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The mixed-lineage kinase 3 inhibitor URM-099 facilitates microglial amyloid- β degradation

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Abstract

Background: Amyloid- β (A β)-stimulated microglial inflammatory responses engage mitogen-activated protein kinase (MAPK) pathways in Alzheimer's disease (AD). Mixed-lineage kinases (MLKs) regulate upstream MAPK signaling that include p38 MAPK and c-Jun amino-terminal kinase (JNK). However, whether MLK-MAPK pathways affect A β -mediated neuroinflammation is unknown. To this end, we investigated if URM-099, a brain-penetrant small-molecule MLK type 3 inhibitor, can modulate A β trafficking and processing required for generating AD-associated microglial inflammatory responses.

Methods: A β 1-42 (A β 42) and/or URM-099-treated murine microglia were investigated for phosphorylated mitogen-activated protein kinase (MKK3, MKK4 (p-MKK3, p-MKK4), p38 (p-p38), and JNK (p-JNK). These pathways were studied in tandem with the expression of the pro-inflammatory cytokines interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α . Gene expression of the anti-inflammatory cytokines, *IL-4* and *IL-13*, was evaluated by real-time quantitative polymerase chain reaction. A β uptake and expression of scavenger receptors were measured. Protein trafficking was assessed by measures of endolysosomal markers using confocal microscopy.

Results: A β 42-mediated microglial activation pathways were shown by phosphorylation of MKK3, MKK4, p38, and JNK and by expression of IL-1 β , IL-6, and TNF- α . URM-099 modulated microglial inflammatory responses with induction of *IL-4* and *IL-13*. Phagocytosis of A β 42 was facilitated by URM-099 with up-regulation of scavenger receptors. Co-localization of A β and endolysosomal markers associated with enhanced A β 42 degradation was observed.

Conclusions: URM-099 reduced microglial inflammatory responses and facilitated phagolysosomal trafficking with associated A β degradation. These data demonstrate a new immunomodulatory role for URM-099 to inhibit MLK and to induce microglial anti-inflammatory responses. Thus, URM-099 may be developed further as a novel disease-modifying AD therapy.

Keywords: Mixed-lineage kinase 3, Alzheimer's disease, Amyloid- β , Microglia, Phagocytosis, Endolysosomal pathway

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Background

Neuroinflammation is a pathogenic driver for Alzheimer's disease (AD) [1–3]. Amyloid- β (A β)-activated microglia secrete pro-inflammatory neurotoxins that are strongly linked to AD-associated neural injury. Such inflammatory responses are notably tied beyond AD to common age-related neurodegenerative, neuroinfectious, and neuroinflammatory disorders [4–13]. Activated microglia, in turn, stimulate neurons to produce more A β and the microtubule-associated protein tau, in a vicious paracrine loop [14]. The events change the brain's microenvironment and further affect microglial activation, leading to progressive neuronal injuries. The end result is a paracrine feedback of neurotoxin amplification that drives disease with A β serving as the principal inducer of innate immune activation [15–17]. This has led many researchers to develop the means to harness immunity for therapeutic gain. Notably, A β vaccination can effectively clear A β in both mouse models of human disease and AD patients [18, 19]. For treatment of AD, A β immunization reduces the A β 42 load with immunomodulation, including microglial phagocytosis and changed lysosomal and scavenger markers [20, 21]. However, such immunizations can also result in the development of meningoencephalitis (~6 %) [22], spongiosis, and neuronal loss [23]. Since removal of plaques at later stages of disease marked by neurofibrillary tangle formation was not proven to be beneficial, alternative treatment strategies are likely required. These might improve clinical outcomes if administered years before clinical signs and symptoms emerge. Indeed, such a therapeutic approach could speed clearance of A β and modify inflammatory activities before disease develops.

Prior therapeutic approaches focused on development of immunomodulatory agents that affect AD microglial responses. For example, nonsteroidal anti-inflammatory drugs (NSAIDs) or antioxidants reduce harmful microglial inflammatory activities and protect neurons in animal models of human disease [24, 25]. While the drugs are effective in converting microglial polarization from an M1 (classical activation) to an M2 (alternative activation) phenotype and attenuating neurotoxin production, successful human therapeutic translation remains out of reach [1, 25, 26].

While the “selectively non-selective” brain-penetrant mixed-lineage kinase type 3 (MLK3) inhibitor, URM-099, can attenuate neuroinflammatory responses and facilitate the actions of long-acting nanoformulated anti-retroviral drugs during human immunodeficiency virus-1 (HIV-1) infection [27–29], it is not known whether it could also harness autophagy. If operative, this could occur as a secondary response to phagocytosis of A β . Here, we show yet another novel therapeutic role of URM-099 as an immune modulator in microglial inflammation and phagocytosis through accelerating phagolysosomal pathway

A β degradation. In this manner, we posit that the drug could be developed as a novel AD therapeutic candidate.

