

Supplementary Materials for

Interferon- β Therapy Against EAE Is Effective Only When Development of the Disease Depends on the NLRP3 Inflammasome

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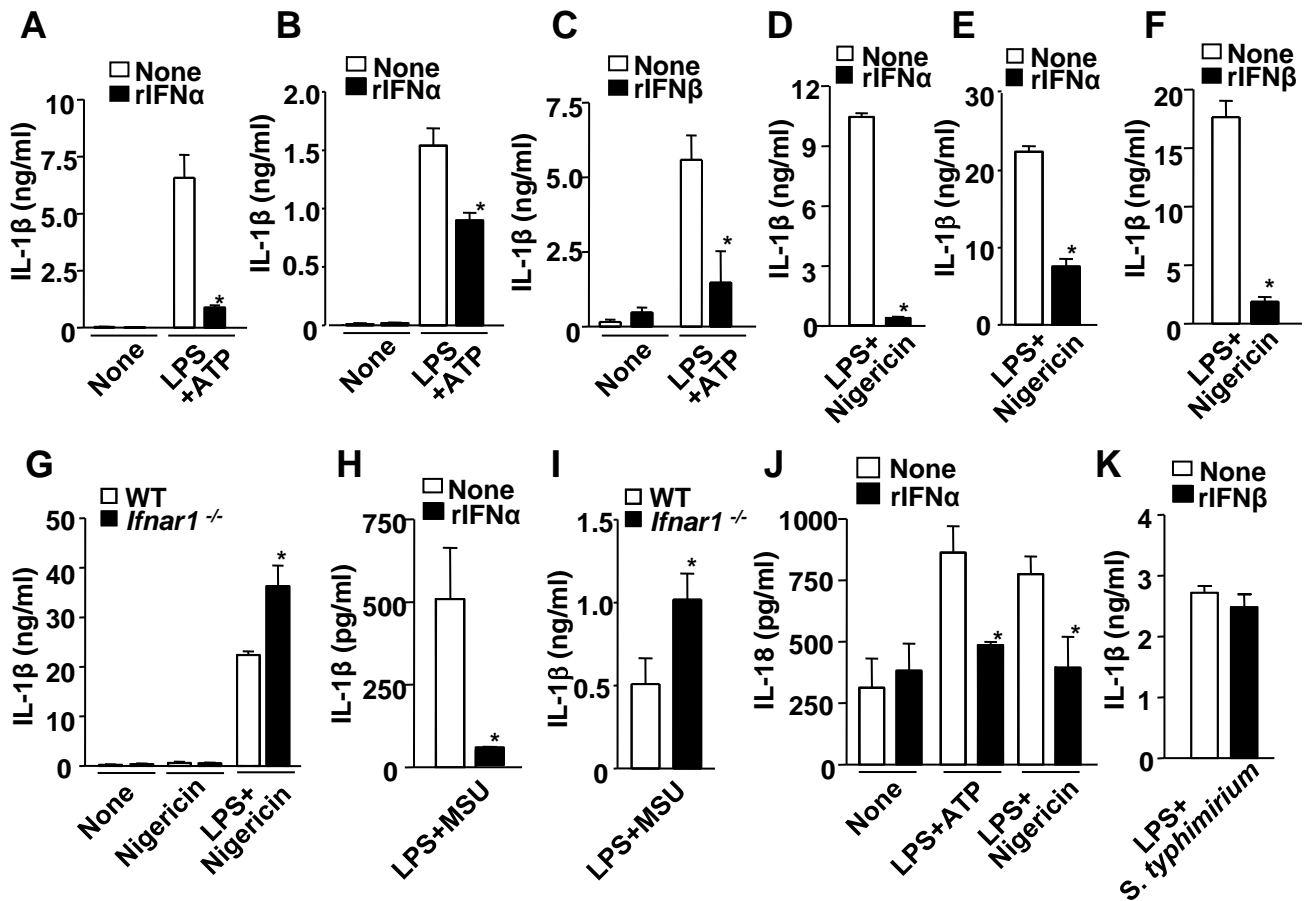


