *Neuropharmacology* **55**, 780-787 (2008).
6. M. Pepys, M. L. Baltz, Acute phase proteins with special reference to C-reactive protein and related proteins (pentaxins) and serum amyloid A protein. *Adv. Immunol.* **34**, 141-212 (1983).
7. K. Zahedi, A. Whitehead, Acute phase induction of mouse serum amyloid P component. Correlation with other parameters of inflammation. *The Journal of Immunology* **143**, 2880-2886 (1989).
8. S. J. Campbell, P. M. Hughes, J. P. Iredale, D. C. Wilcockson, S. Waters, F. Docagne, V. H. Perry, D. C. Anthony, CINC-1 is an acute-phase protein induced by focal brain injury causing leukocyte mobilization and liver injury. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **17**, 1168-1170 (2003).
9. F. Clausen, A. Hanell, M. Bjork, L. Hillered, A. K. Mir, H. Gram, N. Marklund, Neutralization of interleukin-1beta modifies the inflammatory response and improves histological and cognitive outcome following traumatic brain injury in mice. *The European journal of neuroscience* **30**, 385-396 (2009).
10. S. J. Campbell, D. C. Anthony, F. Oakley, H. Carlsen, A. M. Elsharkawy, R. Blomhoff, D. A. Mann, Hepatic nuclear factor kappa B regulates neutrophil recruitment to the injured brain. *Journal of neuropathology and experimental neurology* **67**, 223-230 (2008).
11. A. Ottani, D. Giuliani, C. Mioni, M. Galantucci, L. Minutoli, A. Bitto, D. Altavilla, D. Zaffe, A. R. Botticelli, F. Squadrito, Vagus nerve mediates the protective effects of melanocortins against cerebral and systemic damage after ischemic stroke. *Journal of Cerebral Blood Flow & Metabolism* **29**, 512-523 (2008).
12. D. C. Anthony, Y. Couch, P. Losey, M. C. Evans, The systemic response to brain injury and disease. *Brain, behavior, and immunity* **26**, 534-540 (2012).
13. Y. Jiang, R. Deacon, D. Anthony, S. Campbell, Inhibition of peripheral TNF can block the malaise associated with CNS inflammatory diseases. *Neurobiology of disease* **32**, 125-132 (2008).

14. S. J. Campbell, R. M. J. Deacon, Y. Jiang, C. Ferrari, F. J. Pitossi, D. C. Anthony, Overexpression of IL-1 β by adenoviral-mediated gene transfer in the rat brain causes a prolonged hepatic chemokine response, axonal injury and the suppression of spontaneous behaviour. *Neurobiology of disease* **27**, 151-163 (2007).
15. D. C. Wilcockson, S. J. Campbell, D. C. Anthony, V. H. Perry, The systemic and local acute phase response following acute brain injury. *Journal of Cerebral Blood Flow & Metabolism* **22**, 318-326 (2002).
16. D. Brough, P. J. Tyrrell, S. M. Allan, Regulation of interleukin-1 in acute brain injury. *Trends Pharmacol. Sci.* **32**, 617-622 (2011).
17. R. E. Mrak, W. S. T. Griffin, Interleukin-1, neuroinflammation, and Alzheimer's disease. *Neurobiology of Aging* **22**, 903-908 (2001).
18. S. J. Campbell, D. C. Anthony, F. Oakley, H. Carlsen, A. M. Elsharkawy, R. Blomhoff, D. A. Mann, Hepatic Nuclear Factor [kappa] B Regulates Neutrophil Recruitment to the Injured Brain. *J. Neuropathol. Exp. Neurol.* **67**, 223 (2008).
19. S. J. Campbell, P. M. Hughes, J. P. Iredale, D. C. Wilcockson, S. Waters, F. Docagne, V. H. Perry, D. C. Anthony, CINC-1 is identified as an acute-phase protein induced by focal brain injury causing leukocyte mobilization and liver injury. *The FASEB Journal*, (2003).
20. S. Mathias, A. Younes, C.-C. Kan, I. Orlow, C. Joseph, R. N. Kolesnick, Activation of the sphingomyelin signaling pathway in intact EL4 cells and in a cell-free system by IL-1 beta. *Science* **259**, 519-522 (1993).
21. I. Aubin, C. P. Adams, S. Opsahl, D. Septier, C. E. Bishop, N. Auge, R. Salvayre, A. Negre-Salvayre, M. Goldberg, J. L. Guenet, C. Poirier, A deletion in the gene encoding sphingomyelin phosphodiesterase 3 (Smpd3) results in osteogenesis and dentinogenesis imperfecta in the mouse. *Nature genetics* **37**, 803-805 (2005).
22. R. Uchida, H. Tomoda, Y. Dong, S. Omura, Alutenusin, a specific neutral sphingomyelinase inhibitor, produced by *Penicillium* sp. FO-7436. *The Journal of antibiotics* **52**, 572 (1999).
23. M. Oyama, Z. Xu, K.-H. Lee, T. D. Spitzer, P. Kitrinis, O. B. McDonald, R. R. Jones, E. P. Garvey, Fungal metabolites as potent protein kinase inhibitors: identification of a novel metabolite and novel activities of known metabolites. *Letters in Drug Design & Discovery* **1**, 24-29 (2004).
24. J. Waltenberger, A. Uecker, J. Kroll, H. Frank, U. Mayr, J. D. Bjorge, D. Fujita, A. Gazit, V. Hombach, A. Levitzki, Original Contributions-Vascular Biology-A Dual Inhibitor of Platelet-Derived Growth Factor b-Receptor and Src Kinase Activity Potently Interferes With Motogenic and Mitogenic Responses to PDGF in. *Circ. Res.* **85**, 12-22 (1999).
25. O. Engel, L. Akyuz, A. C. da Costa Goncalves, K. Winek, C. Dames, M. Thielke, S. Herold, C. Bottcher, J. Priller, H. D. Volk, U. Dirnagl, C. Meisel, A. Meisel, Cholinergic Pathway Suppresses Pulmonary Innate Immunity Facilitating Pneumonia After Stroke. *Stroke; a journal of cerebral circulation* **46**, 3232-3240 (2015).

