AIM2 controls microglial inflammation to prevent experimental autoimmune encephalomyelitis

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The role of the PYHIN family member absent in melanoma 2 (AIM2), another important inflammasome sensor, in EAE remains unclear. In this study, we found that AIM2 negatively regulates the pathogenesis of EAE independent of inflammasome activation. AIM2 deficiency enhanced microglia activation and infiltration of peripheral immune cells into the CNS, thereby promoting neuroinflammation and demyelination during EAE. Mechanistically, AIM2 negatively regulates the DNA-PK–AKT3 in microglia to control neuroinflammation synergistically induced by cGAS and DNA-PK. Administration of a DNA-PK inhibitor reduced the severity of the EAE. Collectively, these findings identify a new role for AIM2 in controlling the onset of EAE. Furthermore, delineation of the underlying inflammasome-independent mechanism highlights cGAS and DNA-PK signaling as potential targets for the treatment of heterogeneous MS.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS) and affects ∼2.5 million people globally. MS causes the dysfunction in motor, sensory, visual, and autonomic systems of patients, and its neuropathological features include inflammatory cell infiltration, chronic axonal damage, and demyelination of the CNS (Dendrou et al., 2015). As a multifactorial, heterogeneous disease, MS is difficult to treat. Thus, the etiology and underlying basis of this heterogeneous disease remain largely unknown.

Experimental autoimmune encephalomyelitis (EAE) is a commonly used experimental animal model of MS study and recapitulates many of neuropathological features of MS (Ransohoff, 2012). Myelin oligodendrocyte glycoprotein (MOG)–induced EAE in C57BL/6 mice is often used to explore multiple facets of the mechanism surrounding immune-mediated demyelination, especially in transgenic/KO mice (Constantinescu et al., 2011; Procaccini et al., 2015). Front-line clinical treatments for MS including IFN-β, glatiramer acetate, anti–VLA-4 integrin mAb, and fumaric acid esters have been developed, tested, and validated in the MOG-induced EAE model (Aharoni et al., 2008; Galligan et al., 2010; Humphries et al., 2020; Mindur et al., 2014). Such models can be induced in different ways through innate immune activation. Low doses of the adjuvant (mycobacteria [Mtbc]) are able to induce a subtype of EAE termed type B, which occurs independently of NLRP3 inflammasome (Inoue et al., 2016). Thus, to elucidate the key molecules and underlying mechanisms involved in the distinct MOG-EAE subtypes may provide new insights into developing diagnostic indicators and treatments for heterogeneous MS.

NLRP3 is assembled into multimeric complexes with apoptosis-associated speck-like protein containing CARD (ASC) and pro-inflammatory caspases (caspase-1 and -11) known as an inflammasome. Inflammasome assembly leads to caspase activation and the subsequent cleavage of pro–IL-1β and pro–IL-18 precursors into their mature forms. Inflammasome activation also results in an inflammatory form of cell death known as pyroptosis (Rathinam et al., 2012). Several independent studies have reported a critical role for the NLRP3 inflammasome and its related proteins such as ASC and gasdermin D in the development of type A EAE (Inoue et al., 2012; Li et al., 2019b; Martin et al., 2016). Absent in melanoma 2 (AIM2) is a PYHIN (pyrin and HIN domain containing) family member and an important inflammasome sensor that detects cytosolic double-stranded DNA via its HIN200 domain (Hornung et al., 2009). The AIM2 inflammasome is also essential for host defense against bacterial and viral pathogens, such as Francisella tularensis, vaccinia virus, and mouse cytomegalovirus (Rathinam et al., 2010). In addition, some studies...
have identified important CNS roles for AIM2 such as antibacterial infection, brain injury, and shaping neurodevelopment (Denes et al., 2015; Hanamsagar et al., 2014; Lammert et al., 2020). However, the role of AIM2 in EAE and MS remains poorly understood.

Here, we report a previously unknown and inflammasome-independent protective role for AIM2 in EAE. Microglial AIM2 prevents the development of EAE by negatively regulating antiviral inflammatory signaling during neuroinflammation.

Results
AIM2 deficiency facilitates the development of both types A and B EAE
MOG-induced EAE has become a very well characterized model for investigating key components of the immune system in the pathogenesis of MS (Constantinescu et al., 2011) and has been separated into A and B subtypes to explore the heterogeneity in MS (Inoue et al., 2016). To more comprehensively elucidate the role of AIM2 in heterogeneous MS, we induced both type A and B EAE in WT and AIM2−/− mice using different doses of Mtbg. ASC−/− mice were used as inflammasome-dependent type A EAE controls. Clinical scores and the levels of infiltrating inflammatory cells and demyelination in the spinal cord of ASC−/− mice were lower in type A EAE (Fig. 1, A and B) but comparable in type B EAE relative to WT mice (Fig. 2, A and B), which is consistent with previous reports showing ASC as an essential mediator of type A EAE but not type B EAE. Interestingly, we found AIM2−/− mice displayed higher clinical scores compared with WT mice in both types of EAE. In addition, CNS pathology was also elevated in AIM2−/− mice during both types of EAE (Fig. 1, A and B; and Fig. 2, A and B). These data suggest that unlike other inflammasome proteins, AIM2 plays a protective role in the development of different types of EAE. Consistently, FACS analysis revealed a significantly increase in the infiltration of T cells (CD45+CD4+ and CD45+CD8+), myeloid cells, and activated microglia cells (CD11c+CD11b+) in the CNS of AIM2−/− mice during both types of EAE when compared with WT or ASC−/− mice (Fig. 1, C and D; and Fig. 2, C and D). We also measured the release of mature IL-1β in serum during EAE. Low comparable levels of IL-1β were detected in WT, ASC−/−, and AIM2−/− mice during type B EAE (Fig. 2 E). However, in type A EAE, AIM2−/− mice showed higher levels of IL-1β in serum, but the levels of IL-1β in ASC−/− mice were lower compared with WT mice (Fig. 1 F). Taken together, these results indicated that AIM2 controls the development of both types of EAE in an inflammasome-independent manner. To better explore the underlying inflammasome-independent mechanism by which AIM2 controls neuroinflammation during EAE, we used the type B EAE model in our subsequent experiments.