Fig. S1. IFNAR signaling suppresses activation of the NLRP3 inflammasome. (A to I) We measured the IL-1 β concentration in culture supernatants of (A, C, and E to I) BMMs and (B and D) peritoneal macrophages. Cells were incubated with LPS (100 ng/ml) for 3.5 hours together with (A to C) 5 mM ATP, (D to G) 2.5 μ M nigericin, or (H and I) MSU (all added for the last 30 min) in the presence or absence of (A, B, D, E, and H) rIFN- α (1000 U/ml) or (C and F) rIFN- β (1000 U/ml). The rIFN- α or rIFN- β was added 24 hours before starting the treatment with ATP, nigericin, or MSU. (G and I) Comparison of the IL-1 β production of WT and *Ifnar1*^{-/-} peritoneal macrophages. (J) IL-18 concentrations in BMM culture supernatants were measured. WT BMMs were incubated with LPS (100 ng/ml) and either 5 mM ATP or 2.5 μ M nigericin for 3.5 hours in the presence or absence of rIFN- α (1000 U/ml). (K) rIFN- β does not suppress NLRC4-mediated IL-1 β production. BMMs were incubated with LPS (100 ng/ml) and *S. typhimurium* [at a multiplicity of infection (MOI) of 40] for 3.5 hours (cells were treated with *S. typhimurium* for the last 30 min) in the presence or absence of rIFN- β (1000 U/ml). Data are the mean \pm SEM from four to six experiments. * P < 0.05.

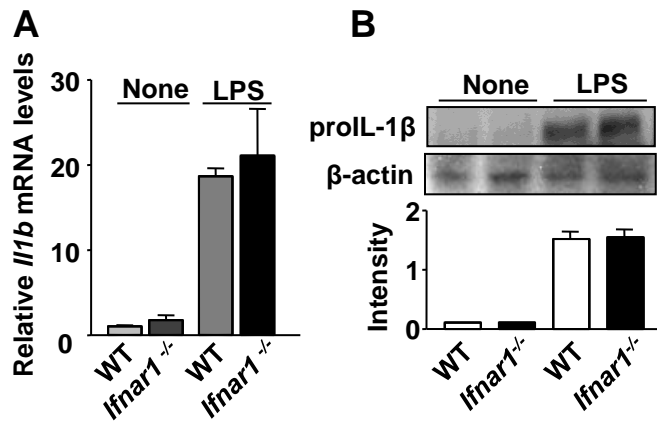


Fig. S2. IFNAR signaling does not inhibit pro-IL-1 β production. **(A and B)** The amounts of **(A)** *Il1b* mRNA and **(B)** pro-IL-1 β protein in WT and *Ifnar1*^{-/-} peritoneal macrophages were determined by qPCR and Western blotting, respectively. Cells were stimulated for 3 hours with LPS. Data in **(A)** are the mean \pm SEM from four experiments. * $P < 0.05$. Data in **(B)** are representative of two independent experiments.

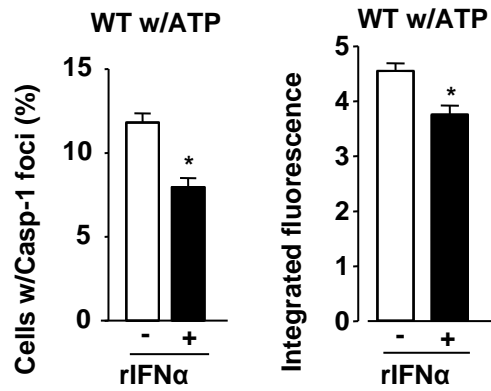


Fig. S3. IFNAR signaling inhibits the formation of caspase-1 foci. The frequency of caspase-1 foci (left) and the integrated fluorescence intensity of caspase-1 foci (right) were measured in ATP-treated WT macrophages that were treated with or without rIFN- α (1000 U/ml) for 24 hours. Data are the mean \pm SEM from four to six mice. * $P < 0.05$.

