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SARM Is Required for Neuronal Injury and Cytokine Production in Response to Central Nervous System Viral Infection

Ying-Ju Hou,* Rebecca Banerjee,† Bobby Thomas,‡,§ Carl Nathan,* Adolfo García-Sastre,¶,‖,# Aihao Ding,* and Melissa B. Uccellini‡

Four of the five members of the Toll/IL-1R domain–containing adaptor family are required for signaling downstream of TLRs, promoting innate immune responses against different pathogens. However, the role of the fifth member of this family, sterile α and Toll/IL-1R domain–containing 1 (SARM), is unclear. SARM is expressed primarily in the CNS where it is required for axonal death. Studies in Caenorhabditis elegans have also shown a role for SARM in innate immunity. To clarify the role of mammalian SARM in innate immunity, we infected SARM−/− mice with a number of bacterial and viral pathogens. SARM−/− mice show normal responses to Listeria monocytogenes, Mycobacterium tuberculosis, and influenza virus, but show dramatic protection from death after CNS infection with vesicular stomatitis virus. Protection correlates with reduced CNS injury and cytokine production by nonhematopoietic cells, suggesting that SARM is a positive regulator of cytokine production. Neurons and microglia are the predominant source of cytokines in vivo, supporting a role for SARM as a link between neuronal injury and innate immunity. The Journal of Immunology, 2013, 191: 000–000.

The innate immune system relies on TLRs as well as a number of other pattern recognition receptors to detect pathogen-associated molecular patterns. The TLRs signal through the Toll/IL-1R (TIR) domain–containing adaptor protein family, which includes MyD88, TRIF, MAL, and TRAM. Each of these family members plays positive roles in innate immunity by inducing the expression of IFN-β or activation of NF-κB, and deficiency leads to increased susceptibility to infection. Sterile α and TIR domain–containing 1 (SARM) is the fifth member of the family to be identified, and it is composed of seven N-terminal HEAT/ARMadillo motifs, two central sterile α motifs, and a C-terminal TIR domain (1), and is highly conserved between fly, worm, and mammals (2).

In Caenorhabditis elegans, TOL-1 is the sole TLR homolog and the SARM homolog TIR-1 is the sole cytoplasmic TIR-domain containing protein (3). TIR-1 knockdown worms display increased susceptibility to fungal infection and decreased antimicrobial peptide synthesis, supporting a positive role for SARM in innate immunity in worms. However, this susceptibility was not dependent on TOL-1 (4), suggesting that SARM may not only function as a TLR adaptor. TIR-1 is also expressed in C. elegans olfactory neurons where it regulates olfactory patterning. Genetic evidence suggests TIR-1 mediated patterning through a UNC-43(CaMKII)→TIR-1(SARM)→NSY(ASK1 MAPKKK)→PMK-1(p38/JNK) pathway (5). Whether this pathway is also involved in the innate immune phenotype is unknown.

In contrast with results in C. elegans, human SARM has been suggested to function as a negative regulator of TRIF signaling in myeloid cells. Overexpression of SARM in 293T cells was shown to inhibit TRIF-dependent TLR signaling. In addition, LPS treatment of human PMBCs led to increased expression of SARM (6). However, when SARM−/− mouse macrophage TLR responses were tested, they failed to show defects in cytokine production. This may be explained by the predominant expression of SARM at the RNA level in human and mouse brain, and relatively low expression in myeloid cells (7). Consistent with CNS expression, SARM has been reported to mediate stress-induced cell death, as neurons from SARM−/− mice are protected from glucose deprivation–induced death (7). In addition, SARM−/− mice infected with West Nile virus showed decreased TNF-α production and increased susceptibility to infection supportive of a positive role for SARM in innate immunity in mammals (8). Thus, SARM expression can be either detrimental or protective depending on the context.

Recent evidence has identified SARM as a mediator of an active axonal destruction program termed Wallerian degeneration (9). After injury to axons, neurons undergo degeneration distal to the injury site and a coordinated sequence of events leads to clearance of necrotic debris, degeneration, and subsequent axonal regeneration (10). SARM−/− axons were protected from Wallerian degeneration, and the synaptic termini at the neuromuscular junctions were preserved after transection (9). After trauma, cytokines and chemokines are
produced locally by cells including microglia and oligodendrocytes, leading to the infiltration of glial cells and macrophages that remove axonal and myelin debris. This process is thought to aid in cytoskeletal rearrangements leading to growth cones and regeneration (11). The Wld<sup>+</sup> mouse, which displays a delayed Wallerian degeneration phenotype similar to SARM<sup>−/−</sup> mice, is also deficient in cytokine production, suggesting that the processes are intricately linked (12). In addition to promoting repair of damaged brain tissue, glial activation and cytokine production in the CNS may injure bystander cells (13, 14). In other organs, this collateral damage is typically reversible, because of the regenerative capacity of the tissue, but repopulation of cells is limited in the CNS (15). Therefore, establishing a balance between the protective and destructive effects of the neuroinflammatory response in the brain is critical (16, 17), and SARM may play a role in this balance.

