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ORIGINAL ARTICLE

Pyroptotic neuronal cell death mediated by the AIM2 inflammasome

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The central nervous system (CNS) is an active participant in the innate immune response to infection and injury. In these studies, we show embryonic cortical neurons express a functional, deoxyribonucleic acid (DNA)-responsive, absent in melanoma 2 (AIM2) inflammasome that activates caspase-1. Neurons undergo pyroptosis, a proinflammatory cell death mechanism characterized by the following: (a) oligomerization of apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC); (b) caspase-1 dependency; (c) formation of discrete pores in the plasma membrane; and (d) release of the inflammatory cytokine interleukin-1β (IL-1β). Probenecid and Brilliant Blue FCF, inhibitors of the pannexin1 channel, prevent AIM2 inflammasome-mediated cell death, identifying pannexin1 as a cell death effector during pyroptosis and probenecid as a novel pyroptosis inhibitor. Furthermore, we show activation of the AIM2 inflammasome in neurons by cerebrospinal fluid (CSF) from traumatic brain injury (TBI) patients and oligomerization of ASC. These findings suggest neuronal pyroptosis is an important cell death mechanism during CNS infection and injury that may be attenuated by probenecid.

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INTRODUCTION

Neuronal cell death is traditionally classified into two broad categories: oncosis, an accidental or passive cell death, and apoptosis, a programmed cell death. Recent findings in the biochemical exploration of cell death suggest that additional cell death mechanisms may exist in the central nervous system (CNS). Pyroptosis is an inflammatory, caspase-1-dependent, programmed cell death mechanism distinct from apoptosis and oncosis in that pyroptosis: (a) does not depend on the effector caspases-3, 6, and 7 that trigger apoptosis; (b) is inhibited with the selective caspase-1 inhibitor peptide tyrosine-valine-alanine-aspartic acid (YVAD); (c) results in 1 to 2 nm pores in the plasma membrane with lysis of the cell and release of its proinflammatory contents; and (d) progresses with an intact mitochondria.[1] The proinflammatory effects of pyroptotic cell death are due in part to the maturation and release of interleukin-1β (IL-1β).

Pyroptosis is induced by the inflammasome, a multiprotein complex consisting of apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), an adaptor protein, and caspase-1, an inflammatory cysteine–aspartic protease.[2] With regard to CNS pathology, the NAcht leucine-rich-repeat protein 1 (NLRP1) inflammasome has been shown to have a role in pathomechanisms; however, new studies have emerged implicating the NLRP3 inflammasome[3–5] in these processes. Absent in melanoma 2 (AIM2) is a member of the hemopoietic IFN-inducible nuclear 200 (HIN-200) family of proteins and was recently discovered to form an inflammasome.[6] AIM2 is activated by viral, bacterial, and host ectopic dsDNA and induces cleavage of procaspase-1, maturation of IL-1β, and pyroptosis.[7] Although AIM2 is expressed in tissue outside the immune system,[8] its functional role in either the recognition of pathogenic DNA, or autoimmune reactions to host nucleic acids, has only been established in innate immune cells. It is unknown if AIM2 regulates the innate immune response to dsDNA in other cell types, such as neurons. Furthermore, it is unknown if AIM2 inflammasome activation induces pyroptosis in non-immune cells.

Here, we investigated whether embryonic cortical neurons challenged with poly(deoxyadenylic-deoxythymidylic) acid sodium salt, (poly(dA:dT)), a synthetic dsDNA activate the AIM2 inflammasome leading to pyroptotic cell death. We report that cortical neurons express a functional AIM2 inflammasome that recognizes aberrant dsDNA. Upon engagement of dsDNA, the neuronal AIM2 inflammasome induced oligomerization of ASC, activation of caspase-1, the production and secretion of IL-1β, formation of discrete pores in the plasma membrane, and ultimately neuronal pyroptosis. Cerebrospinal fluid (CSF) collected from human subjects after traumatic brain injury (TBI) contained significantly higher levels of nucleic acids than CSF from nontrauma control subjects; and application of CSF from TBI patients to primary cortical neurons induced AIM2 inflammasome activation. Moreover, pore formation and cell death were blocked by the pannexin1 inhibitors probenecid and Brilliant Blue FCF (BB FCF), identifying pannexin1 as an effector of neuronal pyroptosis and probenecid as a novel inhibitor of neuronal pyroptosis.

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MATERIALS AND METHODS

Subjects

All animal procedures were approved by the University of Miami Institutional Animal Care & Use Committee (IACUC). The IACUC is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All procedures were in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*. Time pregnant Sprague-Dawley rats were killed using exposure to high carbon dioxide. Embryos were placed in cold isotonic saline and the cerebral cortex was surgically dissected to produce neuronal cultures. Table 1 describes the demographics of TBI patients employed for this single-center prospective, observational study who were recruited from Jackson Memorial Hospital (Miami, Florida). Patients presented with the following inclusion criteria: severe or moderate head trauma (GCS 3 to 7); age 16 to 36 years; and ventriculostomy. Cerebrospinal fluid from control nontraumatized patients; ages 61 to 65 was obtained by ventriculostomy as part of their diagnostic and or prognostic clinical workup. Patients with acute meningitis, cerebral vasculitis, or other recent CNS infection were excluded. Written informed consent was obtained from all patients. The University of Miami's Institutional Review Board approved the study.

Antibodies

Immunocytochemistry was performed with the following primary (1:200) and secondary (1:500) antibodies: anti-AIM2 (Deciphergen, Aurora, CO, USA), anti-ASC (Santa Cruz, Dallas, TX, USA), anti-caspase-1 (Millipore, Billerica, MA, USA), Alexa Fluor 488 anti-mouse IgG (Invitrogen, Grand Island, NY, USA), and Alexa Fluor 594 anti-rabbit IgG (Invitrogen). Immunoblot analysis was performed with the following primary and secondary antibodies at 1:1,000 dilution: anti-AIM2 (Santa Cruz), anti-ASC (Bethyl Laboratories, Montgomery, TX, USA), anti-caspase-1 (Epitomics, Burlingame, CA, USA), anti-IL-1β (Cell Signaling, Danvers, MA, USA), anti-caspase-3 (Abcam, Cambridge, MA, USA), anti-mouse IgG (Cell Signaling), and anti-rabbit IgG (Cell Signaling). Immunoprecipitation was performed with anti-AIM2 (Abnova, Walnut, CA, USA).

Cell Culture and Inflammasome Stimulation

Embryonic neuronal cell cultures were prepared by dissociation of cerebral cortices from E18-19 rat embryos as previously described.[9–11] Cortical tissue was disrupted by gentile trituration and seeded onto poly-L-lysine-coated dishes at a density of $\sim 2 \times 10^6$ cells per well of a standard 6-well cell culture plate. Neuronal cultures were maintained for 7 days in N5 medium[12] with 5% (1 mg/mL protein) of an acid stable fraction of horse serum.[13] Cell cultures were then treated with different doses of poly(dA:dT) complexed with *LyoVec*, a cationic lipid-based transfection reagent, ranging from 2.5 to 20 μg/mL (Invivogen, San Diego, CA, USA) for 3 hours. Controls cells were unstimulated or stimulated with LyoVec alone. In an independent experiment to examine immunogenicity of CSF from TBI patients, neurons were incubated with a 1:1 ratio of media and CSF from a control or TBI patient for 24 hours.