26. H. Offner, A. A. Vandenbark, P. D. Hurn, Effect of experimental stroke on peripheral immunity: CNS ischemia induces profound immunosuppression. *Neuroscience* **158**, 1098-1111 (2009).
27. K. Trajkovic, C. Hsu, S. Chiantia, L. Rajendran, D. Wenzel, F. Wieland, P. Schwille, B. Brügger, M. Simons, Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* **319**, 1244-1247 (2008).
28. B. B. Guo, S. A. Bellingham, A. F. Hill, The neutral sphingomyelinase pathway regulates packaging of the prion protein into exosomes. *Journal of Biological Chemistry* **290**, 3455-3467 (2015).
29. G. Lachenal, K. Pernet-Gallay, M. Chivet, F. J. Hemming, A. Belly, G. Bodon, B. Blot, G. Haase, Y. Goldberg, R. Sadoul, Release of exosomes from differentiated neurons and its regulation by synaptic glutamatergic activity. *Molecular and Cellular Neuroscience* **46**, 409-418 (2011).
30. I. Potolicchio, G. J. Carven, X. Xu, C. Stipp, R. J. Riese, L. J. Stern, L. Santambrogio, Proteomic analysis of microglia-derived exosomes: metabolic role of the aminopeptidase CD13 in neuropeptide catabolism. *The Journal of Immunology* **175**, 2237-2243 (2005).
31. M. Guescini, S. Genedani, V. Stocchi, L. F. Agnati, Astrocytes and Glioblastoma cells release exosomes carrying mtDNA. *J. Neural. Transm.* **117**, 1-4 (2010).
32. E. M. Krämer - Albers, N. Bretz, S. Tenzer, C. Winterstein, W. Möbius, H. Berger, K. A. Nave, H. Schild, J. Trotter, Oligodendrocytes secrete exosomes containing major myelin and stress - protective proteins: Trophic support for axons? *Proteomics-Clinical Applications* **1**, 1446-1461 (2007).
33. M. V. Sofroniew, H. V. Vinters, Astrocytes: biology and pathology. *Acta neuropathologica* **119**, 7-35 (2010).
34. D. Wheeler, E. Knapp, V. V. R. Bandaru, Y. Wang, D. Knorr, C. Poirier, M. P. Mattson, J. D. Geiger, N. J. Haughey, Tumor necrosis factor- α -induced neutral sphingomyelinase-2 modulates synaptic plasticity by controlling the membrane insertion of NMDA receptors. *J. Neurochem.* **109**, 1237-1249 (2009).
35. H. Xu, M. Bae, L. B. Tovar-y-Romo, N. Patel, V. V. R. Bandaru, D. Pomerantz, J. P. Steiner, N. J. Haughey, The human immunodeficiency virus coat protein gp120 promotes forward trafficking and surface clustering of NMDA receptors in membrane microdomains. *The Journal of Neuroscience* **31**, 17074-17090 (2011).
36. K. Trajkovic, C. Hsu, S. Chiantia, L. Rajendran, D. Wenzel, F. Wieland, P. Schwille, B. Brügger, M. Simons, Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* **319**, 1244-1247 (2008).
37. G. Wang, M. Dinkins, Q. He, G. Zhu, C. Poirier, A. Campbell, M. Mayer-Proschel, E. Bieberich, Astrocytes secrete exosomes enriched with proapoptotic ceramide and prostate apoptosis response 4 (PAR-4): potential mechanism of apoptosis induction in Alzheimer disease (AD). *The Journal of biological chemistry* **287**, 21384-21395 (2012).