AIM2 deficiency in microglia exacerbates neuroinflammation and the pathogenesis of EAE
Next, we measured AIM2 protein expression in a variety of immune and nerve cells. Immunoblotting analysis showed that AIM2 was highly expressed in bone marrow–derived macrophages (BMDMs), bone marrow–derived dendritic cells (DCs), and microglia. AIM2 was expressed at a lower level in T cells, and was barely detectable in astrocytes, oligodendrocytes, and neurons (Fig. 3 A). To further determine whether AIM2 deficiency in CNS resident cells or peripheral immune cells contributes to the regulation of EAE, we performed bone marrow chimera experiments by adoptively transferring WT or AIM2−/− bone marrow cells into lethally irradiated WT recipient mice to determine the function of AIM2 in peripheral cells on EAE. Comparable clinical scores (Fig. 3 B) and infiltration of immune cells (Fig. 3 C) were observed between these two recipients, indicating that AIM2 in peripheral cells is not involved in protecting against EAE. We next performed a reverse bone marrow transfer experiment by reconstituting lethally irradiated WT or AIM2−/− mice with CD45.1 WT bone marrow cells. We found that AIM2−/− mice reconstituted with CD45.1 WT bone marrow showed higher clinical scores (Fig. 3 D) and more CNS infiltration of immune cells (Fig. 3, F and G). H&E and Luxol fast blue (LFB) staining also showed more inflammatory cell recruitment and increased demyelination in the spinal cord of AIM2−/− recipient mice (Fig. 3 D). Thus, these results suggested that AIM2 deficiency in CNS-resident cells promotes the pathogenesis of EAE.

We also crossed AIM2fl/fl mice with GFAP-Cre mice to generate astrocyte conditional AIM2 KO mice (Fig. S1, A–C). During EAE, AIM2−/−GFAP-Cre and littermate control AIM2−/− mice had comparable susceptibility to EAE (Fig. S2, A–C). Consistent with low expression of AIM2 in astrocytes, these results demonstrate that AIM2 deficiency in astrocytes has no effect on EAE. To determine the role of AIM2 in microglia, we crossed AIM2fl/fl mice with Cx3cr1-Cre mice to generate microglia and macrophage conditional AIM2 KO mice. Clinical scores and immune cell infiltration were significantly increased in AIM2fl/Cx3cr1-Cre mice compared with Cx3cr1-Cre mice (Fig. S2, D–H). Given AIM2−/−Cx3cr1-Cre mice deleted both microglia and peripheral macrophages, we crossed AIM2−/− mice with Cx3cr1-CreERT2 mice to further generate microglia–specific conditional AIM2 KO mice. Tamoxifen was administered to these mice to specifically delete AIM2 in microglia. Short-lived blood monocytes can be renewed within 6 wk after administration of tamoxifen. However, the long-lived microglia are unable to regenerate. AIM2−/−Cx3cr1-CreERT2 mice developed more severe EAE with higher clinical scores, increased pathological features, and more immune cell infiltration when compared with Cx3cr1-CreERT2 mice (Fig. 3, H–K). Furthermore, the percentages and absolute numbers of both T helper type 1 (Th1) and Th17 cells were also significantly increased in the CNS of AIM2−/−Cx3cr1-CreERT2 mice (Fig. S3, A and C). However, the absolute numbers of Th1 and Th17 cells in the spleen of AIM2−/−Cx3cr1-CreERT2 mice were decreased, while percentages were comparable to control mice (Fig. S3, B and D). Thus, these results suggest that the recruitment of pathogenic T cells into the CNS is substantially enhanced in AIM2−/−Cx3cr1-CreERT2 EAE mice. Consistently, the relative expression of inflammatory cytokines and chemokines in CNS of AIM2−/−Cx3cr1-CreERT2 mice was markedly increased relative to Cx3cr1-CreERT2 mice during EAE (Fig. S3 E). Collectively, these data demonstrated that the specific loss of AIM2 in microglia is sufficient to exacerbate type B EAE.
AIM2 controls the microglial phenotypic switch from homeostasis to disease during EAE

It is clear from the above results that microglia-intrinsic AIM2 regulates the development of EAE. To further dissect the mechanistic role of microglial AIM2 in controlling EAE, we performed microarray analysis using CD45lowCD11b+ microglial cells sorted from the CNS of WT and AIM2−/− mice on day 18 after EAE immunization (Fig. 4 A). Interestingly, volcano plot analysis displayed significantly increased expression in many genes related to a disease-associated...
microglia signature, such as Apoe, Axl, Siglec1, Cybb, and Clec7a, in AIM2−/− cells, whereas many genes associated with a homeostatic microglia (M0) signature, such as Fcrls, Gpr34, and Tgfbr1, were down-regulated (Fig. 4 B). The heatmap and real-time PCR analysis confirmed these changes (Fig. 4, C and D). Consistently, immunofluorescence analysis showed that APOE expression was increased in microglia of AIM2−/− mice compared with WT mice during EAE (Fig. 4 E). Together, these data suggest that AIM2 plays an important role in controlling the phenotypic switch of homeostatic microglia to inflammatory microglia during EAE.

Figure 2. AIM2 inhibits type B EAE development. (A) Mean clinical scores of age-matched female WT, AIM2−/−, and ASC−/− mice subjected to type B EAE (n = 8 mice per group). (B) Representative H&E and LFB staining and histology scores of spinal cord sections from type B EAE−induced WT, AIM2−/−, and ASC−/− mice, showing inflammatory cell infiltration and demyelination (arrowheads). Scale bar, 500 µm. (C and D) Flow-cytometric analysis of immune cells (including CD45+CD4+ T cells, CD45+CD8+ T cells, and CD45+CD11b+ microglia and myeloid cells) infiltrated to the spinal cord and brain of type B EAE−induced WT, AIM2−/−, and ASC−/− mice at day 18 after immunization (n = 5 mice per group). Data are presented as representative plots (C) and summary graphs of quantified percentages and absolute cell numbers (D). (E) ELISA analysis of IL-1β production in serum from indicated mice in type B EAE (n = 5 mice per group). Data are pooled from three independent experiments. *, P < 0.05; **, P < 0.01. Error bars show mean ± SEM. Unpaired t test for A and E, and multiple unpaired t test for D.