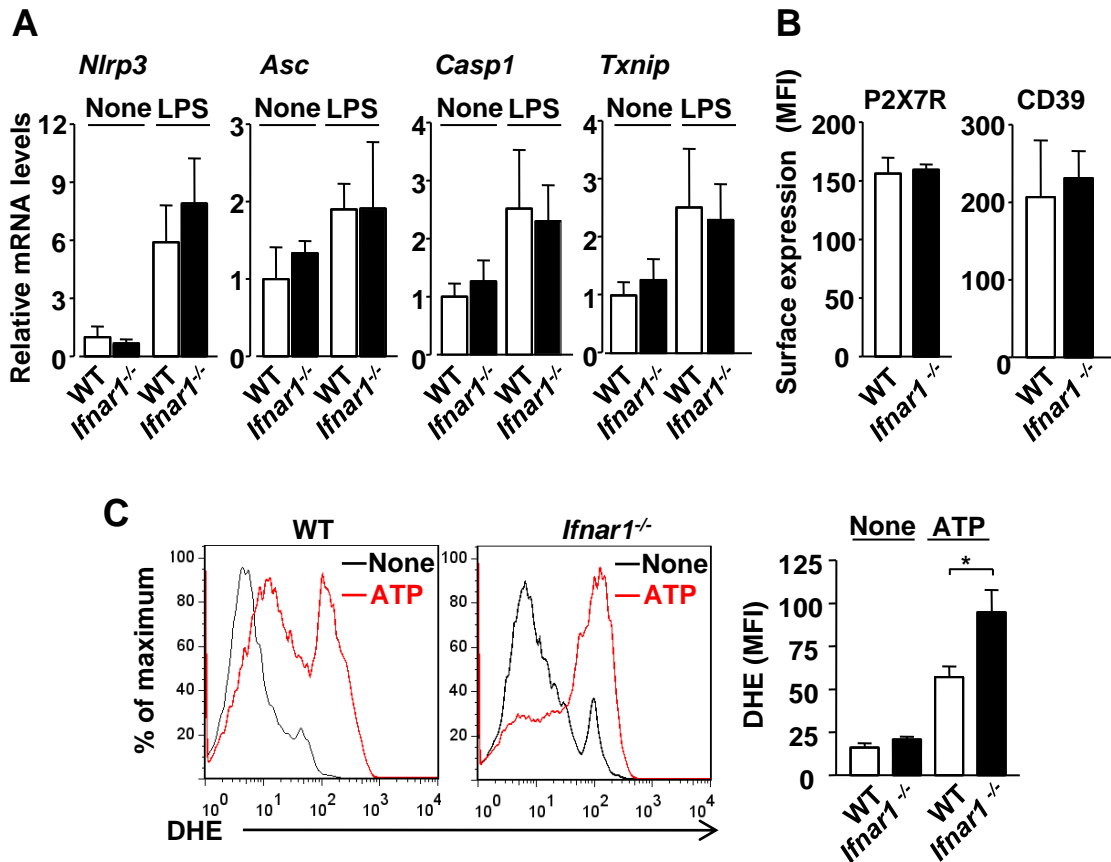


Fig. S4. IFNAR signaling does not affect expression of *Nlrp3*, *Asc*, *Casp-1*, and *Txnip* or the abundance of P2X7R and CD39, but does induce ROS generation. **(A)** Evaluation of *Nlrp3*, *Asc*, *Casp1*, and *Txnip* mRNA abundance by qPCR. WT and *Ifnar1*^{-/-} macrophages were incubated with or without LPS (100 ng/ml) for 3 hours. **(B)** The cell-surface abundance of P2X7R and CD39 on unstimulated WT and *Ifnar1*^{-/-} macrophages was determined by flow cytometry. **(C)** Representative histograms of ROS production as detected with DHE. WT and *Ifnar1*^{-/-} macrophages were incubated with (red line) or without (black line) 5 mM ATP for 30 min. ROS generation was detected with DHE by flow cytometry. Data are the mean \pm SEM from four to six experiments. * $P < 0.05$.