In this study, we challenged SARM<sup>−/−</sup> mice with various pathogens to better understand the role of SARM in innate immune responses and disease. Consistent with a role of SARM in neurodegeneration, we found that vesicular stomatitis virus (VSV)–infected SARM<sup>−/−</sup> mice were protected from neurodegeneration and neuropathology. In addition, SARM<sup>−/−</sup> mice showed dramatically reduced cytokine and chemokine production in the brain after VSV challenge, supporting a positive role for SARM in cytokine production similar to its role in the CNS (16). The Wld<sup>+</sup> mouse, which displays a delayed Wallerian degeneration phenotype similar to SARM<sup>−/−</sup> mice, is also deficient in cytokine production, suggesting that the processes are intricately linked (12). In addition to promoting repair of damaged brain tissue, glial activation and cytokine production in the CNS may injure bystander cells (13). In other organs, this collateral damage is typically reversible, because of the regenerative capacity of the tissue, but repopulation of cells is limited in the CNS (15). Therefore, establishing a balance between the protective and destructive effects of the neuroinflammatory response in the brain is critical (16, 17), and SARM may play a role in this balance.

Materials and Methods

Mice

SARM<sup>−/−</sup> mice on the C57BL/6J background were generated previously (7) and compared with wild-type (WT) C57BL/6J mice purchased from The Jackson Laboratory. Animal studies were approved by the Institutional Animal Care and Use Committee of Weill Medical College of Cornell University and/or Icahn School of Medicine at Mount Sinai.

Bacterial infections

Eight-week-old mice were i.v. infected with 5 × 10<sup>8</sup> L. monocytogenes. Livers were homogenized in a 0.3% collagenase solution (Collagenase Type IV; Worthington) and plated on Brain Heart Infusion agar plates to determine bacterial burden. Using an Inhalation Exposure System (GlassCol), 6 ml Mycobacterium tuberculosis at OD = 0.15<sub>580</sub> (~7.5 × 10<sup>6</sup> bacteria) was nebulized for 40 min, which correlates with ~10<sup>8</sup> bacteria implanting. Lungs were homogenized and plated on 7H11 agar to determine bacterial burden.

Viral infections

Six- to 8-wk-old animals were anesthetized with ketamine/xylazine and infected intranasally with 10<sup>7</sup> PFU VSV-Indiana or 100 PFU influenza A/H3N2 virus (A/PR/8/34) virus in 20 µl PBS. For intracranial infections, 5-wk-old mice were injected with 50 PFU in 30 µl in the parietal lobe, slightly in front of the bregma. Mice were monitored daily for weight and sacrificed when exhibiting severe paralysis or >25% weight loss. Organs were homogenized (MP Biomedical) in 0.2% BSA/PBS. VSV titers were determined on MDCK cells with oxoid agar overlay and crystal violet staining.

Pathology

Mouse were perfused with 4% PFA/PBS. Brains were fixed overnight (ON) in 4% PFA/PBS, paraffin embedded, and sectioned at the Histology Shared resource facility at Icahn Medical School at Mount Sinai. Serial sections were stained for H&E, VSV, or TUNEL. For VSV immunohistochemistry, sections were deparaffinized followed by Ag retrieval in Citra plus solution (Biogenex). Sections were blocked with 4% goat serum and incubated with rabbit polyclonal anti–VSV-G (Abcam) followed by anti-rabbit IgG(H+L)-bio, ABC kit, Nova red staining (Vector Laboratories), and hematoxylin counterstain. TUNEL staining was performed using the DeadEnd Col...