Ma et al. Journal of Experimental Medicine 4 of 17
AIM2 prevents EAE independent of inflammasome https://doi.org/10.1084/jem.20201796
Figure 3. AIM2 deletion in microglia promotes the development of EAE. (A) Immunoblot analysis of the protein expression of AIM2 in peripheral cells (BMDMs, bone marrow–derived DCs [BMDCs], and T cells) and CNS resident cells (microglia, astrocytes, oligodendrocytes, and neurons) from WT mice. (B and C) Mean clinical scores (B) after EAE induction and summary graph of CNS-infiltrating immune cells (C) at day 18 after immunization in WT mice adoptively.

Ma et al. Journal of Experimental Medicine 50 of 17
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AIM2 deficiency enhances the microglial antiviral pathway–related inflammation during EAE

To further identify the pathways involved in AIM2 controlling microglial inflammation, we did in-depth analysis of the microarray data. Notably, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed the up-regulation of many viral-related pathways in AIM2−/−/CD45lowCD11b+ cells, including herpes simplex infection, influenza A, and cytosolic DNA–sensing pathway (Fig. 5 A). Gene ontology biological process (GO) analysis further confirmed the top biological processes up-regulated in AIM2−/− microglia were related with defense response to virus and cellular response to interferon-β (Fig. 5 B). Additionally, gene network and gene set enrichment analysis (GSEA) highlighted the regulated role of AIM2 in genes involved in the defense response to virus pathway (Fig. 5, C and D). Consistently, the heatmap and real-time PCR analysis displayed significantly increased expression of many genes associated with antiviral pathways and DNA-sensing pathways, such as Ddx58, Ifit1, Mx1, Oas1b, Ifi30, Stali, Cxcl10, Ccl5, and Il6 in AIM2−/− cells (Fig. 5, E and F). Thus, these genomic analyses clearly demonstrate that AIM2 restricts antiviral inflammatory signaling pathways in microglia.

AIM2 targets the DNA-PK (DNA-dependent protein kinase)–AKT3 axis to inhibit the microglial antiviral pathway–related inflammation

Poly(dA:dT) (poly(deoxyadenylic-deoxythymidylic)) transfection can strongly activate antiviral inflammatory pathways (Lees-Miller et al., 1990; Li et al., 2013), but barely do AIM2 inflammasome activation without first signal to prime (Hornung et al., 2009). We next investigated the inflammasome-independent effect of AIM2 in microglia on antiviral pathway–associated inflammation in response to poly(dA:dT) transfection. Indeed, we detected higher levels of antiviral-related cytokines and chemokines, such as IFN-β, CXCL10, CCL5, CCL2, IL-6, and TNF-α, in response to poly(dA:dT) in primary microglia from AIM2−/− mice than those from WT mice (Fig. 6 A; and Fig. S4, A and B). AIM2 has been reported to restrict colon tumorigenesis by suppression of the DNA-PK–AKT axis in an inflammasome-independent manner (Wilson et al., 2015). Additionally, a recent study reported that AKT3 can phosphorylate IRF3 at Ser385, which facilitates TBK1-induced phosphorylation of IRF3 on Ser396 and enhances the antiviral inflammatory signaling (Xiao et al., 2020). We speculated that AIM2 in microglia may negatively regulate the DNA-PK–AKT3 axis to control antiviral pathway–related inflammation. To verify this hypothesis, we treated primary microglia from WT and AIM2−/− mice with poly(dA:dT) and analyzed the phosphorylation of IRF3, TBK1, and AKTs. We observed an increase in the phosphorylation of IRF3 at Ser385, Ser396, and AKT, but not the phosphorylation of TBK1, AKT1, and AKT2 (Fig. 6 B) in AIM2−/− microglia compared with controls after treatment. Since there is no commercial antibody to specifically detect p-AKT3, we silenced AKT1 and AKT2 in microglia, and immunoprecipitated AKT3 to detect AKT3 phosphorylation by using an anti-phosphorylated AKT antibody after poly(dA:dT) treatment. We found that AIM2 deficiency markedly enhanced poly(dA:dT)–induced AKT3 phosphorylation (Fig. 6 C), suggesting a negative role for AIM2 in regulating the activation of AKT3. Consistently, we also observed the enhanced phosphorylation of IRF3 at Ser385 in microglia from AIM2−/− mice by comparing to WT mice (Fig. 6 D). To further dissect the mechanism of AIM2 regulation of AKT3, we examined the interaction of the DNA-PK catalytic subunit (DNA-PKcs) with AIM2 and AKT3 (Fig. 6 E), suggesting that AIM2 suppresses recruitment of AKT3 and IRF3 to DNA-PK. Consistently, we observed great colocalization between AIM2 and DNA-PK in the spinal cord of EAE mice compared with untreated controls by using in situ hybridization (Fig. S4 H). To further verify that AIM2 targets the DNA-PK–AKT3 axis, the DNA-PK inhibitor NU7441 (Leahy et al., 2004) was used. WT and AIM2−/− microglia were treated with NU7441 followed by poly(dA:dT) treatment. Treatment with NU7441 significantly reduced the elevated production of IFN-β, CXCL10, CCL5, CCL2, IL-6, and TNF-α in AIM2−/− microglial cells (Fig. 6 F; and Fig. S4, C and D). In addition, NU7441 significantly reduced the phosphorylation of IRF3 at Ser385 and Ser396, and AKT3 in AIM2−/− microglial cells to comparable levels in NU7441–treated WTs (Fig. 6, G and H). Moreover, we observed AKT3 knockdown significantly reduced the elevated production of IL-6, IFN-β, CXCL10, and CCL5 and in AIM2−/− microglial cells to comparable levels in AKT3–silenced WTs (Fig. S4, E–G). These data suggest AIM2 can inhibit the microglial antiviral inflammatory signaling by negatively regulating the recruitment of AKT3 and IRF3 to DNA-PK.
Inhibition of the cGAS (cyclic guanosine monophosphate–adenosine monophosphate synthase)-DNA-PK axis rescues EAE pathogenesis in AIM2-deficient mice