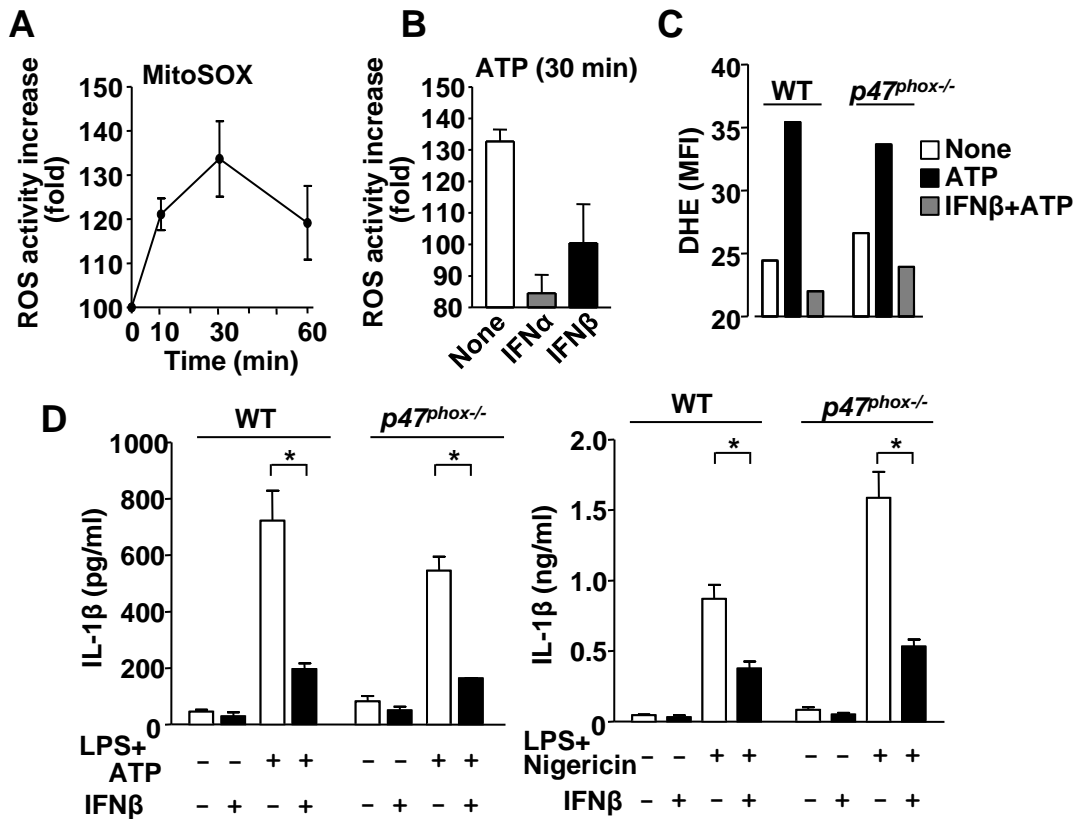


Fig. S5. ROS generated by mitochondria, but not by NADPH oxidase, is suppressed by type I IFN. **(A)** Time course of ATP-induced mitochondrial ROS generation in macrophages. Macrophages were incubated with 5 mM ATP for the indicated times. An increase in ROS activity was calculated as the increase in the MFI value (percentage) of MitoSOX RED staining by flow cytometry. **(B)** Suppression of mitochondrial ROS generation by type I IFN. Cells were incubated with 5 mM ATP for 30 min. rIFN- α or rIFN- β (1000 U/ml) was added 24 hours before treatment with ATP. **(C)** ROS production was detected with DHE by flow cytometry and the MFI of DHE staining was calculated. WT and *p47^{phox}-/-* macrophages were incubated with ATP alone or in the presence of IFN- β . Cells were treated with rIFN- β (1000 U/ml) for 24 hours and with 5 mM ATP for the last 30 min. **(D)** IL-1 β production in *p47^{phox}-/-* deficient BMMs. BMMs were incubated with LPS (100 ng/ml) for 3.5 hours, and 1 mM ATP or 20 μ M nigericin was added for the last 30 min. rIFN- β (1000 U/ml) was added 24 hours before treatment with ATP or nigericin. Data are the mean \pm SEM from four to six experiments.

