Supplementary information to

Amyloid-β activates NLRP3 inflammasomes by affecting microglial immunometabolism through the Syk-AMPK pathway

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Suppl. Figure 1. R406, syk inhibitor, protects Aβ42-induced inflammasome activation in LPS-primed microglia.

(a) LPS primed
R406 - - +
Aβ42 - + +
pSyk (72kDa)
Syk (72kDa)
Pro IL-1β (35kDa)
Pro IL-18 (24kDa)
β-actin (45kDa)

(b) pSyk/Syk
IL-1β (pIg)/actin

(c) IL-1β (pIg)/actin

(d) LPS primed

(e) Veh
LPS+Aβ42
LPS+R406+Aβ42

Caspase-1 (DAPI)
Scale bar: 20 μm

(f) LPS primed

(g) Veh
LPS+Aβ42
LPS+R406+Aβ42

ASC/DAPI
Scale bar: 10 μm

(h) ASC focal/ductal cells (%)
Suppl. Figure 1. R406, Syk inhibitor, protects Aβ42-induced inflammasome activation in LPS-primed microglia. LPS-primed microglia were pre-treated with Syk inhibitor R406 for 1 h followed by treatment with Aβ42 for 24 h. (a-d) Representative image and quantification of western blot for the pSyk, Syk, pro IL-1β, IL-18 and β-actin. (e and f) Representative immunofluorescence image of primary microglia with anti-caspase-1 (green) and quantification of immunofluorescence of caspase-1. Scale bar: 20μm. (g and h) Immunofluorescence staining for ASC (red) speck. Scale bar: 10μm. Percentages of microglia containing ASC foci was quantified. All data are represented with mean ± SEM and analyzed by one-way ANOVA Tukey’s multiple comparisons test. (n=4). *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.
Suppl. Figure 2. Bay inhibits Aβ42-induced mitochondrial fission in LPS-primed microglia.

[Images showing the effects of Veh, LPS+Aβ, and LPS+Bay+Aβ on microglia at 3h, 6h, 12h, and 24h.]
Suppl. Figure 2. Bay inhibits Aβ42-induced mitochondrial fission in LPS-primed microglia. LPS-primed microglia were pre-treated with Bay 61-3606 for 1 h followed by treatment with Aβ42 for 24 h. Cells were used for ICC at 3, 6, 12 and 24 h post Aβ42 treatment and immunostained against TOM20. (a) Representative images of mitochondrial morphology showing the effect of Bay 61-3606. Mitochondria and nuclei were stained with Tom20 (green) and DAPI (blue), respectively. Scale bar: 5μm.
Suppl. Figure 3. Mdivi-1 inhibits Aβ42-induced mitochondrial fission in LPS-primed microglia.
**Suppl. Figure 3. Mdivi-1 inhibits Aβ42-induced mitochondrial fission in LPS-primed microglia.** LPS-primed microglia were pre-treated with Mdivi-1, a selective mitochondrial division inhibitor, for 1 h followed by treatment with Aβ42 for 24 h. (a) Representative images of mitochondrial morphology showing the effect of Mdivi-1. Mitochondria and nuclei were stained with Tom20 (green) and DAPI (blue). Scale bar: 5μm.
Suppl. Figure 4. Flufenamic acid inhibits LPS-induced microglial inflammation and restores altered AKT/AMPK signaling.
Suppl. Figure 4. Flufenamic acid inhibits Aβ42-induced microglial inflammasome activation and restores altered Akt/AMPK signaling. Primary microglia were pre-treated with acetylsalicylic acid (ASA) or ibuprofen (Ibu) or flufenamic acid (FA) for 1 h at concentration of 100 μM followed by treatment with LPS (10 ng/ml) for 24 h. (a-d) Whole protein lysates were analyzed for pro IL-1β, pro IL-18, Caspase-1 and β-actin. (e) Primary microglia were pre-treated with different concentrations of ASA, ibuprofen or FA for 1 h followed by treatment with LPS (10 ng/ml) for 24 h. The expressions of pro IL-1β protein were determined by western blot. (f) Quantification of western blot for FA-pretreated set. (g and h) IL-1β and IL-18 mRNA expressions were analyzed using real-time PCR. 18S rRNA was used for normalization. (i) Microglia morphology was examined under phase contrast microscope. (j-l) Primary microglia were pre-treated with NSAIDs (100 μM) for 1 hr followed by treatment with LPS (10 ng/ml) for 24 h. Protein levels of phospho-Akt (Ser723), total Akt, phospho-AMPK(T1722448), total AMPK, pro IL-1β, COX2, and actin were measured by western blot. All data are represented with mean ± SEM and analyzed by one-way ANOVA Tukey’s multiple comparisons test. (n=2-4). *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.
Suppl. Figure 5. Flufenamic acid attenuates Aβ42-induced inflammasome activation in LPS-primed microglia.