Axonal damage and apoptotic loss of neurons occur in the brain during EAE (Hobom et al., 2004). Indeed, we observed a marked increase of DNA damage marker γH2AX in the spinal cord of mice during EAE (Fig. S5 A). DNA or cyclic dinucleotides released by dying nerve cells have been reported to activate cGAS-STING (stimulator of interferon genes) in microglia (Abdullah et al., 2018; Chin, 2019). To determine if AIM2 can restrict EAE...
by restraining cGAS-mediated inflammation, we crossed AIM2−/− mice with cGAS−/− mice to generate AIM2 and cGAS double-KO mice. During type B EAE, cGAS deficiency significantly alleviated clinical scores, inflammatory cell infiltration, and demyelination when compared with WT mice (Fig. 7, A–C). Furthermore, AIM2−/−cGAS−/− were comparable to cGAS-deficient mice, as indicated by clinical score, inflammatory cell infiltration, and pathological features (Fig. 7, A–C; and Fig. S5 B). Thus, these data suggested that AIM2 plays a protective role in EAE by controlling cGAS-mediated EAE neuroinflammation. To further confirm...
AIM2 regulates EAE severity in development of MS in this study. AIM2 in microglia functions in EAE independently of its inflammasome function. Why AIM2 in microglia protects against neuroinflammation by suppressing the DNA-PKcs (Fig. 7, D–G). Notably, the expression of AIM2 was higher in microglia than in macrophages, DCs, astrocytes, and neurons. Microglia-specific AIM2 plays a dominant role in modulating the pathogenesis of EAE. Although inflammasome proteins such as ASC, caspase-1, and AIM2 are enriched in microglial cells (Singhal et al., 2014), the AIM2 inflammasome requires a higher threshold of DNA for activation to occur (Choubey, 2019; Jones et al., 2010). Conversely, lower levels of DNA can be sensed directly by cGAS and DNA-PKcs (Ablasser and Chen, 2019; Choubey, 2019; Lees-Miller et al., 2017). Moreover, the AIM2 inflammasome requires a higher threshold of DNA for activation to occur. The expression of AIM2 in microglia was higher than in macrophages, DCs, astrocytes, and neurons. Microglia-specific AIM2 plays a dominant role in modulating the pathogenesis of EAE. Although inflammasome proteins such as ASC, caspase-1, and AIM2 are enriched in microglial cells (Singhal et al., 2014), the AIM2 inflammasome requires a higher threshold of DNA for activation to occur (Choubey, 2019; Jones et al., 2010). Conversely, lower levels of DNA can be sensed directly by cGAS and DNA-PKcs (Ablasser and Chen, 2019; Choubey, 2019; Lees-Miller et al., 1990). The amount of DNA released by damaged neurons during EAE nerve injury may not be sufficient to stimulate AIM2-dependent inflammasome activation, which may explain why AIM2 in microglia functions in EAE independently of its inflammasome function. Notably, we performed microarray analysis of microglia (CD45+CD11b+ cells) isolated from the CNS of WT and AIM2–/– mice of EAE and revealed that the most prominent up-regulated responses in AIM2–/– microglia from EAE-treated mice were associated with defense against viral infection. Correspondingly, a recent study showed an inflammasome-independent role of AIM2 in obesity and insulin resistance that is mediated by the up-regulation of Il20rb and antiviral IFN signaling (Gong et al., 2019). Previous studies have implicated viral infections as triggers of neurodegenerative diseases such as Parkinson’s disease, Alzheimer’s disease, and amyotrophic lateral sclerosis (Amor et al., 2010; Deleidi and Isacson, 2012). Consistent with this, the antiviral cytokine IFN-β levels increase with age (Baruch et al., 2014; Yu et al., 2015). Additionally, viruses have been considered to be etiological agents of MS involved in demyelination (Libbey et al., 2014). Although IFN-β is the first-line treatment of relapsed and remitting MS at present, ~50% of MS patients are nonresponsive to IFN-β. Furthermore, in some cases, IFN-β can exacerbate MS and consistently worsens neuromyelitis optica (Axtell et al., 2011; Río et al., 2006). Indeed, it
Figure 7. Inhibition of cGAS-DNA-PK axis restricts EAE phenotype in AIM2<sup>−/−</sup> mice. (A) Mean clinical scores after EAE induction in WT, AIM2<sup>−/−</sup>, cGAS<sup>−/−</sup>, and AIM2<sup>−/−</sup>cGAS<sup>−/−</sup> mice (n = 5 mice per group). (B) Representative H&E and LFB staining and histology score of spinal cord sections harvested from the mice.
has been reported that acute virus infection can induce the development of type II EAE in mice (Inoue et al., 2016). Significant increases in the mRNA levels of antiviral inflammatory genes such as Ddx58, Ifit1, Oas1, Ifr7, Il6, Il12, Ccl22, Cxcl10, and Ccl5 were identified in microglia from AIM2−/− EAE-treated mice. The increased expression of chemokines and cytokines such as Cxcl10, Ccl22, Ccl5, and Il6 in AIM2−/− cells plays a crucial role in T cell infiltration and cell proliferation during EAE (Balashov et al., 2007; Welsh et al., 2016).