38. J. S. Wadia, R. V. Stan, S. F. Dowdy, Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nature medicine* **10**, 310-315 (2004).
39. D. Anthony, R. Dempster, S. Fearn, J. Clements, G. Wells, V. H. Perry, K. Walker, CXC chemokines generate age-related increases in neutrophil-mediated brain inflammation and blood-brain barrier breakdown. *Current biology : CB* **8**, 923-926 (1998).
40. M. Alexander, R. Hu, M. C. Runtsch, D. A. Kagele, T. L. Mosbrugger, T. Tolmachova, M. C. Seabra, J. L. Round, D. M. Ward, R. M. O'Connell, Exosome-delivered microRNAs modulate the inflammatory response to endotoxin. *Nature communications* **6**, 7321 (2015).
41. S. Balusu, E. Van Wonterghem, R. De Rycke, K. Raemdonck, S. Stremersch, K. Gevaert, M. Brkic, D. Demeestere, V. Vanhooren, A. Hendrix, C. Libert, R. E. Vandenbroucke, Identification of a novel mechanism of blood-brain communication during peripheral inflammation via choroid plexus-derived extracellular vesicles. *EMBO molecular medicine* **8**, 1162-1183 (2016).
42. Q. Ji, Y. Ji, J. Peng, X. Zhou, X. Chen, H. Zhao, T. Xu, L. Chen, Y. Xu, Increased Brain-Specific MiR-9 and MiR-124 in the Serum Exosomes of Acute Ischemic Stroke Patients. *PloS one* **11**, e0163645 (2016).
43. W. K. Sumanasekera, E. S. Tien, J. W. Davis, R. Turpey, G. H. Perdew, J. P. Vanden Heuvel, Heat shock protein-90 (Hsp90) acts as a repressor of peroxisome proliferator-activated receptor- α (PPAR α) and PPAR β activity. *Biochemistry* **42**, 10726-10735 (2003).
44. G. Lazennec, L. Canaple, D. Saugy, W. Wahli, Activation of peroxisome proliferator-activated receptors (PPARs) by their ligands and protein kinase A activators. *Mol. Endocrinol.* **14**, 1962-1975 (2000).
45. C. Blanquart, R. Mansouri, R. j. Paumelle, J.-C. Fruchart, B. Staels, C. Glineur, The protein kinase C signaling pathway regulates a molecular switch between transactivation and transrepression activity of the peroxisome proliferator-activated receptor α . *Mol. Endocrinol.* **18**, 1906-1918 (2004).
46. B. Staels, W. Koenig, A. Habib, R. Merval, M. Lebreton, I. P. Torra, P. Delerive, A. Fadel, G. Chinetti, J.-C. Fruchart, Activation of human aortic smooth-muscle cells is inhibited by PPAR α but not by PPAR γ activators. *Nature* **393**, 790-793 (1998).
47. S. Kliewer, B. Forman, B. Blumberg, E. Ong, U. Borgmeyer, D. Mangelsdorf, K. Umesono, R. Evans, Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *PNAS* **91**, 7355-7359 (1994).
48. R. B. Vega, J. M. Huss, D. P. Kelly, The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor α in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. *Mol. Cell. Biol.* **20**, 1868-1876 (2000).
49. D. Deplanque, P. Gelé, O. Pétrault, I. Six, C. Furman, M. Bouly, S. Nion, B. Dupuis, D. Leys, J.-C. Fruchart, Peroxisome proliferator-activated receptor- α activation as a mechanism of preventive neuroprotection induced by chronic fenofibrate treatment. *The journal of neuroscience* **23**, 6264-6271 (2003).