(a) Aβ42, LPS, and FA treatments were applied to microglial cells. Western blot analysis of NLRP3, Pro IL-1β, and Pro IL-18 levels with β-actin as a loading control.

(b) Quantitative analysis of NLRP3 levels normalized to β-actin.

(c) Quantitative analysis of Pro IL-1β levels normalized to β-actin.

(d) Quantitative analysis of Pro IL-18 levels normalized to β-actin.
Suppl. Figure 5. Flufenamic acid attenuates Aβ42-induced inflammasome activation in LPS-primed microglia. Mouse primary microglia cells were primed with LPS (10 ng/ml) for 3 h, and washed with serum-free DMEM. LPS-primed microglia were stimulated with Aβ42 (4 μM) for 24 h or treated with FA (1μm) for 1 h before Aβ42 exposure. (a) Immunoblot analysis of NLRP3, pro IL-1β, pro IL-18 and β-actin in whole cells lysate. (b-d) Densitometric quantification of immunoblots bands. All experiments were performed at least three independent times and representative figures are shown. β-actin was used as loading control. Data are presented as mean ± SEM and data were analyzed by one-way ANOVA Tukey’s multiple comparisons test. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p <0.0001.
Suppl. Figure 6. Flufenamic acid alleviates AD pathology by regulating microglial activation.

(a) Control chow

| Treatment     | Iba1/DAPI |
|---------------|-----------|
| ADLP<sup>WT</sup> | ![Image]   |
| ADLP<sup>APPT</sup>+Saline | ![Image]   |
| ADLP<sup>APPT</sup>+FA | ![Image]   |
| ADLP<sup>APPT</sup>+Saline | ![Image]   |
| ADLP<sup>APPT</sup>+FA | ![Image]   |

(b) Graph showing Iba1 (% of area)

(c) Graph showing % of alternation

(d) Graph showing Biolin-4G8/DAPI

(e) Graph showing Biolin-4G8 (% of area)

(f) Graph showing AT8/DAPI

(g) Graph showing Aβ (% of area)
Suppl. Figure 6. Flufenamic acid alleviates AD pathology by regulating microglial activation. PLX3397 was formulated in chow (300mg/kg) and treated to 6 months-old ADLP^{APT} for 3 months. Post one month from starting chow treatment, FA or saline was i.p. injected daily. (a and b) Hippocampus of mice were immunostained with Iba1. n= 8 for ADLP^{WT}+Saline, n=3 for ADLP^{APT}+saline, n=4 for ADLP^{APT}+FA, n= 7 for ADLP^{APT}+PLX+Saline and ADLP^{APT}+PLX+FA. % of area measured as mean ± SEM. Data were analyzed by one-way ANOVA Tukey’s multiple comparisons test. Scale bar: 100μm. (c) Evaluation of cognitive function by Y-maze task. Alternation rates of mice were presented as mean ± SEM. n=5 for ADLP^{WT}+saline, n=6 for ADLP^{APT}+PLX+Saline and ADLP^{APT}+PLX+FA. (d and e) Hippocampus was immunostained with biotin-4G8 antibody against Aβ (d) and quantified by percentage of area (e). n=7 for ADLP^{APT}+PLX+Saline and ADLP^{APT}+PLX+FA. (f and g) Phosphorylated tau (Ser202/Thr205) were detected with AT8 antibody (f), and quantified by percentage of area (g). n=6 for ADLP^{APT}+PLX+Saline and n=7 for ADLP^{APT}+PLX+FA. Data were analyzed by one-way ANOVA Tukey’s multiple comparisons test. *p < 0.05, ***p < 0.001 and ****p < 0.0001.
Suppl. Video 1. Video file showing mitochondria-labeled (green) primary microglia in vehicle condition. Scale bar, 10µm.

Suppl. Video 2. Video file showing mitochondria-labeled (green) primary microglia in LPS+Aβ condition. Asterisk indicates microglia that show strong mitochondrial fission. Scale bar, 10µm.

Suppl. Video 3. Video file showing mitochondria-labeled (green) primary microglia in LPS+Bay+Aβ condition. Scale bar, 20µm.