oryometric TUNEL System (Promega). Pathology was scored blindly by the Comparative Pathology Diagnostic Laboratory of the Icahn Medical School at Mount Sinai as follows: 1 = mild, few inflammatory cells, focal or multifocal, <5 cells thick; 2 = moderate, multifocally affected, inflammation is 5–10 cells thick; and 3 = severe, multifocally affected, >10 cells thick, spreading into parenchyma. Points were given for necrosis or meningitis detected, and a total score was calculated for sagittal brain sections from each hemisphere. For Fluoro-Jade staining, brains were fixed for 2 h in 4% PFA, ON in 4% PFA/20% sucrose, frozen in OCT, and sectioned. Slides were stained in Fluoro-Jade C (Chemicon) according to the manufacturer’s protocol. For MCP-1 immunohistochemistry, mice were perfused with PBS and 4% PFA/PBS, and brains were fixed ON in 4% PFA/30% sucrose/PBS. Paraffin sections were blocked with 10% goat serum and stained with rabbit polyclonal anti–MCP-1 (Millipore), anti–rab1A (Jackson ImmunoResearch), streptavidin, and 1:200 Diamobenzidine substrate (Sigma). For MCP-1 immunofluorescence, mice were perfused with PBS and formalin-free Zinc fixative (BD Biosciences), and 5-mm sections were incubated ON in fixative. Samples were paraffin embedded and sectioned at the Electron Microscopy & Histology core facility at Weill Cornell Medical College. Sections were deparaffinized and stained with rabbit polyclonal anti–MCP-1 (Millipore), mouse anti-CD11b (Serotec), mouse anti-Gr1 (Covance), and anti-mouse Alexa488 and anti-rabbit IgG Alexa 594 (Molecular Probes).

Quantitative RT-PCR and ELISA

Total RNA was isolated from perfused brain and lung with the RNeasy kit (Qiagen), and reverse transcribed with Oligo dT using MultiRv reverse transcriptase (Perkin Elmer). cDNA was used for PCR with gene-specific primer and probes (BioSearch) using the ABI PRISM 7900HT sequence detection system (Perkin Elmer). MIP-1α, MCP-1, and RANTES ELISAs were from R&D Systems.

Infiltration and flow cytometry analysis

Mice were perfused with PBS, and brains were washed through a 70-µm filter in 0.06% BSA/300 µM EDTA/Hanks. Leukocytes were isolated on a 30%/70% Percoll gradient (GE Healthcare) after centrifugation at 2500 rpm for 30 min. Cells were washed and incubated with UV LIVE/DEAD stain (Invitrogen); anti–CD11b-allophycocyanin-Cy7, anti–CD11b-FITC, anti–CD44-allophycocyanin-Cy7, and anti–CD8-PE-Cy7 (BD Pharmingen); and anti–CD45-allophycocyanin–CD4-allophycocyanin-Cy7, and anti–NK1.1-allophycocyanin (eBioscience). Cell number was quantified by flow cytometry using AccuCount Particles (Spherotech), and plots were gated on live cells. Flow cytometry was performed on a BD LSRII.

Bone marrow chimeras

Six-week-old B6.SJL-Pipp<sup>−/−</sup>Pepe<sup>+/+</sup>/BoyJ (CD45.1) and SARM<sup>−/−</sup> (CD45.2) mice were irradiated with two doses of 600 rad. Bone marrow cells from WT (CD45.2) or SARM<sup>−/−</sup> donor mice were prepared, and 10<sup>5</sup> donor cells were i.v. injected into recipient mice 4 h after irradiation. Reconstitution (95%) was confirmed by flow cytometry 6 wk later, and mice were infected 2 wk later.

Neuronal cell culture

Primary hippocampal neurons were generated from embryonic day 15–17 embryos using previously described methods (19) and plated at 1.5 × 10<sup>5</sup> cells in 24-well PureCoat plates (BD Biosciences) in Neurobasal media supplemented with B27 and GlutaMAX (Invitrogen). Primary microglia were isolated from astrocyte monolayers from postnatal day 1 mice (19) in the presence of 5 ng/ml M-CSF (R&D Systems) and removed by shaking at 125 rpm for 4 h. Astrocytes were isolated from the same cultures by trypsinization after microglia were removed. Bone marrow–derived macrophages were cultured for 6 d in DMEM supplemented with 20% L929 cell media. For mixed cultures, neurons were allowed to differentiate for 3 d in vitro, before microglia, astrocytes, or bone marrow–derived macrophages were directly added to neurons at a 10:1 ratio of neurons to other cells. Eighteen hours after mixing, cells were infected with VSV at multiplicity of infection (MOI) 1 for 30 min, and supernatants were harvested at 8 h postinfection.

Microarray

Mice were infected intranasally with 10<sup>7</sup> PFU VSV; at day 5 postinfection, mice were perfused with PBS, brains were harvested, and RNA was prepared by TRIzol extraction. Triplicate samples of three pooled mice were analyzed at the Biopolymers Facility at Harvard Medical School on the Mouse 430 2.0 chip (Affymetrix). Genes significantly different between WT and SARM<sup>−/−</sup> VSV-infected mice were determined using Genepattern,
and results were deposited in National Center for Biotechnology Information Gene Expression Omnibus (accession no. GSE44331, http://www.ncbi.nlm.nih.gov/geo/).