Immunoblot Analysis

Cells were washed in cold phosphate-buffered saline (PBS), lysed in 400 μL buffer, and the supernatants were resolved by gel electrophoresis and immunoblotted as previously described.[4] To control for protein loading, cell lysates were evaluated using a Bradford assay, and sample data gathered from the optical density assay were utilized to normalize the protein levels across all samples. Quantification of band density was preformed with UN-SCAN-IT gel digitizing software (Silk Scientific, Orem, UT, USA), and data were normalized to β-actin.

Immunocytochemistry

Neurons were grown on poly-L-lysine-coated coverslips and stimulated with 5.0 μg/mL poly(dA:dT) for 3 hours. Cells were fixed in 4% paraformaldehyde, washed in cold PBS, and permeabilized with cold 95% ethanol at -20°C for 2 minutes. Nonspecific antibody binding was blocked by incubating cells with 10% bovine serum albumin[14] in PBS. Cells were incubated with primary antibody diluted 1:200 in 10% BSA–PBS followed by fluorescently labeled secondary antibodies diluted 1:500 in 10% BSA–PBS. Coverslips were mounted onto slides with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole. Images were obtained with a laser scanning confocal microscope (FluoView, Olympus 1000, Center Valley, PA, USA). Secondary antibody alone was used as a negative control.

Modified Chromatin Immunoprecipitation

Neurons were transfected with 5.0 μg/mL of a 300 base pair DNA fragment complexed with LyoVec for 3 hours. Controls were left unstimulated. One group was also treated with 5 units of DNase (Invitrogen) for 10 minutes at 37°C after poly(dA:dT) stimulation. Media was aspirated and cells were fixed in 1% formaldehyde-N5 media and gently rocked at room temperature for 10 minutes. Cells were washed by gentle rocking in ice-cold PBS for 5 minutes followed by a 5-minute wash in 0.125 mol/L glycine stop-fix solution. Cells were washed again in PBS for 5 minutes. Cells were

Table 1. Summary of demographic data and dsDNA levels of patients with TBI

Controls

Age	*Gender*	*Notes*	*DNA quantification*
61	Female	VP shunt revision	68 ng/mL
63	Male	New VP shunt	61 ng/mL
65	Male	New VP shunt	66 ng/mL

TBI

Age	*Gender*	*Notes*	*GCS score*	*GOS score*	*Intracranial pathology*	*DNA quantification*
26	Male	MVA	3	4	SAH, SDH	120 ng/mL
22	Male	Motorcycle accident	3	4	SDH, SAH	103 ng/mL
19	Female	MVA	7	3	SAH	109 ng/mL
30	Female	MVA	8	4	SAH	131 ng/mL
21	Male	Motorcycle accident	5	3	SAH, SDH	188 ng/mL
17	Female	MVA	4	3	Intraparenchymal hemorrhage	179 ng/mL
26	Male	MVA	8	3	SAH, IVH	103 ng/mL
36	Male	ATV accident	3	5	SDH, SAH, diffuse cerebral edema	120 ng/mL
16	Male	MVA	7	5	SDH	181 ng/mL
22	Male	MVA	3	3	SDH	83 ng/mL
36	Male	Gunshot wound	3	1	SDH, bullet fragments in frontal lobe	128 ng/mL

ATV, all terrain vehicle; GCS, Glasgow coma scale; GOS, Glasgow outcome score; IVH, intraventricular hemorrhage; MVA, motor vehicle accident; SAH, subarachnoid hemorrhage; SDH, subdural hematoma; VP, ventriculoperitoneal. GCS was obtained on admission. GOS was assessed at 5 months post injury.

harvested in PBS with 1X protease and phosphatase inhibitors and spun at 1,500 × *g* for 5 minutes at 4°C. Supernatant was aspirated and pellets were resuspended in ice-cold lysis buffer with 1X protease and phosphatase inhibitors. Samples were incubated on ice for 30 minutes and then homogenized with a Type-B dounce homogenizer on ice. After homogenization, samples were centrifuged in microcentrifuge tubes at 2,600 × *g* for 10 minutes at 4°C. Supernatant was removed and diluted twofold with buffer Y (0.01% SDS, 1.1% Triton X-100, 1.2 mmol/L ethylenediaminetetraacetic acid (EDTA), and 167 mmol/L NaCl). Hundred microliters of supernatant was set aside to be used as input in the polymerase chain reaction and the remainder of supernatant was aliquoted 1 mL per tube. Co-immunoprecipitation was performed on aliquoted samples as described below and immune complex-bound beads were washed in a series of three Tris-SDS-EDTA buffers with increasingly stringent salt concentrations (150 mmol/L NaCl, 500 mmol/L NaCl, and 5 mol/L LiCl/300 mmol/L NaCl, respectively) to remove nonspecific DNA binding followed by a final wash in Tris-EDTA (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.1) buffer. Samples were vortexed for 10 seconds, placed on a rotator for 15 minutes, and centrifuged at 1,650 × *g* for 2 minutes. Immune complexes were eluted with 100 μL elution buffer (1% SDS, 0.1 mol/L $NaHCO_3$) for 15 minutes, followed by centrifugation at 1,650 × *g* for 1 minute. After removing supernatant, a subsequent elution was performed with 50 μL elution buffer and supernatant pooled from both elution steps.

Cross-links were reversed by incubating the input with 200 μL deionized water + 12 μL 5 mol/L NaCl and the IP with 6 μL of 5 mol/L NaCl. Samples were incubated for overnight at 65°C. Immunoprecipitation samples were then combined with one part proteinase solution (3 μL 0.5 mol/L EDTA, 3 μL 2 mol/L Tris-Cl pH 6.8, and 3 μg proteinase K) and input samples were combined with two parts proteinase solution. Samples were incubated for 90 minutes at 45°C and DNA was purified using a polymerase chain reaction purification kit (Qiagen, Germantown, MD, USA) according to manufacturer's instructions. Samples were eluted in 50 μL Tris-EDTA buffer.

Purified DNA samples and inputs (1:10) were subjected to polymerase chain reaction amplification. Samples underwent 20 cycles consisting of a 40-second denaturing step at 98°C, a 20-second annealing step at 62°C, and a 30-second synthesis step at 72°C. Samples were run on a 1% agarose gel with 1 μL ethidium bromide per 50 mL TAE (Tris-acetate-EDTA) (40 mmol/L Tris, 20 mmol/L acetic acid, 1 mmol/L EDTA) buffer and visualized using ultraviolet light.