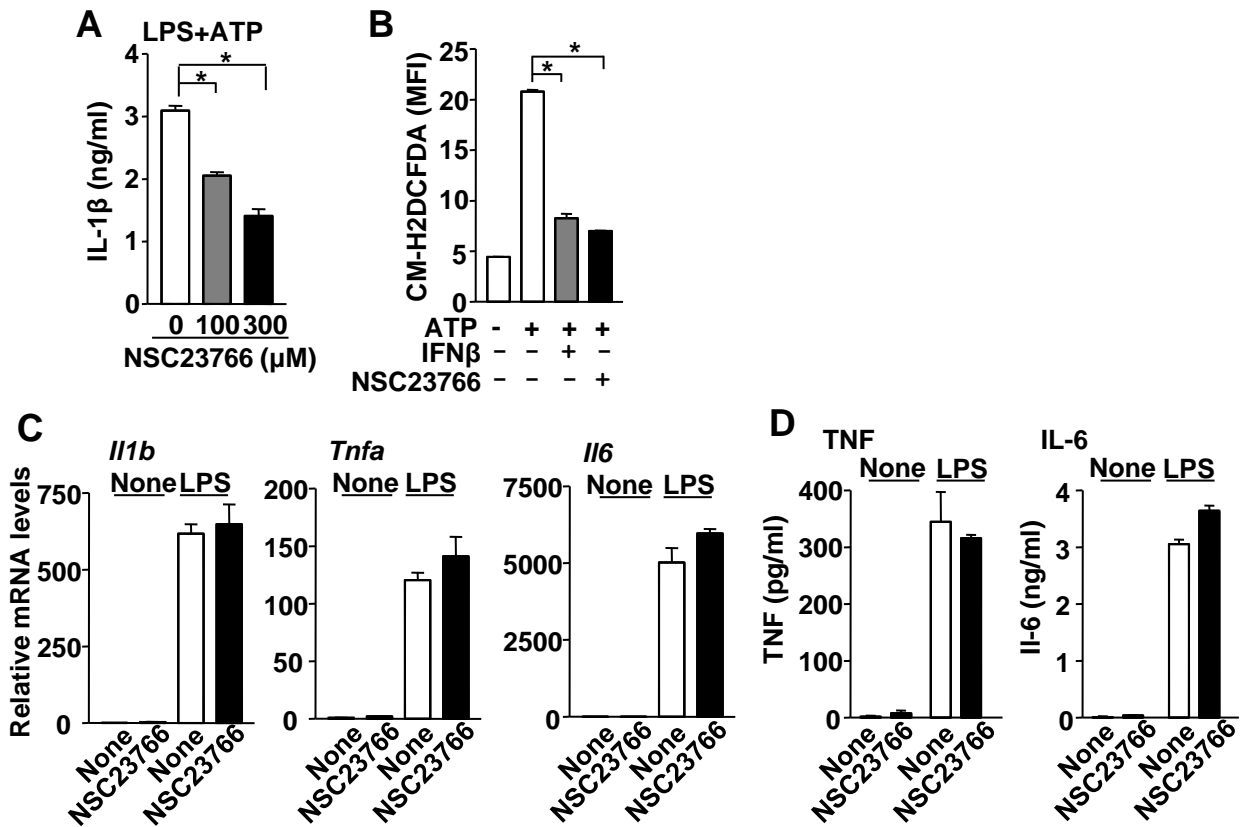


Fig. S6. Inhibition of Rac1 inhibits the production of IL-1 β and ROS, but does not affect the expression of *Tnf*, *Il6*, or *Il1b*. **(A)** Involvement of Rac1 activation in the production of IL-1 β . BMMs were incubated with LPS (100 ng/ml) for 3.5 hours, and 1 mM ATP was added for the last 30 min. Twenty-four hours before ATP was added, NSC23766 was added at the indicated concentrations. **(B)** The suppressive effects of rIFN- β and NSC23766 on ROS generation were confirmed in experiments with CM-H₂DCFDA, another ROS indicator. Cells were pretreated with rIFN- β (1000 U/ml) or 100 μ M NSC23766 24 hours before treatment with ATP. Some samples were further stimulated with 5 mM ATP for the last 30 min. **(C and D)** The amounts of cytokine mRNAs (as detected by qPCR) and proteins (as detected by ELISA) in macrophages treated with or without NSC23766 were determined. Cells were treated with LPS (100 ng/ml) for (C) 3 hours (to detect mRNA) or (D) 6 hours (to detect protein). Twenty-four hours before treatment with LPS, 300 μ M NSC23766 was added. Data are the mean \pm SEM from four to six experiments. * P < 0.05.