Results
SARM$^{-/-}$ mice show normal responses to Listeria. M. tuberculosis, and influenza virus but are protected from VSV

To determine whether SARM has a role in innate immunity similar to the other TIR-domain–containing adapters, we infected SARM$^{-/-}$ mice with various bacterial and viral pathogens. Bacterial burdens of SARM$^{-/-}$ mice in response to M. tuberculosis were similar to WT animals (Fig. 1A), as were responses to Listeria (Fig. 1B). SARM$^{-/-}$ mice also showed similar susceptibility to influenza virus and similar viral titers in the lung (Fig. 1C).

To determine whether SARM plays a unique role in the innate immune response in the brain, given its expression in the CNS (7), we next studied responses to VSV, a member of the family Rhabdoviridae, commonly used as a model to study neurotropic viral infection. Surprisingly, SARM$^{-/-}$ mouse showed dramatic protection from intranasal VSV infection at a range of infectious doses (Fig. 1D, Supplemental Fig. 1). This protection was not due to differences in viral titers in the brain or lung (Fig. 1D). To exclude possible differences in neuroinvasion similar to TLR3 deficiency (20), and to directly address whether the unique phenotype with VSV infection was related to CNS infection, we inoculated mice intracranially with VSV. Using this method, we observed the same enhanced survival phenotype in SARM$^{-/-}$ mice, indicating that CNS infection with VSV results in increased survival in SARM$^{-/-}$ mice and suggesting neuroinvasion did not account for the observed difference in susceptibility. This phenotype was again independent of viral load in the brain (Fig. 1E), suggesting differences in the immune response contributed to the enhanced survival of SARM$^{-/-}$ mice. Both intranasal and intracranial VSV infection led to tail and hind limb paralysis and labored breathing that occurred with rapid onset between days 4 and 9 postinfection. Symptoms were similar in both WT and SARM$^{-/-}$ animals that succumbed to infection; however, the occurrence of symptoms and lethality was less common in SARM$^{-/-}$ mice. Animals that survived infection either showed no symptoms or signs of mild tail or hind-limb paralysis that did not persist beyond 14 d postinfection.

SARM$^{-/-}$ mice show reduced pathology in the brain

To compare the extent of neuronal damage, we next examined histological sections from WT and SARM$^{-/-}$ mice. Because WT mice succumb to disease with variable kinetics, brains were harvested between days 6 and 10 postinfection when animals showed signs of paralysis. SARM$^{-/-}$ animals with similar symptoms were harvested when available. WT animals showed multifocal necrosis and meningitis (11/11 and 10/11, respectively; Fig. 2D), but SARM$^{-/-}$ mice showed reduced incidence of pathology (4/11 necrosis and 6/11 meningitis), and pathology was also less severe when present (Fig. 2B, 2D). In serial sections from WT mice, we observed loss of cell architecture, eosinophilia of the cytoplasm, and appearance of pyknotic and karyorrhectic nuclei under H&E staining (Fig. 2A, top) as well as TUNEL staining indicative of necrosis in sections that stained positive for VSV Ag. However, although we did observe some necrosis in SARM$^{-/-}$ brain (Fig. 2D), we also observed several areas that stained positive for VSV Ag but showed no signs of necrosis under H&E or TUNEL staining (Fig. 2A, middle). In addition, WT mice had a higher

![FIGURE 1: SARM$^{-/-}$ mice are protected from VSV infection. (A) WT and SARM$^{-/-}$ mice infected with nebulized M. tuberculosis have similar bacterial load in the lungs (n = 4 per time point). (B) Similar bacterial burden in livers of WT and SARM$^{-/-}$ mice infected i.v. with 5 $\times$ 10$^3$ CFU L. monocytogenes (n = 4 per time point). (C) WT and SARM$^{-/-}$ mice have similar survival rates (n = 10) and viral titers in lungs (n = 5) after intranasal infection with 100 PFU A/PR/8/34 influenza virus. (D) WT and SARM$^{-/-}$ mice were intranasally infected with 10$^7$ PFU VSV and monitored daily for mortality (n = 20). Brains and lungs were harvested to determine viral burden by plaque assay (n = 9–10). PFU data shown were pooled from two independent experiments. (E) SARM$^{-/-}$ mice intracranially infected with 50 PFU VSV have decreased mortality as compared with WT animals, but viral burdens in the brains of SARM$^{-/-}$ mice were comparable with that of WT (n = 10). Bars represent mean bacterial or viral titers. For (D) and (E), *p < 0.02 and ***p < 0.0002 compared with WT mice, Student t test. n.d., Not detected.](http://www.jimmunol.org/)
incidence of more severe meningitis as indicated by expansion of the leptomeninges and accumulation of high numbers of inflammatory cells (Fig. 2B, top) as compared with milder meningitis observed in SARM<sup>2/2</sup> mice (Fig. 2B, bottom). We also observed less neurodegeneration in SARM<sup>2/2</sup> animals in the olfactory bulb, which is the site of initial VSV replication and spread to the brain (21) (Fig. 2C), suggesting that neural tissue is protected from degeneration in SARM<sup>2/2</sup> mice during VSV infection, as previously reported during axonal injury (9).