Co-Immunoprecipitation

Embryonic cortical neurons were homogenized in lysis buffer. Cell homogenates were incubated on ice for 15 minutes and then centrifuged at 2,600 × *g* for 5 minutes at 4°C to remove cell debris. Immunoprecipitation assays were performed using 1 mg magnetic Dynabeads (Invitrogen) coupled to 7 μg of anti-AIM2 (Abnova) according to the manufacturer's instructions. Briefly, 250 μg of lysate applied to 1.5 mg of anti-AIM2 coupled magnetic beads, incubated overnight at 4°C on a rotator, and eluted in a low pH buffer. Preimmune serum (Abnova) served as a negative control. Elutions were collected and added to 1X Laemmli buffer to dissociate proteins. Five microliters of 1 mol/L Tris, pH 9.8 were added to match the pH of the stacking gel. Samples were then boiled for 5 minutes and subjected to SDS-PAGE and immunoblot analysis.

Partial Purification of Pyroptosome

Apoptosis-associated speck-like protein containing a caspase recruitment domain oligomers were partially purified as described.[15] Briefly, embryonic neuronal cell lysates or rat cortical homogenates were centrifuged at 1,800 r.p.m. for 8 minutes. The supernatant was filtered through a 5 μm polyvinylidene difluoride membrane (Millipore) at 2,000 × *g*. After filtration, 400 μL of 3[(3-cholamidopropyl) dimethylammonio]-propanesulfonic acid (CHAPS) buffer (20 mmol/L HEPES-KOH, pH 7.5, 5 mmol/L $MgCl_2$, 0.5 mmol/L EGTA, 0.1 mmol/L phenylmethylsulfonyl fluoride, protease inhibitor cocktail, and 0.1% CHAPS) was added to samples. The pyroptosome was pelleted by centrifugation at 2,700 × *g* for 8 minutes. The pellet was resuspended in 45 μL of CHAPS buffer and incubated in 2 mmol/L disuccinimidyl suberate (Thermo Scientific, Waltham, MA, USA) 30 minutes at room temperature to cross-link ASC dimers. An equal volume of 2 × Lammeli buffer was added, and samples were boiled for 5 minutes, and subjected to gel electrophoresis and immunoblot analysis.

Cell Viability Assay

Embryonic cortical neurons were grown in a 96-well microplate. Ac-YVAD-CMK (Enzo) was reconstituted in PBS with 1% BSA and cells were pretreated with 50 μmol/L YVAD for 30 minutes before addition of poly(dA:dT) for 3 hours. Vehicle/LyoVec control groups were run in parallel. After stimulation, Alamarblue was added to each well (1:20) and cells were incubated at 37°C for 90 minutes. Absorbance was recorded at 600 nm through a Victor plate reader using Wallace workstation software (Perkin Elmer, Waltham, MA, USA).

Dye Uptake

Neurons were pretreated for 5 minutes with either 500 μmol/L BB FCF, for pannexin1 specificity, or 1 mmol/L probenecid, and then stimulated with 5 μg/mL poly(dA:dT) with fresh BB FCF or probenecid for 3 hours. One group was pretreated with 50 μmol/L YVAD. Triton X-100 detergent (0.1%) was used as a positive control for dye uptake. Unstimulated neurons and neurons incubated with LyoVec only were run in parallel. One micromolar YO-PRO-1 iodide (Invitrogen), 1 μmol/L ethidium homodimer-2(Eth-D2) (Sigma-Aldrich, St Louis, MO, USA), and 2 μmol/L Hoechst 33342 (Immunochemistry Technologies, Bloomington, MN, USA) were added for 15 minutes. Images were captured using an Olympus CKX41 inverted microscope (Olympus). Dye-positive cells were counted in a total of 24 fields per group (6 wells per group, 4 fields per well).

Cerebrospinal Fluid Sample Collection and Analysis

The University of Miami's Institutional Review Board approved the study in accordance with the ethical standards of the responsible committee on human experimentation. Cerebrospinal fluid samples were collected via ventriculostomy from severe (Glasgow coma score ⩽ 8) TBI patients (Table 1), within 12 hours of injury. Control patients required a ventriculostomy for nontraumatic pathology as part of their diagnostic or prognostic clinical workup. Patients with acute meningitis, cerebral vasculitis, or other recent CNS infection were excluded. Written informed consent was obtained from all patients. The University of Miami's Institutional Review Board approved the study. Samples were centrifuged at 2,000 × *g* for 10 minutes at 4°C to pellet cellular bodies and debris, and the supernatants were aliquoted and stored at − 80°C. Before analysis, samples were spun again at 11,000 × *g* for 3 minutes at 4°C. Deoxyribonucleic acid was extracted from 11 TBI CSF samples and three control CSF samples using QIAamp DNA mini purification kit (Qiagen). Deoxyribonucleic acid was eluted from the column with 100 μL of provided buffer AE and centrifugation of 6,000 × *g* for 1 minute. A PicoGreen assay (Invitrogen) was performed to quantify the concentration of DNA present in CSF samples. Twenty microliters of eluate was added to 80 μL Tris-EDTA buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.3) and 100 μL PicoGreen in a 96-well plate. Lambda DNA of known concentrations was assayed in parallel to obtain a standard curve. The plate was incubated in the dark for 5 minutes and fluorescence intensity measured at 485/535 nm through the Victor plate reader and Wallace workstation software (Perkin Elmer). Rat fluid percussion injury was performed as previously described.[3]

Statistical Analysis

When two groups were analyzed, statistical comparisons were made using two-tailed Student's *t*-tests. When more than two groups were analyzed a one-way ANOVA followed by Tukey's multiple comparison tests were used. Data are expressed as mean ± s.e.m. *P* values of significance were $*P \leqslant 0.05$, unless indicated otherwise in figure legend.

RESULTS

Poly(Deoxyadenylic-Deoxythymidylic) Acid Sodium Salt Activates an Inflammasome in Embryonic Cortical Neurons

Embryonic cortical neurons were stimulated with increasing doses of poly(dA:dT), a synthetic dsDNA and ligand of the AIM2 inflammasome, complexed with *LyoVec*, a cationic lipid-based transfection reagent. There was a significant increase in caspase-1 cleavage in neuronal lysates (Figure 1A) and a significant increase in released IL-1β into neuronal supernatants (Figure 1B) in all doses tested. The most robust IL-1β response was observed at 5 and 10 μg/mL. These findings demonstrate that neurons express a functional DNA-responsive inflammasome.