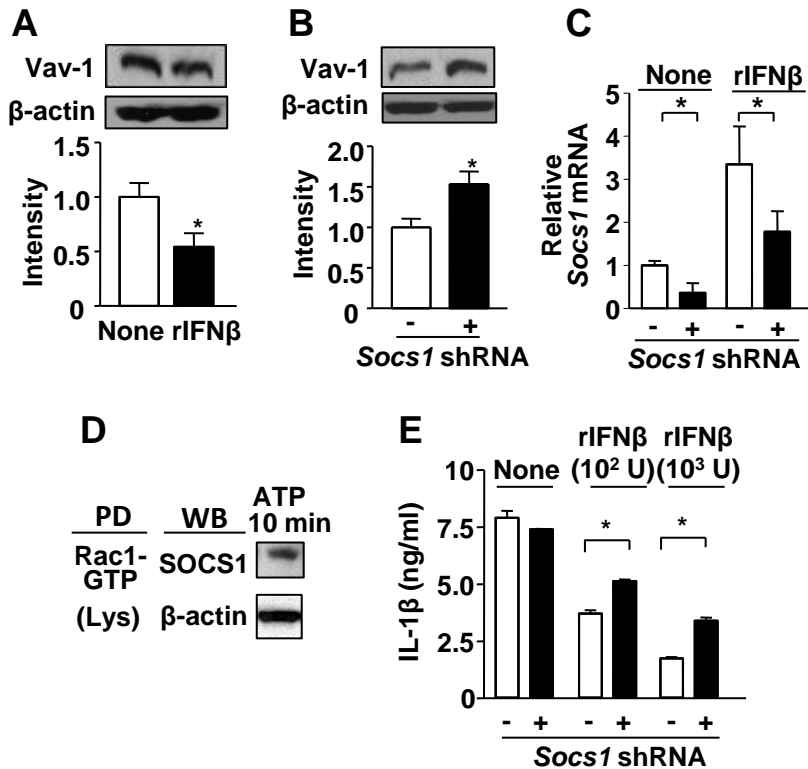


Fig. S7. Involvement of SOCS1 in IFNAR signaling. **(A)** Reduction of Vav1 abundance in BMMS by treatment with rIFN- β (1,000 units/ml) for 24 hours. **(B)** *Socs1* silencing derepresses Vav1 abundance in BMMs. Cells were transfected with constructs encoding *Socs1*-specific shRNA (+) or control shRNA (-). Four hours after transfection, cells were treated with rIFN- β for 24 hours. **(C)** Efficacy of *Socs1* silencing in WT BMMs treated for 24 hours with or without rIFN- β (1000 U/ml), which was added to the cell culture 4 hours after transfection. **(D)** Coimmunoprecipitation of Rac1-GTP and SOCS1 in BMMs 10 min after treatment with ATP. Data are representative of two independent experiments. **(E)** SOCS1 mediates inhibition of IL-1 β production by IFN- β . Cells were pretreated with rIFN- β (100 or 1000 U/ml) for 24 hours before ATP treatment. Western blotting data in (A) and (B) are representative of three independent experiments, and densitometric analysis of all of the experiments are presented in the bar graph. Data shown in (C) and (D) are the mean \pm SEM from four to six experiments. * $P < 0.05$.