SARM<sup>2/2</sup> mice have reduced cytokines and infiltration in the brain

To determine whether there was a difference in the inflammatory response between WT and SARM<sup>2/2</sup> animals post-VSV infection, we isolated RNA from brain and lung homogenates at day 6 postinfection and tested a number of chemokines and cytokines by quantitative RT-PCR (qRT-PCR). Many inflammatory mediators are produced post intranasal infection with VSV including chemokines such as MCP-1 and RANTES, as well as cytokines such as type I IFNs and TNF-α (22, 23). SARM<sup>2/2</sup> mice had severely blunted responses to all cytokines and chemokines examined in the brain (Fig. 3A), but had similar levels to WT in the lung (Supplemental Fig. 2). In addition, levels of MIP-1α, MCP-1, and RANTES protein were also significantly reduced in SARM<sup>2/2</sup> brains compared with WT. In contrast with the RNA data, we were unable to detect TNF-α protein in brain homogenates (Fig. 3B).

Microarray analysis on brain samples also corroborated these results. WT mice showed significantly higher upregulation of a number of chemokines and IFN-inducible genes as compared with SARM<sup>2/2</sup> animals (Fig. 3C, Table I), confirming that SARM expression is important for the initiation of the innate immune response in the brain post VSV infection. Given the lack of type I IFN in the brains of SARM<sup>2/2</sup> mice, it was surprising that we did not observe differences in viral replication (Fig. 1D). Analysis of the microarray data set showed that SARM<sup>2/2</sup> mice did produce levels of IFN-inducible genes above PBS controls, although they failed to upregulate these genes to the same extent as WT mice (Fig. 3C). This may indicate that low levels of type I IFN were present and sufficient to control viral replication. XIAP-associated factor, Xaf1, was highly upregulated in SARM<sup>2/2</sup> animals compared with WT; however, Western blot failed to show any change in Xaf1 levels (data not shown).

Given the differences in chemokines, we next examined recruitment of inflammatory cells into the brain postinfection. Consistent with published results and our histological examination (Fig. 2A), we saw more infiltrating leukocytes in the brains of VSV-infected mice at day 7 postinfection, but there was a decrease in the total number of cells in the brains of infected SARM<sup>2/2</sup> mice (1 × 10<sup>6</sup>) compared with WT mice (1.5 × 10<sup>6</sup>; Fig. 3D). Using flow cytometry, we found significantly fewer macrophages and monocytes in SARM<sup>2/2</sup> brains than in WT brains (Fig. 3E). In addition, SARM<sup>2/2</sup> mice showed a trend of decreased neutrophils, CD4<sup>+</sup> T cells, and CD8<sup>+</sup> T cells (Fig. 3E, 3F), although the differences were not statistically significant at this time point.

Although VSV-associated neuropathogenesis is reported to be T cell independent, CD4<sup>+</sup> and CD8<sup>+</sup> T cells are required for viral...
clearance and host recovery (21). In addition to expression in the CNS, low levels of SARM were also detected in CD3+ splenocytes (7). SARM<sup>−/−</sup> mice also showed no differences in CD4+ or CD8+ T cell numbers in the thymus, spleen, or lymph nodes, and SARM<sup>−/−</sup> splenocytes proliferated normally in response to anti-CD3/anti-CD28 (Supplemental Fig. 3), indicating that SARM is dispensable for T cell development. Decreased numbers of activated microglia have been observed in SARM<sup>−/−</sup> mice during West Nile virus infection (8). We did observe a trend of less activated microglia (CD11b<sup>+</sup>CD45<sup>+</sup>) in SARM<sup>−/−</sup> mice (Fig. 3E, 3G); however, the differences were not statistically significant at this time point.