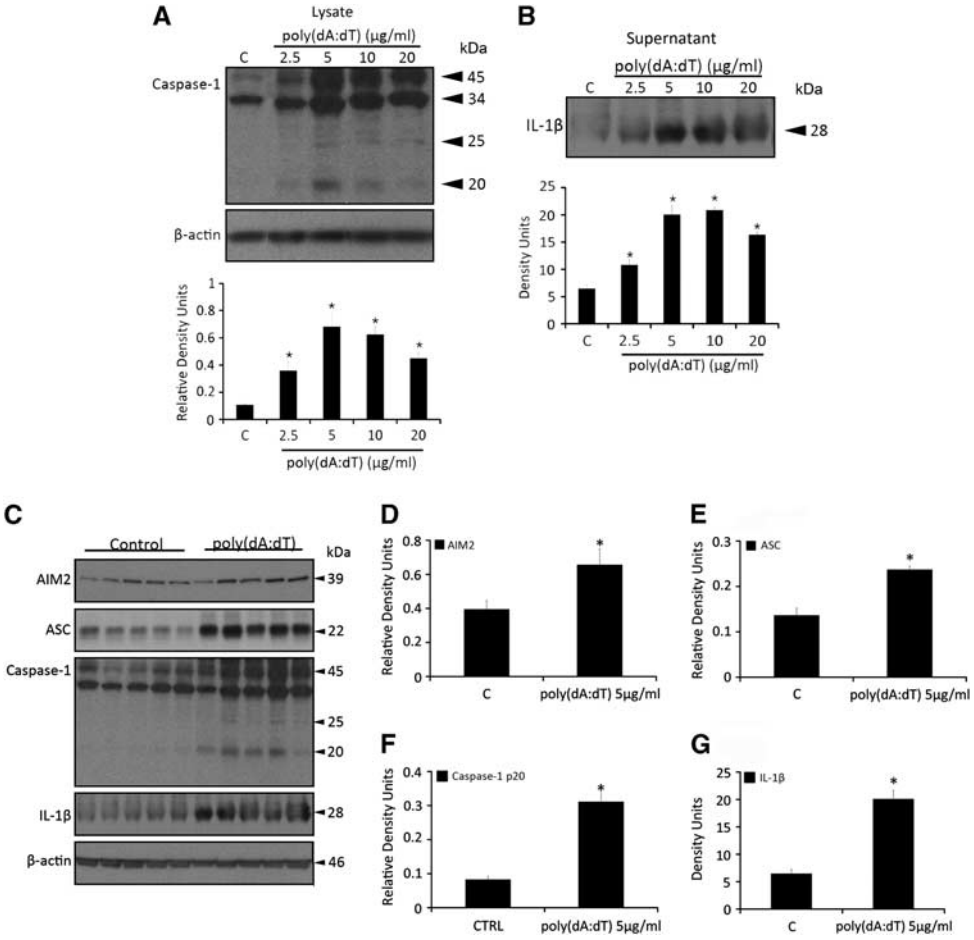

Figure 1. The AIM2 inflammasome ligand, poly(deoxyadenylic-deoxythymidylic) acid sodium salt (poly(dA:dT)), induces processing of caspase-1 and release of mature interleukin-1β (IL-1β) and expression of absent in melanoma 2 (AIM2) inflammasome proteins in embryonic cortical neurons. Immunoblot analysis of neurons stimulated with increasing doses of poly(dA:dT) for 3 hours. Densitometric analysis shows a dose-dependent effect of dsDNA on activation of caspase-1 in lysates (**A**) and release of IL-1β in supernatant (**B**). $N = 5$ to 6 per group. Data were normalized to β-actin and are presented as mean + s.e.m. $^*P < 0.05$ compared with control. Poly(deoxyadenylic-deoxythymidylic) acid sodium salt induces expression of AIM2 inflammasome proteins. Immunoblots of neuronal lysates stimulated with 5.0 μg/mL poly(dA:dT) for 3 hours (**C**) show a significant increase in AIM2 (**D**), ASC (**E**), cleaved caspase-1 (**F**), and mature IL-1β (neuronal supernatants) (**G**) compared with unstimulated controls. β-actin was used as a control for protein loading. Data presented as mean ± s.e.m. $^*P < 0.05$. $N = 5$ to 6.

Absent in Melanoma 2 Inflammasome Proteins are Expressed in Neurons

Absent in melanoma 2 is a recently identified DNA-sensing inflammasome component in macrophages.[16,17] Next, we investigated if neurons express AIM2 inflammasome proteins and if expression of these proteins changed upon stimulation with 5 μg/mL poly(dA:dT) (Figure 1C). Neurons expressed AIM2, ASC, caspase-1, and IL-1β (Figure 1C). Stimulation with poly(dA:dT) increased expression of AIM2 (Figure 1D), ASC (Figure 1E), and caspase-1 (Figure 1F). Moreover, poly(dA:dT) treatment significantly increased levels of p28 IL-1β in neurons (Figure 1G). LyoVec alone did not activate the inflammasome (data not shown).

Subcellular Localization of Absent in Melanoma 2 Inflammasome Proteins in Embryonic Cortical Neurons

To investigate the subcellular localization of AIM2 inflammasome proteins, we performed immunocytochemistry of ASC, caspase-1, and AIM2 in unstimulated and poly(dA:dT)-stimulated neurons. ASC was expressed in the neuronal nucleus and cytoplasm (Figure 2A). Caspase-1 was also expressed in the cytoplasm and nucleus of cortical neurons (Figure 2A top panel). Upon stimulation with poly(dA:dT), punctate caspase-1 immunoreactivity was observed in the neurites, which showed a beaded staining pattern (Figure 2A). Multiple foci of AIM2 staining coincided with the location of the nucleus (Figure 2B bottom panel). These foci oligomerized into a single focus after stimulation with poly(dA:dT).

Absent in Melanoma 2 Forms a Functional, Deoxyribonucleic Acid-Responsive Inflammasome in Neurons

To investigate if AIM2 associated with ASC and caspase-1 in a functional DNA-responsive inflammasome, we performed co-immunoprecipitation with anti-AIM2 or preimmune serum (negative control). Anti-AIM2 immunoprecipitated ASC and caspase-1, but not caspase-3, an apoptotic caspase not directly involved in inflammasome activation and served as a control (Figure 3A). Preimmune serum did not immunoprecipitate the inflammasome-associated proteins demonstrating antibody specificity and serving as control (Figure 3A). Our findings show the AIM2 inflammasome is preassembled in unstimulated neurons. Stimulation with poly(dA:dT) induced an increase in processing of caspase-1 by the AIM2 inflammasome (Figure 3A).

To determine if AIM2 interacts directly with exogenous nucleic acids, neurons were stimulated with a 300 base pair DNA fragment complexed with LyoVec and a modified ChIP assay was