50. H. Shao, J. Chung, K. Lee, L. Balaj, C. Min, B. S. Carter, F. H. Hochberg, X. O. Breakefield, H. Lee, R. Weissleder, Chip-based analysis of exosomal mRNA mediating drug resistance in glioblastoma. *Nature communications* **6**, 6999 (2015).
51. A. M. Andrews, E. M. Lutton, S. F. Merkel, R. Razmpour, S. H. Ramirez, Mechanical Injury Induces Brain Endothelial-Derived Microvesicle Release: Implications for Cerebral Vascular Injury during Traumatic Brain Injury. *Frontiers in cellular neuroscience* **10**, 43 (2016).
52. C. Loov, C. R. Scherzer, B. T. Hyman, X. O. Breakefield, M. Ingelsson, alpha-Synuclein in Extracellular Vesicles: Functional Implications and Diagnostic Opportunities. *Cellular and molecular neurobiology*, (2016).
53. D. Kapogiannis, A. Boxer, J. B. Schwartz, E. L. Abner, A. Biragyn, U. Masharani, L. Frassetto, R. C. Petersen, B. L. Miller, E. J. Goetzl, Dysfunctionally phosphorylated type 1 insulin receptor substrate in neural-derived blood exosomes of preclinical Alzheimer's disease. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **29**, 589-596 (2015).
54. M. S. Fiandaca, D. Kapogiannis, M. Mapstone, A. Boxer, E. Eitan, J. B. Schwartz, E. L. Abner, R. C. Petersen, H. J. Federoff, B. L. Miller, E. J. Goetzl, Identification of preclinical Alzheimer's disease by a profile of pathogenic proteins in neurally derived blood exosomes: A case-control study. *Alzheimer's & dementia : the journal of the Alzheimer's Association* **11**, 600-607 e601 (2015).
55. K. Ridder, S. Keller, M. Dams, A. K. Rupp, J. Schlaudraff, D. Del Turco, J. Starmann, J. Macas, D. Karpova, K. Devraj, C. Depboylu, B. Landfried, B. Arnold, K. H. Plate, G. Hoglinger, H. Sultmann, P. Altevogt, S. Momma, Extracellular vesicle-mediated transfer of genetic information between the hematopoietic system and the brain in response to inflammation. *PLoS biology* **12**, e1001874 (2014).
56. I. Aubin, C. P. Adams, S. Opsahl, D. Septier, C. E. Bishop, N. Auge, R. Salvayre, A. Negre-Salvayre, M. Goldberg, J.-L. Guénet, A deletion in the gene encoding sphingomyelin phosphodiesterase 3 (Smpd3) results in osteogenesis and dentinogenesis imperfecta in the mouse. *Nature genetics* **37**, (2005).
57. L. McCluskey, S. Campbell, D. Anthony, S. M. Allan, Inflammatory responses in the rat brain in response to different methods of intra-cerebral administration. *J Neuroimmunol* **194**, 27-33 (2008).
58. N. J. Haughey, M. P. Mattson, Alzheimer's amyloid beta-peptide enhances ATP/gap junction-mediated calcium-wave propagation in astrocytes. *Neuromolecular medicine* **3**, 173-180 (2003).
59. N. J. Haughey, R. G. Cutler, A. Tamara, J. C. McArthur, D. L. Vargas, C. A. Pardo, J. Turchan, A. Nath, M. P. Mattson, Perturbation of sphingolipid metabolism and ceramide production in HIV-dementia. *Ann Neurol* **55**, 257-267 (2004).
60. D. W. Williams, K. Anastos, S. Morgello, J. W. Berman, JAM-A and ALCAM are therapeutic targets to inhibit diapedesis across the BBB of CD14+ CD16+ monocytes in HIV-infected individuals. *Journal of leukocyte biology* **97**, 401-412 (2015).