SARM expression in nonhematopoietic cells contributes to VSV susceptibility and cytokine production

The increased survival of SARM<sup>−/−</sup> mice compared with WT mice after VSV infection appeared to be correlated with a blunted inflammatory response in the brain. Therefore, we generated BM chimeras to dissect out the contribution of hematopoietic and nonhematopoietic SARM-expressing cells to the inflammatory response in the brain. Strikingly, SARM deficiency in nonhematopoietic cells afforded protection from VSV (Fig. 4A), as SARM<sup>−/−</sup> recipients of either WT or SARM<sup>−/−</sup> BM were better able to survive VSV infection. In addition, cytokine and chemokine production was observed in WT→WT chimeras and SARM<sup>−/−</sup>→WT chimeras, but not in SARM<sup>−/−</sup>→SARM<sup>−/−</sup> or WT→SARM<sup>−/−</sup> chimeras, indicating that WT nonhematopoietic cells were responsible for increased cytokine production in the brain (Fig. 4B). However, WT→WT chimeras showed higher levels for some cytokines compared with SARM<sup>−/−</sup>→WT chimeras, indicating that SARM expression in BM cells may make some contribution to cytokine production. The observed mortality of WT mice is likely related to the inflammatory response generated by the cells of the nonhematopoietic compartment, because mice depleted of macrophages displayed viral replication and encephalitis similar to WT (24).

Neurons and microglia cooperate to induce cytokine production

Because susceptibility to VSV correlated with cytokine and chemokine production in the CNS, we examined the cell types responsible for chemokine production. We chose MCP-1 because it is known to be induced after injury and before neurodegeneration and infiltration (25, 26). Olfactory bulb sections confirmed the presence of MCP-1 staining in VSV-infected WT but not SARM<sup>−/−</sup> mice (Fig. 5A). However, WT→WT chimeras showed enhanced MCP-1 staining in VSV-infected WT but not SARM<sup>−/−</sup> mice (Fig. 5A). Costaining with neuronal nuclei for neurons, CD11b for microglia, and GFAP for astrocytes indicated that both neurons and microglia from WT infected mice produced MCP-1 (Fig. 5B), with neurons being the predominant source.
To determine what cell types are important for cytokine production, we cultured neurons, microglia, and astrocytes in vitro. Neurons and astrocytes produced only low levels of MCP-1 and TNF-α (Fig. 5C) in response to VSV infection. In contrast, microglia produced high levels of MCP-1 and TNF-α. Surprisingly, no difference in cytokine production was observed between WT and SARM+/− cells, suggesting that differences in cytokine production are not cell intrinsic. Consistent with our in vivo results, we did not observe any differences in viral replication in isolated neuron or macrophage cultures (data not shown). Because neuroinflammatory responses often require cooperation between neurons and glia, we infected mixed cell cultures with VSV to assess the production of cytokines. WT neurons cultured at a 10:1 ratio with WT microglia produced high levels of MCP-1 and TNF-α (Fig. 5D). In contrast, SARM+/− neurons cultured with SARM+/− microglia showed greatly diminished MCP-1 production (Fig. 5D, note 10-fold more microglia are present in Fig. 5C). To assess whether the interaction between neurons and microglia was unique, we cocultured neurons and astrocytes or neurons and macrophages. Neither astrocytes nor macrophages were able to reproduce the cytokine production observed when neurons and microglia were cocultured (Fig. 5D), suggesting that a unique interaction between neurons and microglia was required for cytokine production. However, at higher MOIs and later time points, we did observe low levels of TNF-α production and high levels of IFN-α production by macrophages that were not affected by the absence of SARM. The low level of cytokine production is likely due to potent suppression of host protein translation by the M protein because infection with the M51R mutant enhanced cytokine production (Supplemental Fig. 4). Microglia are thought to be maintained in the healthy CNS in an active resting state and express a wide variety of receptors that allow them to very rapidly respond to changes in homeostasis (27), which may explain their ability to produce cytokines during VSV infection.

### Discussion

We have infected SARM+/− mice with a number of bacterial and viral pathogens to investigate a possible role of SARM in innate immunity. We have found that SARM+/− mice display normal responses to most pathogens tested but were dramatically protected from lethality and CNS damage from VSV infection. Normal responses to most infections support a role for SARM in specific CNS pathology consistent with its expression pattern. We also found that expression of SARM in nonhematopoietic cells was critical for lethality and cytokine production.

In contrast with our results, Szretter et al. (8) found that independently generated SARM+/− mice were more susceptible to West Nile virus infection. They similarly found reduced TNF-α production, but in contrast with our results observed more cell death in knockout animals. These results may reflect differences in the nature of the immune response required to clear specific

### Table I. Lack of upregulation of chemokines and IFN-inducible genes in VSV-infected SARM+/− mice

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<td>Ifgpl</td>
<td>IFN inducible GTPase 1</td>
</tr>
<tr>
<td>6.6</td>
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<td>Ms4a4e</td>
<td>Membrane-spanning 4-domains, subfamily A, member 4C</td>
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<tr>
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<td>Ms4a4b</td>
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<td>Chemokine (C-X-C motif) ligand 9</td>
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<td>Placenta-specific 8</td>
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<tr>
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<td>Rsad2</td>
<td>Radical S-adenosyl methionine domain containing 2</td>
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<td>IFN-induced protein 44</td>
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<td>5.1</td>
<td>2.8</td>
<td>Ms4a6d</td>
<td>Membrane-spanning 4-domains, subfamily A, member 6D</td>
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WT and SARM+/− mice were infected intranasally with VSV, and brains were harvested at day 5 postinfection for microarray analysis. Genes showing >2-fold change between WT and SARM+/− mice are shown.