AIM2 prevents EAE independent of inflammasome

Mice

Female mice with the C57BL/6 background were used in this study. The AIM2−/− and ASC−/− mice were a gift from Dr. V. Dixit (Genentech, South San Francisco, CA). The cGAS−/− mice were from The Jackson Laboratory. The AIM2−/− mice were generated using conditional gene targeting methods by Biocytogen. Aim2 conditional KO mice were generated by the CRISPR/Cas9-based approach. Briefly, two single guide RNAs (sgRNAs) were designed by a CRISPR design tool from the Feng Zhang lab (http://crispr.mit.edu; Platt et al., 2014) to target either a region upstream or downstream of exon 5, and then were screened for on-target activity using the Universal CRISPR Activity Assay (Biocytogen). To minimize random integrations, we employ a circular donor vector. The gene-targeting vector containing 5′ homologous arm, target fragment (exon 5), 3′ homologous arm was used as a template to repair the double-strand breaks generated by Cas9/sgRNA. The two LoxP sites were precisely inserted in both sides of the target fragment of the Aim2 gene. T7 promoter sequence was added to the Cas9 or sgRNA template by PCR amplification in vitro. Cas9 mRNA, targeting vector, and sgRNAs were coinjected into the cytoplasm of one-cell-stage fertilized C57BL/6N eggs. The injected zygotes were transferred into oviducts of Kunming pseudopregnant females to generate F0 (numerous founder 0) mice. F0 mice with the expected genotype confirmed by tail genomic DNA PCR and sequencing were mated with C57BL/6 mice to establish germline-transmitted F1 heterozygous mice. F1 heterozygous mice were mated with C57BL/6N mice to establish germline-transmission F0 (numerous founder 0) mice. F0 mice with the expected genotype confirmed by tail genomic DNA PCR and sequencing were mated with C57BL/6N mice to establish germline-transmitted F1 heterozygous mice. F1 heterozygous mice were genotyped by tail genomic PCR, Southern blot, and DNA sequencing. AIM2 floxed mice were crossed with Cx3cr1-Cre (The Jackson Laboratory) to generate mononuclear phagocyte-conditional AIM2 KO mice (AIM2−/− Cx3cr1-Cre) or with GFAP-Cre (The Jackson Laboratory) to generate astrocyte-conditional AIM2 KO mice (AIM2−/− GFAP-Cre). To generate the microglia-conditional AIM2 KO mice, AIM2 floxed mice were crossed with Cx3cr1-CreERT2-EYFP mice (The Jackson Laboratory), and then the mice were i.p injected with 3 mg tamoxifen (T5648; Sigma-Aldrich) dissolved in 200 µl corn oil (C8267; Sigma-Aldrich) for...

Materials and methods

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five consecutive days to induce the expression of Cre recombinase. After 6 wk, the tamoxifen-induced mice were microglia-conditional AIM2 KO mice, and these mice were used for the EAE study. All mice were kept in a barrier facility, and all animal experiments were conducted in accordance with the procedure approved by the Ethical Review Committee for Laboratory Animal Welfare of Nanjing Medical University.

**Antibodies and reagents**

Antibodies to AIM2 (13098s), p-IRF3-S396 (4947s), IRF3 (4302s), p-TBK1 (5483s), TBK1 (3013s), p-AKT1 (9018s), AKT1 (2938s), p-AKT2 (8599s), AKT2 (3063s), p-AKT (4060P), and AKT3 (14982s) were purchased from Cell Signaling Technology. Antibodies to p-IRF3-S385 (D151514) were purchased from Sangon Biotech. Antibody to ionized calcium binding adapter molecule 1 (Iba1; 019–19741) was from Wako. Antibodies to Apoe (ab1906) and γH2AX (ab11174) were from Abcam. Antibody to CD11b (101204) was from Biolegend. Antibody to AKT (21054) was from SAB. Antibodies to actin (A1978) and DNA-PK (SAB4502385) were purchased from Sigma-Aldrich. Antibody to CD4-APC-Cy7 (GK1.5,100414) was from Biolegend. Pertussis toxin (#180) was from List Biological Laboratories. Mycobacterium tuberculosis H37Ra (231141) was from BD. Complete Freund’s adjuvant (F5506) was from Sigma-Aldrich. MOG35-55 peptide (residues 35–55, Met-Glu-Val-Gly-Trp-Tyr-Arg-Ser-Pro-Phe-Ser-Arg-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys) was synthesized by Sangon Biotech (Shanghai).

**Induction and assessment of EAE**

To induce type A EAE, MOG35-55 peptide (300 µg per mouse) was emulsified with complete Freund’s adjuvant (200 µg M. tuberculosis H37Ra [Mtb] per mouse), and then subcutaneously injected in the flanks and neck of mice on day 0 for one immunization. Pertussis toxin (250 ng per mouse) was applied intravenously on days 0 and 2 after immunization. The type B EAE was induced as described above except that M. tuberculosis was administrated at 400 µg per mouse. Mice were assessed daily for clinical signs of EAE in a blinded fashion. EAE score was evaluated as follows: 0.5, partial tail paralysis; 1, tail paralysis; 1.5, reversible corrective reflex impairment; 2, corrective reflex impairment; 2.5, one hindlimb paralysis; 3, both hindlimbs paralysis; 3.5, both hindlimbs paralysis and one forelimb paralysis; 4, hindlimb and forelimb paralysis; and 5, death.

**Bone marrow chimeras**

The recipient mice were subjected to lethal-dose irradiation (10 Gy), and 1 d later, bone marrow cells (10 × 10⁶ derived from the tibiae and femurs of donor mice) were i.v. injected into lethally irradiated mice. Under these conditions, the radio-resistant CNS-resident cells would be retained, whereas bone marrow and peripheral immune cells would be eliminated and replaced.
by bone marrow cells from donor mice. After 8 wk, the chimeric mice were then subjected to EAE induction.

**Histological analysis**

All spinal cord tissue sections used here were 5 µm thick. For paraffin-embedded tissue, spinal cords collected from PBS-perfused mice were fixed in 4% paraformaldehyde overnight. Sections were stained with H&E for evaluation of leukocyte infiltration or with LFB for assessing demyelination. Histology was scored in a double-blinded fashion as followed: 0, no inflammatory cell infiltration and no demyelination; 1, slight inflammatory cell infiltration or demyelination; 2, moderate inflammatory cell infiltration or demyelination in several spots; and 3, substantial inflammatory cell infiltration and large area of demyelination.

**RNA in situ hybridization**

Spinal cord tissue samples were fixed in formalin for 48 h, embedded in paraffin, and cut into 6-µm sections. In situ hybridization was performed according to the manufacturer’s instructions (G3017; Servicebio). Probes recognizing AIM2 RNA (NM_001013779) were multiplexed with probes recognizing DNAPK RNA (NM_011159).