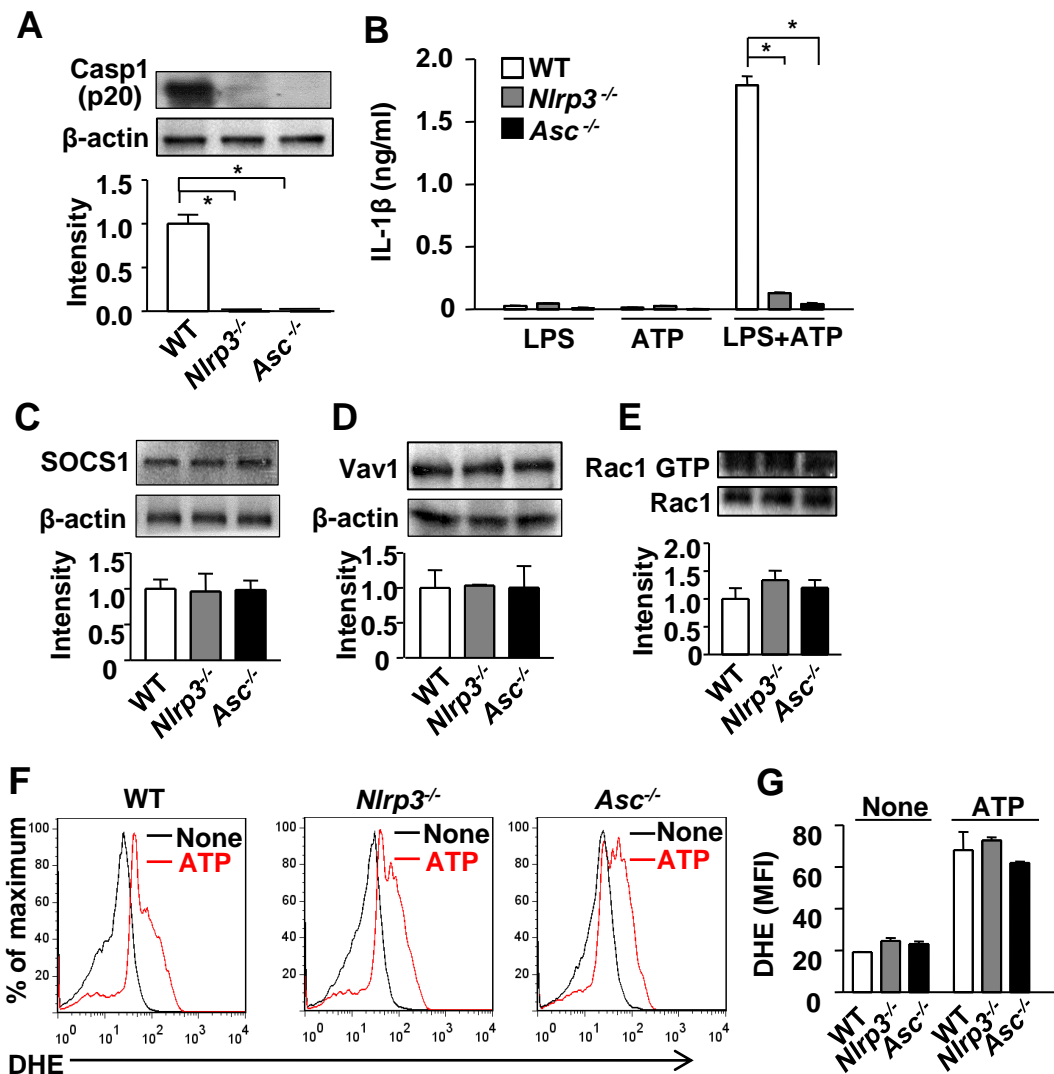


Fig. S8. Events upstream of the NLRP3 inflammasome are intact in NLRP3 inflammasome-deficient macrophages. **(A)** Caspase 1 p20 and β -actin amounts in BMM culture supernatants and cell lysates, respectively. Cells were incubated with LPS (100 ng/ml) for 4 hours and with 5 mM ATP for the last 30 min. **(B)** IL-1 β concentrations in culture supernatants of WT, *Asc*^{-/-}, and *Nlrp3*^{-/-} BMMs. Cells were incubated with LPS (100 ng/ml) for 4 hours, with 5 mM ATP added for the last 30 min, or cells were treated with either LPS or ATP alone. **(C to E)** Western blotting analysis of SOCS1, Vav1, and Rac1-GTP proteins in BMMs. **(E)** BMMs were treated with 5 mM ATP for 10 min. Amounts of β -actin were used for normalization. **(F and G)** ROS production detected with DHE by flow cytometry. WT, *Asc*^{-/-}, and *Nlrp3*^{-/-} BMMs were incubated with or without 5 mM ATP for 30 min. Western blotting data are representative of three independent experiments, and densitometric analysis of all the experiments is presented in the bar graph. Data shown in **(B)** and **(G)** are the mean \pm SEM from four to six experiments. * $P < 0.05$.

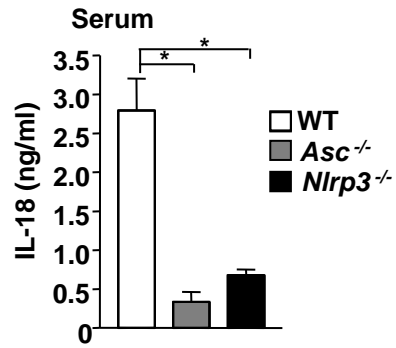


Fig. S9. Serum IL-18 concentrations 9 days after immunization. The amounts of serum IL-18 in WT, *Asc*^{-/-}, and *Nlrp3*^{-/-} mice 9 days after EAE induction were determined by ELISA. Data are the mean \pm SEM from five mice for each group. * $P < 0.05$.

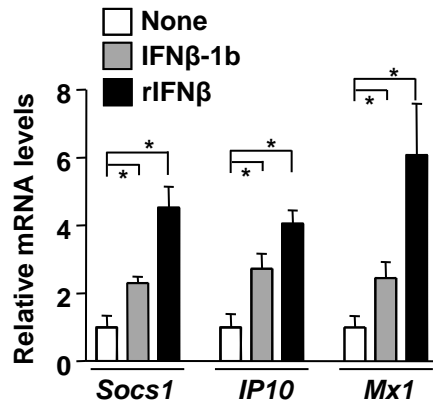


Fig. S10. Evaluation of rIFN- β efficacy in cell culture. Induction of *Socs-1*, *Ip10* (*Cxcl10*), and *Mx1* expression with two different types of rIFN- β was evaluated. WT BMMs were incubated with 10,000 U/ml rIFN- β -1b (human; Betaseron) or 1000 U/ml rIFN- β (mouse) for 6 hours. rIFN- β -1b was used to treat EAE. Mouse rIFN- β was used in ex vivo experiments. Data are the mean \pm SEM from four experiments. * $P < 0.05$.

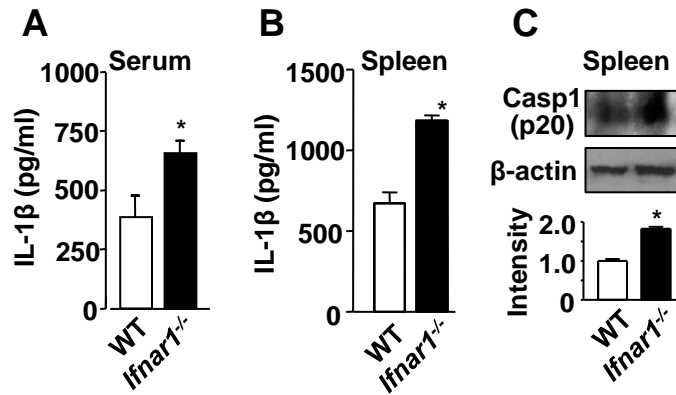


Fig. S11. IFNAR signaling suppresses NLRP3 inflammasome activity in vivo. (A and B) IL-1 β concentrations in (A) serum and (B) culture supernatants of splenocytes (treated as described for Fig. 4C) from WT or *Ifnar1*^{-/-} mice 9 days after induction of EAE. Data are the mean \pm SEM from five mice. (C) Caspase 1 p20 detection was performed (as described for Fig. 4D) with splenocytes isolated from WT or *Ifnar1*^{-/-} mice 9 days after induction of EAE. Data are the mean \pm SEM from five experiments. * $P < 0.05$.