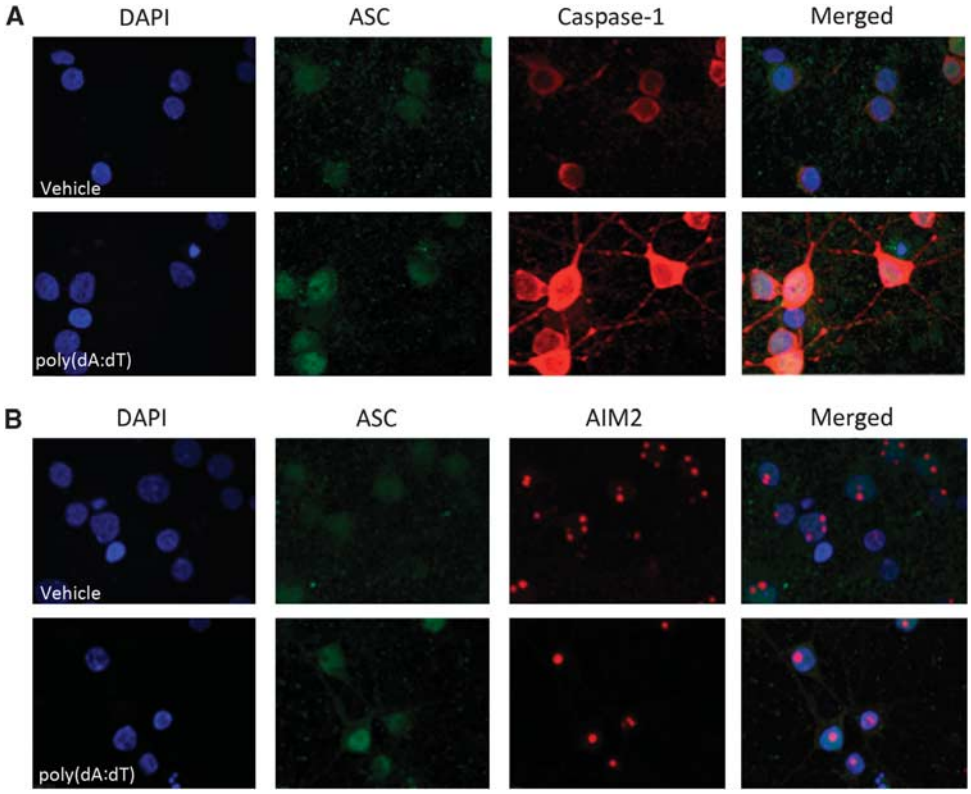

Figure 2. Absent in melanoma 2 (AIM2), ASC, and caspase-1 are present in embryonic cortical neurons and mediates the innate immune response to dsDNA. Panel **A** shows punctate immunoreactivity of caspase-1 in the neurites of poly(deoxyadenylic-deoxythymidylic) acid sodium salt (poly(dA:dT))-stimulated neurons but not in unstimulated neurons. Panel **B** shows oligomerization of AIM2 foci near the nucleus of dsDNA-stimulated neurons. Both panels **A** and **B** show ASC is expressed in the nucleus and cytoplasm of neurons. DAPI, 4′,6-diamidino-2-phenylindole.

performed. Anti-AIM2, but not IgG (control), immunoprecipitated the transfected DNA fragment (Figures 3B and C). To digest away residual DNA that may be left behind before beginning the ChIP assay, we treated one group with DNase. Treatment with DNase decreased the amount of the DNA fragment in the input, but did not digest the AIM2-bound fragments. Our experimental results show that AIM2 forms a functional inflammasome in cortical neurons that recognizes exogenous dsDNA and induces caspase-1 activation and mature IL-1β release.

Stimulation of the Absent in Melanoma Inflammasome Induces Neuronal Pyroptosis

In immune cells, AIM2 activation induces pyroptosis, an inflammatory cell death program executed by caspase-1. To evaluate if activation of the AIM2 inflammasome in embryonic cortical neurons induces cell death, we performed an alamarBlue cell viability assay. Figure 4A shows that there is a significant reduction in the metabolism of alamarBlue after stimulation with poly-(dA:dT), indicating a decrease in cell viability. To establish whether caspase-1 activation contributes to poly(dA:dT)-induced neuronal cell death, neurons were treated with Ac-YVAD-CMK, a cell-permeable irreversible inhibitor of caspase-1 before stimulation with poly(dA:dT). Pretreatment with YVAD blocked cell death induced by dsDNA stimulation (Figure 4A).

To investigate if AIM2-induced, caspase-1-dependent neuronal cell death has other features of pyroptosis, we performed a biochemical assay of ASC pyroptosome formation. Stimulation of embryonic cortical neurons with poly(dA:dT) induced ASC laddering (Figure 4B). ASC monomers, dimers, and trimers are observed at $\sim$25 kDa, $\sim$50 kDa, and $\sim$75 kDa, respectively. These findings indicate assembly of the pyroptosome, a supramolecular complex of ASC oligomers that serves as a platform for caspase-1 activation in pyroptosis.[18]

Pyroptosis is also characterized by the formation of discrete pores in the plasma membrane.[19] To investigate if AIM2-mediated neuronal cell death also shares this feature, we applied two membrane impermeable dyes of different sizes to neurons stimulated with poly(dA:dT). Triton 0.1% served as a positive control for dye uptake. AIM2 inflammasome stimulation induced formation of discrete pores in the plasma membrane, evidenced by an increase in the uptake of YO-PRO-1 iodide, a small (629 Da) membrane impermeable dye, with exclusion of a larger (1,293 Da) membrane impermeable dye, Eth-D2 (Figures 5A and B). LyoVec alone did not induce dye uptake (Figures 5A and B). Pretreatment with YVAD significantly decreased uptake of YO-PRO-1 iodide, indicating pore formation in response to activation of AIM2 depends on caspase-1 (Figures 5A and B).

Pore formation depends on caspase-1, but the identity of the pore is unknown.[19] Pannexin1, an ATP release channel, is involved in the activation of the NLRP1 inflammasome.[20] We therefore investigated if pannexin1 is the pore responsible for the uptake of small dyes during AIM2-mediated pyroptosis by pretreating neurons with BB FCF, a selective inhibitor of pannexin1,[21] before stimulation with poly(dA:dT). We also tested probenecid before stimulation with poly(dA:dT). Independently, both BB FCF and probenecid significantly decreased the uptake of YO-PRO-1 during AIM2 inflammasome stimulation, suggesting pannexin1 forms a plasma membrane pore during pyroptosis (Figures 5A and B). It is hypothesized that opening of discrete plasma membrane pores during pyroptosis is directly responsible for lysis of the cell.[19] We therefore investigated whether probenecid, a drug already