**Immunofluorescence staining**

Tissue sections were incubated at 4°C overnight with primary antibody to IBA1, APOE, and CD11b. Slides were then incubated with indicated secondary antibodies. The nuclei were counterstained with DAPI (Sigma-Aldrich). Slides were dried and mounted using ProLong Antifade mounting medium (Beyotime Biotechnology). Slides were visualized by a Nikon 50i fluorescent microscope.

**Isolation of CNS immune cells and FACS analysis and sorting**

For preparation of immune cells, brains and spinal cords from MOG35-55-immunized mice were excised and digested at 37°C with DNase I (10 U/ml; Roche) and collagenase type IV (0.5 mg/ml; Sigma-Aldrich) in RPMI 1640 under agitation (200 rpm) conditions for 60 min. Single-cell suspensions were obtained by grinding through a 70-µm cell strainer. Subsequently, homogeneous cell suspensions were centrifuged over the Percoll density gradient (GE Healthcare) and separated by collecting the interface fractions between 37 and 70% Percoll. Mononuclear cells were isolated from the interface. After intensive washing, single-cell suspensions were stained with FVD eFluor 506, anti-CD45, anti-CD4, anti-CD8, and anti-CD11b for FACS analysis. For intracellular cytokine staining, cells were stimulated with phorbol 12-myristate 13-acetate (Multi Sciences), ionomycin (Multi Sciences), and brefeldin A (Invitrogen) for 4 h of culture. Cells were fixed and permeabilized with the Intracellular Fixation & Permeabilization Buffer Set (eBioscience) and then subjected to cytokine staining flow cytometry analyses. All flow cytometry was performed on an Attune NxT flow cytometer (Thermo Fisher Scientific), and data were analyzed by FlowJo 10.0.7 software. For FACS sorting, single cell suspensions were stained with FVD eFluor 506, anti-CD45, and anti-CD11b and sorted on a BD FACS Aria.

**Primary microglia culture**

Cerebral cortices from neonatal mice age 1–3 d were collected and carefully stripped of their meninges and the blood vessels. Following dissection, the tissues were digested with 0.25% trypsin–EDTA and washed in HBSS containing FBS. Then the single-cell suspensions were obtained by passing through a cell strainer (70 µm). The cell suspensions were seeded into poly-D-lysine precoated plates and cultured in DMEM/F12 supplemented with 10% FBS at 37°C and 5% CO₂. Medium was replaced every 4–5 d. After 10–14 d, microglia were separated from the underlying astrocytic layer by gentle shaking of the flask, plated overnight in poly-D-lysine precoated plates, and transfected with Lipofectamine 2000 (2 µl/ml)-complexed poly(dA:dT) (1 µg/10⁶ cells). For the DNA-PK inhibition experiment, the microglia were primed with 1 nM NU7441 (DNA-PK inhibitor) for 1 h before poly(dA:dT) transfection. The conditioned media were collected and measured for cytokine production by ELISA, and the cells were collected for cytokine gene expression by RT quantitative PCR (RT-qPCR) or protein activation by Western blotting.

**siRNA-mediated gene silences in microglia**

Primary microglia were plated in poly-D-lysine precoated plates and were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s guidelines. The siRNA sequences were: siAKT1-1 (sense: 5′-CAUGAAGG AGUUGGAGA-3′; antisense: 5′-GUAUCUUGCUCAAACCAU-3′), siAKT1-2 (sense: 5′-CUUUCUCCUAAAGCAAU-3′; antisense: 5′-GAAGAGGAGUUGUUGCA-3′), siAKT2-1 (sense: 5′-GGA GGAGCUUGUCAAAG-3′; antisense: 5′-CUUUGUAGAGC UACCUC-3′), siAKT2-2 (sense: 5′-GCAATAAGGCAGCUCACUG A-3′; antisense: 5′-UCAGUAGGGCUCUUGUCG-3′); and siAKT3 (sense: 5′-CUCAUUCUAAGGCUAAAC-3′; antisense: 5′-UA UAGCCUAUAGAGC-3′). The siRNA and negative control siRNA were from GenePharma.

**RT-qPCR**

Total RNA was extracted by using TRIzol reagent (Life Technologies) and subjected to cDNA synthesis. RT-qPCR was performed using SYBR Green Supermix (Vazyme). The expression of a single gene was calculated by a standard curve method and standardized to the expression of Hprt. The primers used are listed in Table 1.

**Immunoprecipitation and immunoblot analysis**

Primary microglia were transfected with Lipofectamine 2000 (2 µl/ml)-complexed poly(dA:dT) (1 µg/10⁶ cells). Cells were collected in NP-40 lysis buffer (2 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 0.5% [vol/vol] IGEPAL, 10% [wt/vol] glycerol, 50 mM NaF, 1 mM Na₃VO₄, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, and complete protease inhibitor cocktail (Sigma-Aldrich)), followed by incubation for 40 min at 4°C. The lysates were centrifuged for 15 min at 14,000 rpm for removal of cell debris and nuclei. Supernatants were incubated with the indicated antibody overnight at 4°C. Then an aliquot (40 µl) of protein A/G-agarose was added to each sample, followed by incubation for 4 h at 4°C. The beads were washed three times with washing buffer and heated in 2× SDS sample buffer for 3 min at 95°C.
times with 500 ml lysis buffer. An aliquot (50 µl) of SDS-PAGE sample buffer was added to the beads to separate protein. Samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by immunoblot with the appropriate antibodies.

**ELISA**
Primary mouse microglia were stimulated as indicated. Conditioned media were collected and measured for levels of TNF-α according to the manufacturer (R&D Systems). IFN-β ELISA capture antibody (sc-57201) was from Santa Cruz; IFN-detection antibody (32400–1) and standard protein (12400–1) were from R&D Systems. The level of IFN-β was assayed by an in-house sandwich ELISA system as described (Li et al., 2019a).