To determine which cell types are important for cytokine production, we cultured neurons, microglia, and astrocytes in vitro. Neurons and astrocytes produced only low levels of MCP-1 and TNF-α (Fig. 5C) in response to VSV infection. In contrast, microglia produced high levels of MCP-1 and TNF-α. Surprisingly, no difference in cytokine production was observed between WT and SARM+/− cells, suggesting that differences in cytokine production are not cell intrinsic. Consistent with our in vivo results, we did not observe any differences in viral replication in isolated neuron or macrophage cultures (data not shown). Because neuroinflammatory responses often require cooperation between neurons and glia, we infected mixed cell cultures with VSV to assess the production of cytokines. WT neurons cultured at a 10:1 ratio with WT microglia produced high levels of MCP-1 and TNF-α (Fig. 5D). In contrast, SARM+/− neurons cultured with SARM+/− microglia showed greatly diminished MCP-1 production (Fig. 5D, note 10-fold more microglia are present in Fig. 5C). To assess whether the interaction between neurons and microglia was unique, we cocultured neurons and astrocytes or neurons and macrophages. Neither astrocytes nor macrophages were able to reproduce the cytokine production observed when neurons and microglia were cocultured (Fig. 5D), suggesting that a unique interaction between neurons and microglia was required for cytokine production. However, at higher MOIs and later time points, we did observe low levels of TNF-α production and high levels of IFN-α production by macrophages that were not affected by the absence of SARM. The low level of cytokine production is likely due to potent suppression of host protein translation by the M protein because infection with the M51R mutant enhanced cytokine production (Supplemental Fig. 4). Microglia are thought to be maintained in the healthy CNS in an active resting state and express a wide variety of receptors that allow them to very rapidly respond to changes in homeostasis (27), which may explain their ability to produce cytokines during VSV infection.

### Table I. Lack of upregulation of chemokines and IFN-inducible genes in VSV-infected SARM+/− mice

<table>
<thead>
<tr>
<th>Score</th>
<th>Fold Change</th>
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<th>Gene Name</th>
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<td>42.0</td>
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<td>XIAP associated factor 1</td>
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<td>3.5</td>
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<td>WD repeat and FYVE domain containing 1</td>
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<tr>
<td>13.5</td>
<td>3.8</td>
<td>Transcribed locus, moderately similar to XP_001477892.1</td>
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<td>11.8</td>
<td>3.8</td>
<td>Wdylf1</td>
<td>WD repeat and FYVE domain containing 1</td>
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</table>
FIGURE 4. SARM expression is critical in nonhematopoietic cells. (A) WT recipients of bone marrow, irrespective of the genotype of bone marrow, were more susceptible to intranasal VSV infection. Thirteen of 30 SARM−/−→WT mice survived VSV infection, as compared with 14/19 WT→SARM−/− mice, p < 0.02. Thirteen of 31 WT→WT and 15/19 SARM−/−→SARM−/− mice survived VSV infection, p < 0.02. (B) qRT-PCR results of VSV-infected BM chimeras. WT recipients of BM express higher levels of cytokines at day 6 after VSV infection than SARM−/− (n = 5 per time point). Bars represent mean expression. WT and SARM−/− PBS controls from Fig. 3A are shown for comparison. *p < 0.05, Student t test.

pathogens (VSV versus West Nile virus) whereas limiting damage to the CNS. Differences in lethality may be a result of direct viral damage or immune-mediated damage (28). Cytokine production and infiltration of the CNS may be beneficial for clearance of WNV infection but may contribute to pathology during VSV infection. However, both studies support a positive role for SARM in cytokine production in the brain, with the outcome of infection differing depending on the viral infection. Mukherjee et al. (29) have also recently reported that SARM−/− mice are resistant to La Crosse virus infection and neuronal damage induced by the virus via a mechanism dependent on MAVS.