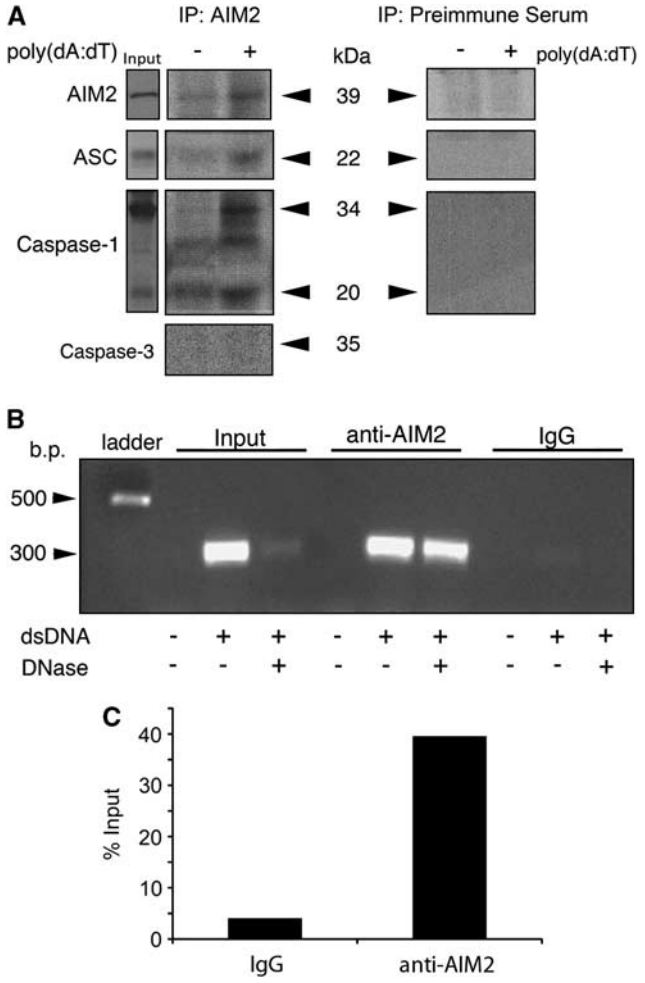

Figure 3. Absent in melanoma 2 (AIM2) forms protein–protein interactions with ASC and caspase-1 in embryonic cortical neurons. There is an increase in active caspase-1 immunoprecipitated with anti-AIM2 in lysates stimulated with poly(deoxyadenylic-deoxythymidylic) acid sodium salt (poly(dA:dT)). Input: + poly(dA:dT). (**A**). In panel **B** neurons were unstimulated, stimulated with dsDNA (5 μg/mL), or stimulated with dsDNA followed by treatment with DNase I. Lysates (input) were immunoprecipitated with anti-AIM2 or immunoglobulin G (IgG). Electrophoresis of deoxyribonucleic acid (DNA) purified from the precipitated immune complex reveals the 300 bp fragment used to stimulate the neurons was precipitated with anti-AIM2, but not with IgG. The DNA fragment in the input was partly digested by DNase, but still precipitated with anti-AIM2 and not with IgG. Panel **C** shows % of input pulled down with IgG and anti-AIM2.

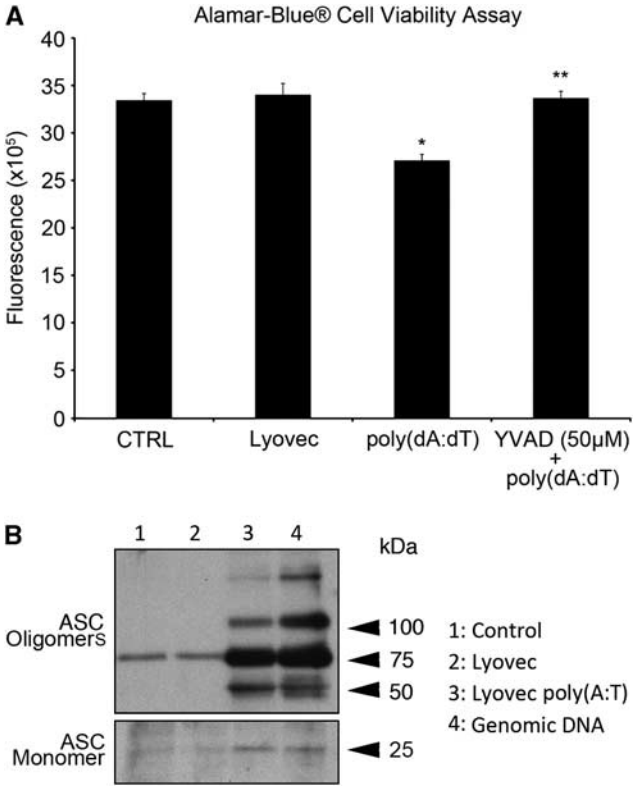

Figure 4. Activation of the AIM2 inflammasome induces neuronal pyroptosis. In panel **A**, embryonic cortical neurons were stimulated with poly(deoxyadenylic-deoxythymidylic) acid sodium salt (poly(dA:dT)) (5 μg/mL; 3 hours) in the presence or absence of YVAD, a caspase-1 inhibitor. There is approximately a 20% reduction in cell viability in neurons stimulated with poly(dA:dT) in the absence of YVAD. Pretreatment with YVAD blocks poly(dA:dT)-induced cell death. LyoVec alone does not decrease cell viability. Data presented as mean +/− s.e.m. *$P<0.0001$ compared with control (CTRL). **$P<0.0001$ compared with poly(dA:dT). $N=12$. In panel **B**, poly(dA:dT) also induced laddering of ASC, indicating formation of the pyroptosome, a supramolecular assembly of ASC dimers that activates caspase-1 and induces pyroptosis. Lane 4 shows genomic DNA induces oligomerization of ASC as well.

approved by the FDA for use in humans, could inhibit cell death during AIM2 inflammasome stimulation. There is a significant increase in cell viability in neurons pretreated with probenecid before stimulation with poly(dA:dT) (Figure 5C).

Collectively, these findings indicate stimulation of the AIM2 inflammasome in cortical neurons by dsDNA induces pyroptosis, a caspase-1-dependent inflammatory mode of cell death. Our findings are also the first to identify pannexin1 as a plasma membrane pore in inflammasome-mediated pyroptosis and probenecid as a novel inhibitor of neuronal pyroptosis.

Human Cerebrospinal Fluid from Traumatic Brain Injury Patients Activates the Absent in Melanoma 2 Inflammasome

The AIM2 inflammasome is unable to distinguish between host and pathogenic nucleic acids,[22] creating the potential for auto inflammatory responses. Host nucleic acids are released from necrotic cells during traumatic and ischemic CNS injury.[23] Cell-free nucleic acids circulate in the plasma of TBI patients and serve as biomarkers of injury severity and outcome.[24] Therefore, we investigated whether the CSF of TBI patients also contains cell-free DNA fragments that may be immunogenic. We detected a significant elevation of cell-free dsDNA in the CSF of TBI patients compared with nontrauma controls (Figure 6A). To investigate if CSF from a TBI patient stimulates an innate immune response in neurons, we incubated embryonic cortical neurons with 50% media–50% CSF from a TBI or control patient. Traumatic brain injury CSF induced AIM2 expression and cleavage of caspase-1, suggesting danger signals released into the CSF after TBI can activate the AIM2 inflammasome and caspase-1, potentially inducing pyroptosis in neighboring cells (Figures 6B–D).

DISCUSSION

Our results show that embryonic cortical neurons express a functional AIM2 inflammasome that is activated by dsDNA and induces the inflammatory cell death modality pyroptosis. Moreover, we identified pannexin1 as a cell death effector channel in pyroptosis and demonstrate that inhibition of pannexin1 by