61. J. M. Westberry, A. L. Trout, M. E. Wilson, Epigenetic regulation of estrogen receptor α gene expression in the mouse cortex during early postnatal development. *Endocrinology* **151**, 731-740 (2010).
62. K. J. Livak, T. D. Schmittgen, Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *methods* **25**, 402-408 (2001).
63. V. A. Swiss, T. Nguyen, J. Dugas, A. Ibrahim, B. Barres, I. P. Androulakis, P. Casaccia, Identification of a gene regulatory network necessary for the initiation of oligodendrocyte differentiation. *PLoS One* **6**, e18088 (2011).
64. X. Chen, L. Hui, N. H. Geiger, N. J. Haughey, J. D. Geiger, Endolysosome involvement in HIV-1 transactivator protein-induced neuronal amyloid beta production. *Neurobiology of aging* **34**, 2370-2378 (2013).
65. L. Tovar-y-Romo, D. Kolson, V. Bandaru, J. Drewes, D. Graham, N. Haughey, Adenosine Triphosphate Released from HIV-Infected Macrophages Regulates Glutamatergic Tone and Dendritic Spine Density on Neurons. *Journal of Neuroimmune Pharmacology* **8**, 998-1009 (2013).
66. E. L. Craig, W. J. Frajola, M. H. Greider, An embedding technique for electron microscopy using Epon 812. *The Journal of cell biology* **12**, 190-194 (1962).
67. V. V. R. Bandaru, M. M. Mielke, N. Sacktor, J. C. McArthur, I. Grant, S. Letendre, L. Chang, V. Wojna, C. Pardo, P. Calabresi, S. Munsaka, N. J. Haughey, A lipid storage-like disorder contributes to cognitive decline in HIV-infected subjects. *Neurology* **81**, 1492-1499 (2013).
68. E. G. Bligh, W. J. Dyer, A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology* **37**, 911-917 (1959).
69. A. Kumar, D. Baycin-Hizal, D. Wolozny, L. E. Pedersen, N. E. Lewis, K. Heffner, R. Chaerkady, R. N. Cole, J. Shiloach, H. Zhang, M. A. Bowen, M. J. Betenbaugh, Elucidation of the CHO Super-Ome (CHO-SO) by Proteoinformatics. *Journal of proteome research* **14**, 4687-4703 (2015).
70. H. Mi, S. Poudel, A. Muruganujan, J. T. Casagrande, P. D. Thomas, PANTHER version 10: expanded protein families and functions, and analysis tools. *Nucleic acids research* **44**, D336-342 (2016).
71. N. Marchesini, W. Osta, J. Bielawski, C. Luberto, L. M. Obeid, Y. A. Hannun, Role for mammalian neutral sphingomyelinase 2 in confluence-induced growth arrest of MCF7 cells. *Journal of Biological Chemistry* **279**, 25101-25111 (2004).
72. R. Kolde, Pheatmap: pretty heatmaps. *R package version* **61**, (2012).

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vivo work was performed by AMD, LBT, and S-WY, DCA. Cell culture experiments were done by ALT and LBT. Exosome isolations were done by ALT and LBT. ALT performed all rt-PCR experiments. Immunohistochemistry, WB experiments and quantification were performed by AMD, LBT, ALT, MK, NT, MB, and S-W-Y. VVRB and NT ran and analysed the lipidomic samples. Proteomic data was acquired by RC and data analysed by AMD. miRNA data analyses was performed by ALT and KW. ChIP assay was performed by MG-M and PC. All BBB modelling was performed by BM, DWW, and JWB. AMD and NJH wrote the manuscript. All authors commented and revised the manuscript.

. **Competing interests:** Patrizia Casaccia is the founding director of the ASRC Neuroscience Initiative, CUNY.. **Data and materials availability:** The proteomics data from astrocytic EVs have been deposited to the PRIDE database, accession ID: PXD006087. The miRNA Nanostring data have been deposited to XXXX, accession ID: XXXX.

Figure Legends

Figure 1: Brain neutral sphingomyelinase regulates the peripheral acute cytokine response, and leukocyte transmigration following central administration of IL-1 β .