**Microarray analysis**
For microarray analysis, CD45lowCD11b+ microglial cells were isolated from CNS of WT and AIM2+ /− mice on day 18 after immunization by sorting on a BD FACS Aria. Total RNA was extracted with Trizol reagent. Samples were analyzed by Beijing Cnkingbio Biotechnology with Affymetrix Mouse Clarion S Array, and all arrays were scanned by using Affymetrix GeneChip Command Console, which was installed in a GeneChip Scanner 3000 7G. The data were analyzed with Robust Multichip Analysis algorithm using Affymetrix default analysis settings and global scaling as a normalization method. Values presented are log2 Robust Multichip Analysis signal intensity. Differentially expressed genes were identified based on the Student’s t test for comparison of the two groups. The threshold set for up- and down-regulated genes was a fold change >1.2 and a P value <0.05. KEGG pathway analysis and GO analysis were performed using the R package, using significantly differentially expressed genes (P < 0.05) as target genes. Gene network analyses were done by the Beijing Genomics Institute in-house customized data mining system called Dr. Tom. GSEA was performed as previously described (Subramanian et al., 2005) using the Gene Ontology Biological Process Database. Raw data files and processed files have been deposited in GEO under accession no. GSE151636.

**Statistical analyses**
The data were analyzed by GraphPad Prism 7.0 software and are presented as mean ± SEM. The statistics were analyzed by using a two-tailed unpaired t test for two groups and a multiple unpaired t test for multiple groups. P values are *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

**Online supplemental material**
Fig. S1 shows the AIM2 conditional KO mice strategy. Fig. S2 shows that AIM2 deficiency in microglia but not in astrocytes promotes the development of EAE. Fig. S3 shows that AIM2 deficiency in microglia promotes the neuroinflammation of EAE. Fig. S4 shows that AIM2 negatively regulates the DNA-PK–AKT3 in microglia to control inflammation. Fig. S5 shows that AIM2 protects against cGAS-mediated neuroinflammation and AIM2 expression is increased in the brain tissues of MS patients.

### Table 1. Primers used for RT-qPCR

|   | S                        | AS                        |
|---|--------------------------|---------------------------|
| Siglec1 | 5′-CAGGACATCTCAGCTGC-3′ | 5′-GGACATCGTGAAGTTGTC-3′ |
| Ad   | 5′-ATGGCGGACTTGGGCTTTC-3′ | 5′-CCATGACTGGAATCTAGTGC-3′ |
| Cybb  | 5′-TGGGTGCGAATGTCTTTC-3′ | 5′-CCATGACTGGAATCTAGTGC-3′ |
| Clec7a | 5′-GACTTCAGCCTCAAGACATCC-3′ | 5′-TTGTGTCGCAAAATGCTAGG-3′ |
| Gpr34 | 5′-CTCACGAGTGCCAAATGCTAC-3′ | 5′-GCCGAAATAATACAGAGGCA-3′ |
| Tgfbr1 | 5′-TCTGAGGACTTGATTGTCCG-3′ | 5′-AAAGGGACATCGATGAGTA-3′ |
| Cxcl10 | 5′-AAGTGCTGCGCTCTTTGTC-3′ | 5′-ATTCTATGGGCCCTTCTTC-3′ |
| Il-6  | 5′-CTTGGGACTGCTGCTGAGC-3′ | 5′-GCCATTGCAAACTCTTCTTC-3′ |
| Tnf   | 5′-TACCTGAGGCTGGAATCTAGTGC-3′ | 5′-ACACTTGGCGTCTTTCCG-3′ |
| Cdl5  | 5′-GACACACCCTCCCTGCTT-3′ | 5′-TGGTCTCTCCGCTGAGAC-3′ |
| If7   | 5′-TCCTGAGGCTGGAATCTAGTGC-3′ | 5′-ACACTTGGCGTCTTTCCG-3′ |
| Fcrsl | 5′-CCTCTGCTTCCCTTGTTC-3′ | 5′-ATGGTGACTGGAAGCATGC-3′ |
| Ddx58 | 5′-CACATTGCGGAAATAACAGG-3′ | 5′-TGCTGACTTCCGAGACATC-3′ |
| Ifit1 | 5′-TGTCTTCCGAGACATC-3′ | 5′-AACCTTGAGGCTGGAATCTAGTGC-3′ |
| Oas12 | 5′-TTGTGCGCAGGATCGACT-3′ | 5′-TGATGTTGCGACCTTCTTC-3′ |
| Stat1 | 5′-GTCAAGAGTGGCTGCTTCTTC-3′ | 5′-GCGAAGCAAGACATCAGAC-3′ |
| Hprt | 5′-GTCAGAGGGCTGGGACTGGAATCTAGTGC-3′ | 5′-TGTCGTCTCTCCGCTGAGAC-3′ |
| Apoe | 5′-CTGACAGGATGCGCTTACGGC-3′ | 5′-GCAGGTAATCCGAGAGC-3′ |

AS, antisense; S, sense. 

**Ma et al.**
AIM2 prevents EAE independent of inflammasome

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Author contributions: C. Ma, S. Li, Y. Hu, Y. Ma, Y. Wu, C. Wang, and X. Liu designed and performed the experiments, analyzed the data, and prepared the figures; B. Wang, G. Hu, and J. Zhou provided the key technique mentoring, research reagents, and mice; and C. Ma, S. Li, and S. Yang wrote the paper.