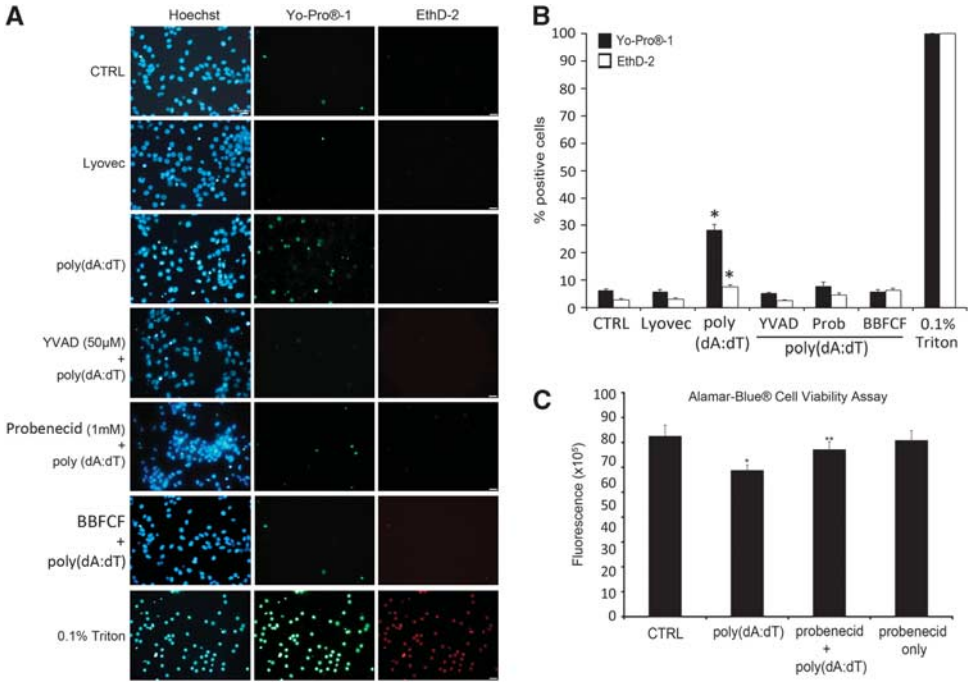

Figure 5. Neuronal pyroptosis is inhibited by probenecid and the pannexin1 inhibitor Brilliant Blue FCF (BB FCF). In panel **A**, embryonic neonatal cortical neurons were stimulated with poly(deoxyadenylic-deoxythymidylic) acid sodium salt (poly(dA:dT)) in the presence or absence of YVAD, probenecid, or BB FCF, and stained with a small (629 Da) membrane impermeable dye, YO-PRO-1, (green) and a larger (1,293 Da) membrane impermeable dye, ethidium homodimer-2 (Eth-D2), (red) to identify neurons with discrete membrane pores. Hoechst stain (blue) shows total cells in each field. Small, 1 to 2 nm, caspase-1-dependent pores form in a subpopulation (~20%) of cortical neurons stimulated with poly(dA:dT). Selective inhibition of pannexin1 by either probenecid or BB FCF abolishes pore formation and uptake of the smaller dye after activation of the AIM2 inflammasome. Panel **B** shows percentage of cells that stained positive for YO-PRO-1 and Eth-D2 counted from four fields per well, six wells per group. Data presented as mean ± s.e.m. $^{*}P<0.01$ compared with control (CTRL). In panel **C**, embryonic cortical neurons were stimulated with poly(dA:dT) in the presence or absence of 1 mmol/L probenecid. There is approximately a 20% reduction in cell viability in neurons stimulated with poly(dA:dT). Pretreatment with probenecid inhibits poly(dA:dT)-induced cell death. Probenecid alone does not decrease cell viability. Data presented as mean ± s.e.m. $^{*}P<0.01$ compared with CTRL. $^{**}P<0.05$ compared with poly(dA:dT). $N=12$.

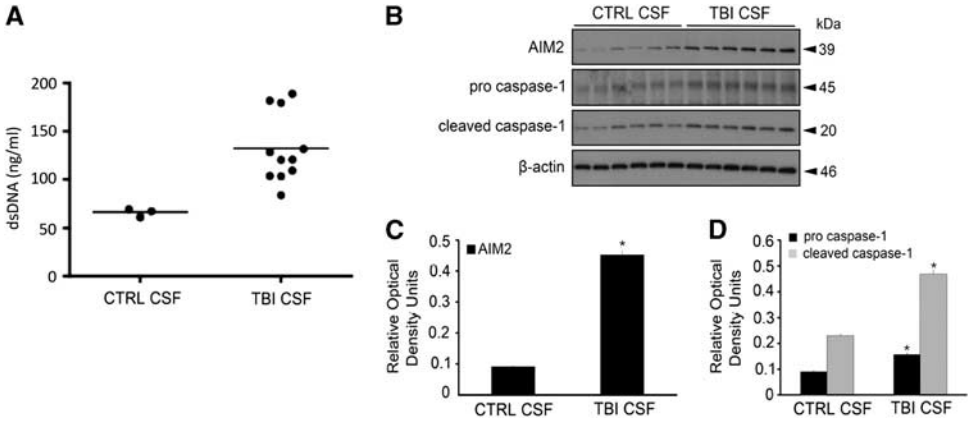

Figure 6. Traumatic brain injury (TBI) results in release of cell-free nucleic acids into the cerebrospinal fluid (CSF) and assembly of the pyroptosome. Panel **A** shows dsDNA is elevated in the CSF of TBI patients compared with nontrauma controls ($P=0.01$). In panel **B**, immunoblots of neuronal lysates incubated in 50% media–50% CSF from a TBI patient for 24 hours show a significant increase in AIM2 expression (**C**) and cleavage of caspase-1 (**D**) compared with neurons incubated in 50% media–50% CSF from a nontrauma control patient. β-actin was used as protein loading control. Data presented as mean ± s.e.m. $^{*}P<0.0001$ compared with control. $N=6$.

probenecid attenuates pyroptosis in neurons. Our findings are the first to report expression of AIM2 inflammasome proteins in the CNS and the first to identify pyroptosis as a cell death mechanism in neurons. There is ~20% reduction in cell viability, indicating a subpopulation of embryonic cortical neurons is vulnerable to pyroptotic cell death. These findings are consistent with selective neuronal loss after TBI, and with reports of pyroptosis in other cell types, in which ~20% to 30% of the cells form discrete pores and undergo cell death in response to activation of the AIM2 inflammasome.[6,19]

The key features of pyroptosis (i.e., plasma membrane pore formation, nuclease-mediated DNA fragmentation, and nonclassical secretion of inflammatory cytokines) all depend on caspase-1, but the mediators of these events downstream of caspase-1 are unknown. The identity of the plasma membrane pore in pyroptosis has remained elusive. Here, using two different selective inhibitors, we show pannexin1, an ATP release channel linked to activation of the NLRP1 and NLRP3 inflammasomes, opens during pyroptosis. Furthermore, we show inhibition of pannexin1 by probenecid attenuates pyroptosis, suggesting pannexin1 is a cell death effector in pyroptosis. Our studies do not determine how pannexin1 is activated during poly(dA:dT)-induced pyroptosis. However, our findings demonstrate that opening of the pannexin1 channel during poly(dA:dT)-induced pyroptosis requires active caspase-1, suggesting caspase-1 is upstream of pannexin1 during this stimulus. In cells undergoing pyroptosis, caspase-1 activation mediates calcium influx[25] and pannexin1 can be activated by increased intracellular calcium;[26] one possible mechanism explaining our findings. Once open, pannexin1 may perpetuate caspase-1 activation in a positive feedback loop. Caspase-7 cleaves pannexin1 into a constitutively open channel.[27] Alternatively, our findings are explained when caspase-7 is activated by caspase-1 during AIM2 inflammasome stimulation, leading to activation of pannexin1. Indeed, caspase-7 is a substrate of caspase-1.[28]

Poly(dA:dT) activated the neuronal AIM2 inflammasome in a dose-dependent manner, with a peak response observed at 5 and 10 μg/mL. This response, along with our finding that anti-AIM2 pulled down caspase-1 cleavage products in unstimulated neurons, is consistent with previous reports in macrophages, in which AIM2 inflammasome proteins are preassembled and 5 μg poly(dA:dT) stimulation for three hours induces caspase-1 processing.[29] There was a less robust response at 20 μg/mL, the highest concentration tested. As with any innate immune response, the host is tasked with eradication of the offending agent while minimizing inflammation and tissue damage. This balance may be accomplished by other pattern recognition receptors that bind ectopic DNA at a lower affinity than AIM2 and attenuate inflammasome activation. One such candidate is P202, a member of the mouse HIN-200 family that contains a DNA-binding HIN domain but lacks an ASC-binding pyrin domain and is unable to form an inflammasome. P202 sequesters DNA and interferes with activation of the AIM2 inflammasome,[30] which may account for the decrease in IL-1β activation at higher doses of DNA.