(A) Representative photomicrographs of Ly6b+ cells in striatum 24 hours after injection of saline or IL-1 β (0.1 ng in 3 μ l) in wild type (Wt), or mSmpd3 mice, and in Wt mice following co-injection of IL-1 β + GW4869 (20 μ M), or altenusin (Alte; 50 μ M). Insets are magnifications of the indicated regions and scale bar = 250 μ m. **(B and C)** Stereological quantitation of leukocytes for the indicated conditions. **(D)** Quantitative mass spectrometry of long-chain ceramides in Wt mice and mice expressing a deletion mutation in mSmpd3 mice 2 hours after striatal injection of IL-1 β or saline. **(E)** Stereological quantitation of

activated IBA-1+ microglia and GFAP+ astrocytes in striatum of Wt mice following striatal injection of IL-1 β , or IL-1 β + altenusin. **(F)** Striatum cytokine expression in Wt mice 2h following striatal injection of IL-1 β , or IL-1 β + altenusin. **(G-I)** Cytokine expression in liver (G), lung (H), and spleen (I) measured 2 hours after intra-striatal injection of IL-1 β , or IL-1 β + altenusin. Data are mean \pm SEM of n = 4-5 mice per condition for ceramide and PCR, and n= 3 mice per condition for leukocyte staining. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to IL-1 β .

Figure 2: EV adoptively transfer an acute peripheral cytokine response. (A) Pictorial representation of EV adoptive transfer experiments. EV were harvested from plasma of donor mice 2h following intra-striatal administrations of IL-1 β , or saline. EV (3.0×10^9) were infused into the tail veins of recipient animals co-injected into striatum with IL-1 β (0.1 ng in 3 μ l) + altenusin (50 μ M). As an additional control, recipient mice were infused with protein and RNA depleted EV (reconstituted liposomes were 80 nm) **(B)** qRT-PCR data of liver cytokine expression 2h following infusions of EV from donor mice into recipient mice. **(C and D)** Representative photomicrographs (C) and stereological quantitation (D) of Ly6b+ leukocytes in striatum 24h following venous infusion of EV, or liposomes. **(E)** Representative photomicrographs of ICAM-1 staining in the striatum of mice injected with saline, IL-1 β , or IL-1 β + altenusin. Data are mean \pm SEM of n = 4-5 mice per condition for PCR, and n = 3 mice per condition for leukocyte quantitation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to saline, and # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to IL-1 β . ANOVA with Tukey post hoc comparisons.

Figure 3: IL-1 β stimulation of astrocytes results in the formation of membrane microdomains that are EV release sites. (A) Representative fluorescence images from

FM 1-43 labeled astrocytes. The white arrows highlight the release of EVs into the media and the red arrows highlight EV's trapped in the cell. **(B-C)** Representative immunofluorescent overlays of CTX555 (labels GM1; red), and ceramide (green) dual immunopositive plasma membrane microdomains. Insets show magnification of the indicated region. Scale bar = 50 μ m. **(D and E)** Quantitative data of the percent of plasma membrane dual positive for (D) ceramide and GM1, or (E) nSMase2 and GM1 for the indicated treatment conditions. **(F)** Immunofluorescent images from mouse striatum 72h following injection of an adenoviral vector expressing a GFAP driven shRNA directed against nSMase2 (GFAP sh-nSMase2; 5×10^9 particles in 0.5 μ l). Images of DAPI, GFAP, the mCherry expressing vector, and merged image obtained from mouse striatum. Insets are magnifications of the indicated astrocytes. **(G and H)** Representative western blot (G) and density quantitation (H) of nSMase2 from the striatum expressing GFAP sh-nSMase2 and the contralateral striatum. **(I)** Sterological quantitation of Ly6b+ leukocytes in striatum 24h following injection of saline, or IL-1 β in the indicated treatment conditions. **(J)** Pictorial representation of experimental design for infusions of astrocyte-shed EV. Astrocytes in fresh plasma-free media were stimulated with IL-1 β for 2h. EV were isolated from the media and 2.78×10^9 EV were infused into the tail vein of mice administered IL-1 β (0.1 ng in 3 μ l) + alenusa (50 μ M). **(K)** qRT-PCR data of liver cytokine expression 4h following infusions of EV or control EV (liposomes) depleted of protein and RNA for the indicated treatment conditions. **(L and M)** Representative photomicrographs (L) and sterological quantifications (M) of Ly6b+ leukocytes in striatum 24h following infusion of astrocyte-shed EV or liposomes. Data are mean \pm SEM of 35-60 cells from 3-4 independent experiments per condition for immunofluorescence, n = 3-5 mice per condition for GFAP sh-nSMase2 expression, PCR, and quantitation of leukocyte influx. * $p < 0.01$, ** $p < 0.05$, *** $p < 0.001$

compared to control., # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to IL-1 β or contralateral, †† = $p < 0.05$ compared to IL-1 β +scRNA. ANOVA with Tukey post hoc comparisons.