Osterloh et al. (9) have recently reported that both Drosophila and mouse SARM are required for injury-induced axonal cell death. Wallerian degeneration is an active cell death program akin to apoptosis with a well-established innate immune component that allows for clearance of debris from damaged neurons and subsequent nerve regeneration (12). Consistent with this, we observed less overall pathology and less neurodegeneration in SARM−/− VSV-infected animals. Importantly, we also observed a dramatic reduction in cytokine production, with neurons being the predominant source of cytokines. This supports a role for SARM in linking neuronal damage with the innate immune response. It is unclear whether neurodegeneration or cytokine production and infiltration are more relevant for in vivo protection, and this is difficult to assess experimentally because they are likely to be linked. Future work will address whether a specific cytokine or chemokine is responsible for pathogenesis, or a number of factors create a proinflammatory environment that leads to pathogenesis.

In addition to expression in neurons, low-level expression of SARM was observed in T cells from SARM-GFP BAC transgenic mice (7). Pameerselvam et al. (30) have recently reported that overexpression of SARM in 293T cells and CD8+ T cells causes increased apoptosis via the intrinsic mitochondrial pathway. In addition, knockdown of SARM in CD8 T cells led to enhanced survival. Localization of SARM at the mitochondria and mitochondrial clustering have been reported when SARM is overexpressed (7). However, Osterloh (9) et al. report that endogenous Drosophila SARM-GFP is broadly localized to axons and not preferentially located at the mitochondria, and the poor quality of mouse Abs make localization hard to access. We did not observe any defects in T cell proliferation in vitro (Supplemental Fig. 3) or increased T cell accumulation in vivo (Fig. 5E), although differences may be apparent at later time points postinfection.

In vivo, we observed that neurons were the predominant source of cytokines, but we also observed cytokine production by microglia. In addition, it is important to note that microglia are predominantly of host origin in radiation bone marrow chimeras (31), so microglia may contribute to cytokine production. In isolated neuronal and microglial cultures, we were unable to observe differences in cytokine production in response to VSV infection, but our in vitro mixed neuronal cultures revealed a novel interaction between neurons and microglia leading to cytokine production. Activated microglia have been implicated in the pathogenesis of various CNS diseases, including Alzheimer’s disease (32) and Parkinson’s disease (33), as well as variety of different viral infections (34–36). Microglia have been described as the resident macrophage of the CNS because they share many similar characteristics and effector functions (37), but they are a distinct population arising from different precursors than macrophages. Recent lineage tracing has shown that mouse adult microglia are derived from primitive myeloid precursors that arise from the extraembryonic yolk sac and seed in the mouse brain (38) in a process that occurs before definitive hematopoiesis (39). Unlike adult mouse macrophages, microglia are self-renewing and are resistant to high doses of gamma-ray irradiation (40). Although astrocytes can also initiate and enhance inflammation in the CNS, our coculture experiments did not reveal a role for astrocytes in VSV-induced cytokine production.

The nature of the interaction between neurons and microglia leading to cytokine production requires further investigation. Microglia are thought to be the predominant source of cytokines in the brain, but recent evidence suggests that neurons are also capable of producing cytokines (41). Our in vivo cytokine staining suggests that both neurons and microglia produce cytokines during infection. Glial cells can contribute to neuroinflammatory responses through the generation of proinflammatory mediators, but they can also communicate with neurons bidirectionally via contact-mediated or se-
creted factors to regulate the level of inflammatory responses. Many microglia-derived molecules can promote neurodegeneration, including IL-1β (42), reactive oxygen species (43), NO (44), glutamate (45), and ATP (46). In addition to soluble mediators, there could be a cell–cell contact requirement between neurons and microglia that is disrupted in SARM-/- neurons and microglia. The neuroimmunoregulatory CX3CL1–CX3CR1 interaction is a mechanism used by neurons to prevent aberrant microglia activation. Another neuroimmunoregulatory molecule is CD200, which is expressed on neurons. By interacting with its receptor, CD200R, on microglia, microglia are kept quiescent in the normal state and prevent phagocytosis (13). It is possible that loss of any of these cell–cell interactions can lead to microglia activation and an inflammatory response. Future experiments will address whether soluble factors or cell–cell contact are required for cytokine production and whether viral pathogen-associated molecular pattern or endogenous danger signals are upstream of SARM.

In summary, our data demonstrate that mammalian SARM plays a role in neurodegeneration during viral CNS infection. In addition, SARM plays a positive role in cytokine production similar to its C. elegans homolog. This is likely limited to infections of the CNS consistent with its expression pattern and its importance on nonhematopoietic cells. The data suggest that SARM is crucial for CNS injury and cytokine production in the CNS and may provide a link between neurodegeneration and the innate immune response. Therapeutic targeting of SARM may be beneficial in neurodegenerative diseases and CNS viral infection.
References

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Disclosures
The authors have no financial conflicts of interest.