One novel finding of our studies is the nuclear localization of AIM2. Others have previously reported ASC and caspase-1 expression in the neuronal nucleus.[4] In unstimulated macrophages, AIM2 is diffusely cytoplasmic. Upon stimulation with poly(dA:dT), AIM2 oligomerizes into a single cytoplasmic focus.[31] One other report demonstrates an inflammasome assembling in the nucleus. IFI16, also a member of the HIN-200 family of proteins, forms an inflammasome in the nucleus of endothelial cells and mediates anti-viral innate immunity to nuclear replicating viruses.[32] Our immunocytochemistry findings, coupled with the immunoprecipitation data, suggest AIM2 may function in a similar manner, forming an inflammasome in the neuronal nucleus and recognizing ectopic DNA. In support of this idea, we observed multiple foci of AIM2 in the nucleus that upon stimulation with poly(dA:dT) appear to coalesce into a single focus. These findings are consistent with previous reports of homotypic interactions between AIM2 proteins upon binding to DNA.[17,33]

ASC staining is diffuse in unstimulated macrophages, but upon stimulation with poly(dA:dT), ASC coalesces into a single focus previously called the pyroptosome.[18] Although we observed oligomerization of ASC upon stimulation with poly(dA:dT) in neurons, ASC staining remained diffuse. Rather than facilitate pyroptosis, the ASC focus in macrophages may sequester caspase-1 into a single location, restricting its access to substrates that are effectors of pyroptosis. It is proposed that the ASC focus is more important for activation of cytokines, while diffuse ASC is critical for execution of pyroptosis.[34] Diffuse ASC staining of neurons undergoing pyroptosis observed in our studies supports this conclusion.

The biochemical techniques employed here to evaluate cell death are specific to pyroptosis; i.e. ASC oligomerization, plasma membrane pore formation, caspase-1 dependency, and release of IL-1β. Such endpoints are less prone to misinterpretation by the investigator than nonspecific assays such as terminal deoxynucleotidyl transferase dUTP nick end labeling staining and lactate dehydrogenase release. Nonetheless, cross talk between the different cell death mechanisms (i.e., pyroptosis, apoptosis, oncosis) is a consideration when interpreting our findings. Although conveniently classified as separate processes, the limits of each cell death mechanism are increasingly indistinct. It is more likely that *in vivo*, pyroptosis is not an isolated path to cellular demise. Caspase-1 deficient macrophages stimulated with poly(dA:dT) undergo a caspase-8-dependent form of cell death characterized by lactate dehydrogenase release, propidium iodide uptake, and a much slower progression than pyroptosis.[35] Thus pyroptosis and apoptosis are not always mutually exclusive events, and inhibition of one may drive the cell toward the other. In our experiments, caspase-1-inhibition completely blocked poly(dA:dT)-induced cell death, suggesting apoptosis did not compensate for inhibition of pyroptosis in neurons. We performed the cell death assays up to 24 hours after stimulation with poly(dA:dT). Future studies should include extended time points to investigate if other cell death pathways are recruited when pyroptosis is blocked.

We also report an elevation of cell-free DNA in the CSF of TBI patients compared with nontrauma controls. Circulating host DNA fragments accumulate in plasma in an array of pathologic conditions, including neurologic injury, as early as 45 minutes after injury.[36,37] Cell-free DNA is elevated in the plasma of TBI patients compared with healthy controls, and the degree of elevation correlates significantly with mortality.[24] Similar findings are reported in a rodent model of TBI, where cell-free plasma DNA is elevated after injury and correlates with brain edema and the neurologic severity score.[38] In addition, elevated levels of caspase-1 have been reported in human cerebral contusions after TBI,[39] supporting the idea that inflammasomes are activated during the acute phase after TBI. Our findings are the first to report an elevation of cell-free DNA in the CSF from a TBI patient applied to cultured embryonic cortical neurons activated the AIM2 inflammasome, suggesting CSF from injured patients is immunogenic and may induce pyroptosis in surrounding cells.

Our findings establish pyroptosis as a neuronal cell death mechanism induced by activation of the AIM2 inflammasome, providing additional evidence that neurons are not passive bystanders during injury or infection, but possess functional PRRs that recognize and respond to pathogen- and damage-associated molecular patterns. The implications are widespread, as characterization of a neuronal DNA-sensing inflammasome reveals the pathomechanisms of neurotropic viruses and potentially sheds light on aberrant autoimmune reactions to host nucleic acids. Based upon these findings, we propose a mechanism for neuronal AIM2 inflammasome activation and pyroptosis in which the AIM2 inflammasome assembles in the nucleus. Upon activation by host or pathogenic DNA, AIM2 oligomerizes and activates ASC, which activates caspase-1 in the nucleus. ASC dissociates from the inflammasome and translocates to the cytoplasm, where it oligomerizes into the pyroptosome, a molecular platform for cytoplasmic caspase-1 activation. Active caspase-1 cleaves inflammatory cytokines, opens the pannexin1 pore, and induces cell death. Moreover, neuronal pyroptosis was inhibited by probenecid, a nonselective pannexin1 channel blocker but more importantly by the specific pannexin1 channel blocker BB FCF.[21] However, it should be noted that our studies used embryonic

cortical neurons to evaluate neuronal pyroptosis. As neuronal plasticity and the immune profile of neurons change during maturation, it will be of interest to determine whether mature adult neurons or neurons in different regions of the CNS exhibit similar innate immune responses as those displayed by embryonic cortical neurons.

Further characterization of the neuronal AIM2 inflammasome and pyroptosis will provide an important knowledge base, upon which therapies to limit the pathologic sequelae of neuroinflammation can be developed. We also identify probenecid and BB FCF as novel inhibitors of pyroptosis. Probenecid is approved to treat gout in humans, and its safety is well established.[40] Future studies should explore whether probenecid, BB FCF or derivatives of these compounds may be used as a therapeutic agents in infectious, traumatic, and neurodegenerative pathologies that involve inflammatory cell death. Collectively, the data illustrate that resident CNS cells actively participate in the innate immune response.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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