Figure 4: EV released from IL-1 β -stimulated astrocytes rapidly cross the blood-brain-barrier and localize to peripheral organs. (A). Quantification of number of GFP positive particles that were transported through an in vitro model of the BBB under the described conditions **(B and C)** Representative electron micrographs of immunogold labeled GFP+ EV located in (B) the cytoplasm of an endothelial cell from the BBB model, and (C) in the cytoplasm of vascular endothelial cells of a GFP-GFAP transgenic mouse following striatal injection of IL-1 β (red arrows indicated immunogold labeled GFP). **(D)** GFP+ immunogold labeling was not observed in endothelial cells of Wt animals injected with IL-1 β , but luminal release of EV was observed (white arrows). **(E - M)** Representative immunofluorescent images (2h) and stereological quantitation (2h, 24h) of GFP+ EV from (E-G) liver, (H-J) lung, and (K-M) spleen following striatal injections of saline, IL-1 β , or IL-1 β + altenusin in the indicated strain of mice. Overlay images show F-actin labeled cells (red), DAPI labeled nuclei (blue), and GFP+ EV (green). **(N)** Immunofluorescent images from the liver of a GFAP-GFP mouse injected in striatum with the membrane probe FM-143 followed by IL-1 β . Images show GFAP, FM143, and the merged image with DAPI. Inset shows magnification of the indicated region. Scale bar = 1 μ m. Data are mean \pm SEM of $n = 5$ culture for BBB, and $n = 5$ mice per condition. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ increase compared to saline. ## $p < 0.01$, ### $p < 0.001$ decrease compared to IL-1 β . ANOVA with Tukey post hoc comparisons.

Figure 5. The cargo of EV shed from astrocytes in response to IL-1 β regulates the liver cytokine response through modulation of PPAR α . (A) Representative electron

micrograph of EV isolated from astrocytes 2h following stimulation with IL-1 β (100 ng/ml). **(B)** Quantitation of astrocyte-shed EV size and quantity. **(C)** Representative Western Blots from astrocyte-shed EVs stained for the tetraspan protein CD63, and the lipid raft marker flotillin. **(D)** Venn diagram comparing protein content of EV constitutively released from astrocytes (Control) with EV released following a 2h stimulation with IL-1 β (100 ng/ml). **(E)** Ingenuity Pathway Analysis was conducted using the protein and miRNA content of astrocyte EV shed in response to IL-1 β . Red fill indicates negative regulation, green fill indicates positive regulation, blue outline shows components identified by the protein content of EV, and a double purple outline shows components targeted by multiple miRNAs. **(F - I)** Chromatin immunoprecipitation (ChIP) assays results of NF- κ B binding to (F) *Ccl2*, (G) *Il-1 β* , (H) *Tnf α* and (I) *Il17* promoter regions in liver 2h following striatal injections of saline, or IL-1 β (0.1 ng in 3 μ l). **(J and K)** Representative photomicrographs (J) and stereological quantitation (K) of Ly6b+ leukocytes in striatum 24h following striatal injection of saline, IL-1 β , IL-1 β + tail vein infusion of the PPAR α agonist fenofibrate (10mg/kg). **(L and M)** Representative photomicrographs (L) and stereological quantitation (M) of Ly6b+ leukocytes 24 hours after striatal injection of IL-1 β , IL-1 β + altenusin (50 μ M), or IL-1 β + altenusin + tail vein administration of the PPAR α antagonist GW6471 (20 mg/kg). Data are mean \pm SEM. n = 3-6 mice per condition. ** $p < 0.01$, *** $p < 0.001$ compared to saline, * $p < 0.05$ compared to IL-1 β + altenusin. ## $p < 0.01$ ### $p < 0.001$ compared to IL-1 β . ANOVA with Tukey post-hoc comparisons.