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Figure S1. **AIM2 conditional KO mice strategy.** (A) Targeting vector design for generation of a mouse strain with AIM2 exon 5 flanked by LoxP sites. (B) Regions selected for Southern blotting and the result of Southern blotting analysis. (C) Genotyping strategy and representative image for genotyping analysis of AIM2 LoxP sites.
AIM2 deletion in microglia but not in astrocyte promotes the development of EAE. (A) Mean clinical scores after EAE induction in AIM2^fl/fl and AIM2^fl/GFAP-Cre mice (n = 5 mice per group). (B and C) FACS analysis of immune cells infiltrated to the CNS of the mice in A at day 18 after immunization (n = 5 mice per group). Data are presented as a representative plot (B) and quantified percentage and absolute cell numbers (C). (D) Mean clinical scores after EAE induction in Cx3cr1-Cre and AIM2^fl/Cx3cr1-Cre mice (n = 6 mice per group). (E and F) FACS analysis of immune cells infiltrated to the CNS of the mice in D at day 18 after immunization (n = 6 mice per group). Data are presented as a representative plot (E), quantified percentage, and absolute cell numbers (F). (G) Representative H&E and LFB staining and histology score of spinal cord sections from EAE-induced Cx3cr1-Cre and AIM2^fl/Cx3cr1-Cre mice, showing inflammatory cell infiltration and demyelination (arrowheads). Scale bar, 200 µm. (H) Immunoblot analysis of AIM2 expression in BMDMs and microglia from indicated mice. Data are pooled from three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Error bars show mean ± SEM. Unpaired t test for A and D, and multiple unpaired t test for C and F.
AIM2 deletion in microglia promotes the development of EAE. (A and C) FACS analysis of Th1 (IFN-γ+) and Th17 (IL-17A+) cells from CD4+ T cells infiltrated to the CNS of type B EAE-induced AIM2fl/fl Cx3cr1-CreERT2 and Cx3cr1-CreERT2 mice at day 18 after immunization (n = 6 mice per group). Data are presented as a representative plot (A), quantified percentage, and absolute cell numbers (C). (B and D) FACS analysis of Th1 (IFN-γ+) and Th17 (IL-17A+) cells from CD4+ T cells infiltrated to the spleens of type B EAE-induced AIM2fl/fl Cx3cr1-CreERT2 and Cx3cr1-CreERT2 mice at day 18 after immunization (n = 6 mice per group). Data are presented as a representative plot (B), quantified percentage, and absolute cell numbers (D). SP, spleen. (E) Quantitative PCR analysis of the relative mRNA expression of proinflammatory cytokines and chemokines in the spinal cord of indicated mice (n = 5 mice per group) at the EAE peak. Data were normalized to a reference gene, Hprt. Data are pooled from three independent experiments. *, P < 0.05; **, P < 0.01. Error bars show mean ± SEM. Unpaired t test.
Figure S4.  

AIM2 inhibits the microglial antiviral pathway-related inflammation by targeting DNA-PK.  

(A) RT-qPCR analysis of Ccl5 and Cxcl10 mRNA expression of WT and AIM2−/− microglia transfected with poly(dA:dT) for 8 h.  

(B) ELISA analysis of CCL5 and CXCL10 in the supernatants of WT and AIM2−/− microglia transfected with poly(dA:dT) for 8 h.  

(C) WT and AIM2−/− microglia were treated with DNA-PK inhibitor NU7441 (1 nM) and then were transfected with poly(dA:dT) for 8 h. The mRNA expression of indicated cytokines and chemokines was analyzed by RT-qPCR.  

(D) WT and AIM2−/− microglia were treated with DNA-PK inhibitor NU7441 (1 nM) and then were transfected with poly(dA:dT) for 8 h. The production of indicated chemokines was analyzed by ELISA.  

(E and F) WT and AIM2−/− microglia were transfected with siRNAs to silence Akt3 for 48 h, and then were transfected with poly(dA:dT) for 4 and 8 h. The mRNA expression (F) or protein levels (G) of indicated cytokines and chemokines were analyzed by RT-qPCR or ELISA.  

(G) Immunoblot analysis of AKT3 expression in WT microglia transfected with siRNAs to silence Akt3 for 48 h.  

(H) Fluorescence in situ hybridization staining of Aim2 and Dnkap mRNA in spinal cord from control and EAE-induced WT mice at day 18 after immunization. Quantification of Aim2 and Dnkap mRNA puncta colocalization (solid arrowheads) or in close proximity (hollow arrowheads) per 200× image (n = 3 mice per group). Scale bar, 100 μm. Data are pooled from three independent experiments. *, P < 0.05; **, P < 0.001. Error bars show mean ± SEM. Unpaired t test. NT, no treatment.
Figure S5. **AIM2 protects against cGAS-mediated neuroinflammation and AIM2 mRNA expression is increased in the brain tissues of MS patients.**

(A) Spinal cord sections from control and EAE-induced WT mice at day 18 after immunization were evaluated for levels of DNA damage (γH2AX, green). Data are presented as representative images (left) and quantification of relative integrated optical density (IOD) of γH2AX (right; n = 3 mice per group). Scale bar, 200 µm.

(B) Flow-cytometric analysis of immune cells (including CD45+CD4+ T cells, CD45+CD8+ T cells, and CD45+CD11b+ microglia and myeloid cells) infiltrated to the spinal cord and brain of EAE-induced WT, AIM2−/−, cGAS−/−, and AIM2−/−cGAS−/− mice at day 18 after immunization (n = 5 mice per group). Data are presented as representative plots.

(C) AIM2 mRNA expression in hippocampus, frontal cortex, internal capsule, corpus callosum, and parietal cortex of frozen autopsy samples from five female MS patients (average age, 57.6 yr) and five female age-matched healthy controls (average age, 57.2 yr); the data were from an RNA-seq database (accession no. GSE123496).

(D) AIM2 mRNA expression in subpial gray matter lesions of the frontal gyri from 10 healthy (two female and eight male; average age at death, 53.1 yr) and 10 MS patients (five female and five male; average age at death, 49.4 yr), who were selected retrospectively on the basis of a confirmed clinical and neuropathological diagnosis, and snap-frozen brain blocks were provided by various tissue banks within the BrainNet Europe network; the data were from a microarray database (GEO accession no. GSE26927).

(E) The AIM2 mRNA expression in a total of 25 white matter (WM) samples from five healthy controls without neurological disease, and 21 normal-appearing white matter (NAWM) and 16 active lesions from 10 progressive MS patients; the data were from a RNA-seq database (GEO accession no. GSE138614).

(F) The AIM2 mRNA expression in peripheral blood mononuclear cells (PBMCs) from eight MS patients and four healthy controls (HC); the data were from microarray database (GEO accession no. GSE23832). * P < 0.05; ** P < 0.01; *** P < 0.001. Error bars show mean ± SEM. Unpaired t test. CTRL, control.