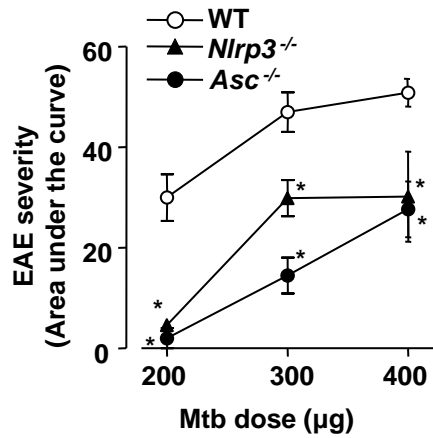


Fig. S12. NLRP3 inflammasome–dependent and –independent EAE. EAE was induced in mice by immunization with MOG₃₅₋₅₅ peptide emulsified in CFA (including titrated amounts of Mtb, 200 to 400 µg). Mice were assessed daily for clinical signs of EAE. EAE severity was evaluated as the area under the curve (AUC) from time-course experiments between days 0 and 30. Data are the mean ± SEM from five mice. * $P < 0.05$.

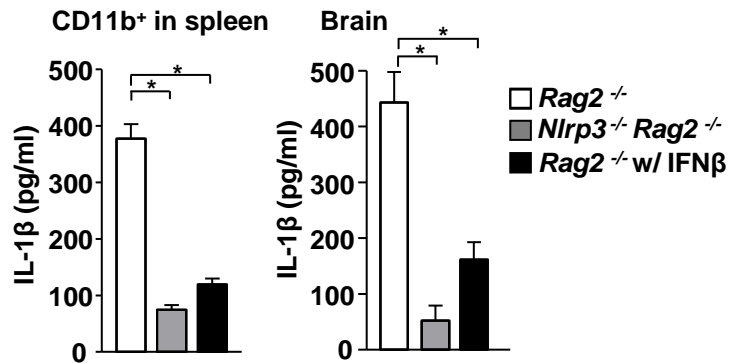


Fig. S13. Inflammasome activity in mice with passive EAE. IL-1 β production by CD11b⁺ cells (mainly macrophages) from spleens and by leukocytes from brains. Cells were isolated from recipient mice 22 days after adoptive transfer of CD4⁺ T cells (see Fig. 6). CD11b⁺ cells (2×10^6 cells) and leukocytes from brains (3×10^5 cells) were treated with LPS (1 μ g/ml) for 24 hours. Data are the mean \pm SEM from three mice. * $P < 0.05$.

Table S1. Sequences of primers used for qPCR analysis.

Gene	Sequence
<i>Asc</i>	forward 5'-AAA GAA GAG TCT GGA GCT GTG GCA-3' reverse 5'- ACT TCT GTG ACC CTG GCA ATG AGT-3'
<i>Nlrp3</i>	forward TCA GAT TGC TGT GTG TGG GAC TGA reverse AGC TCA GAA CCA ATG CGA GAT CCT
<i>Casp1</i>	forward 5'-GAA GGC CCA TAT AGA GAA AGA TTT TAT TG-3 reverse 5'-GAC AGG ATG TCT CCA AGA CAC ATT-3'
<i>Txnip</i>	forward, 5'-ACC TAA ACA TCC CAG ATA CCC-3' reverse, 5'-TTG AGA GTC GTC CAC ATC G-3'
<i>Il1b</i>	forward, 5'-CGC AGC AGC ACA TCA ACA AGA GC-3' reverse, 5'-TGT CCT CAT CCT GGA AGG TCC ACG-3
<i>Tnfa</i>	forward, 5'-CAT CTT CTC AAA ATT CGA GTG ACA A-3' reverse, 5'-TGG GAG TAG ACA AGG TAC AAC CC-3'
<i>Il6</i>	forward, 5'-GAG GAT ACC ACT CCC AAC AGA CC- reverse, 5'-AAG TGC ATC ATC GTT GTT CAT ACA-3'
<i>Ip10</i>	forward, 5'-GTG TTG ACA TCA TTG CCA CG-3' reverse, 5'-GCT TAC AGT ACA GAG CTA GG-3
<i>Mxl1</i>	forward, 5'-GAT CCG ACT TCA CTT CCA GAT GG-3' reverse, 5'-CAT CTC AGT GGT AGT CAA CCC-3'
<i>Socs1</i>	Qiagen (Mm_Socs1_1_SG)
<i>β-actin</i>	forward, 5'-TGT TAC CAA CTG GGA CGA CA-3' reverse: 5'-CTG GGT CAT CTT TTC ACG GT-3'