Methods

Microglia isolation and cultivation

Housing, care, and breeding of non-transgenic mice (B6.129 hybrid background) were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. Primary cultured mouse microglia were prepared from the postnatal day 1 newborn mouse brains [30–32]. Meninges-free newborn mouse cortices were minced and trypsinized, followed by mechanical dissociation and filtration to remove tissue chunks. Cells were plated onto plastic tissue culture bottles as mixed glial cultures in Dulbecco's modified eagle medium supplemented with heat-inactivated 10 % fetal bovine serum, 50 μ g/ml penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA) and macrophage colony-stimulating factor (MCSF). Microglia were released from astrocytes in the tissue culture media by shaking. Non-adherent cells were collected 7–14 days after plating.

A β activation and URM-099 treatment

Microglia were plated at a density of 2×10^5 onto 24-well plates for RNA/protein extraction or onto 24-well plates with glass coverslips for confocal microscopy. 2×10^4 cells were placed in each 96-well plate for immunofluorescence studies. One day before A β exposure and URM-099 treatment, media was replaced without MCSF. Microglia were pre-incubated for 30 min with URM-099 (100 nM) [28]. Notably, URM-099 administered at 100 nM did not affect microglial viability as measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Additional file 1: Figure S1) that was performed as previously described [33]. After pre-incubation with URM-099, microglia were incubated with media containing the monomeric A β 42 (10 μ M, Life Technologies, Carlsbad, CA, USA) prepared as previously described [32]. Since monomeric rather than oligomeric A β 1-42 (A β 42) peptides are effectively phagocytosed by human monocyte-derived macrophages [34], monomeric A β 42 was used. After A β treatment, microglia were harvested for total RNA or protein extraction or fixed with freshly depolymerized 4 % paraformaldehyde (PFA) in PBS for 15 min for immunofluorescence assays.

Immunoblot and ELISA tests

Microglial cells were harvested using ice-cold RIPA buffer with protease and phosphatase inhibitor cocktail at end points and centrifuged at 15,000 \times g for 10 min at 4 $^{\circ}$ C to collect cell lysates. Protein concentrations were determined using a Micro BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA). For immunoblots, protein lysates were diluted 1:1 with Laemmli buffer containing

β -mercaptoethanol, incubated at 100 °C for 5 min, electrophoresed on sodium dodecyl sulfate-polyacrylamide gels, and electroblotted to 0.45- μ m pore size polyvinylidene fluoride membranes (Immobilon-P, Millipore, Billerica, MA, USA). Membranes were blocked in 3 % bovine serum albumin/TBST or 5 % skim milk/TBST and incubated with antibodies (Abs) to phospho-MKK3 (p-MKK3), total MKK3, phospho-MKK4 (p-MKK4), total MKK4, phospho-p38 (p-p38), total p38, phospho-JNK (p-JNK), total JNK (1:1000, Cell Signaling Technology, Danvers, MA), CD36, CD47 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and A β (6E10, 1:1000, Covance, Emeryville, CA, USA), at 4 °C for overnight, followed by 30-min incubation in 3 % bovine serum albumin/TBST or 5 % skim milk/TBST with HRP-conjugated anti-goat, mouse, or rabbit IgG Abs (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were detected with SuperSignal West Pico or Femto Chemiluminescent substrate and captured using a myECL Imager (Thermo Fisher Scientific, Waltham, MA, USA). After detection of the bands, membranes were incubated with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, MA, USA) and were then used to detect β -actin for normalization using HRP-conjugated anti- β -actin monoclonal (Sigma, St. Louis, MO, USA). For quantitative analysis, ImageJ software (NIH, Bethesda, MD, USA) was used to quantify band intensities relative to total proteins or control β -actin expression. For ELISA, tissue culture media were subjected to capture antibodies for IL-1 β , IL-6, and TNF- α (PeproTech, Rocky Hill, NJ, USA) according to the manufacturer's instructions. For A β 42 ELISA, cell lysates and tissue culture media were subjected to A β 42 ELISA (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

Immunofluorescence and confocal microscopy

Immunofluorescence was performed using pan-A β Ab (rabbit polyclonal, 1:100) and Alexa Fluor[®]488-conjugated anti-rabbit IgG (1:1000), followed by 30 min counterstaining with 4',6-diamino-2-phenyl-indole (DAPI) (all from Life Technologies, Carlsbad, CA, USA). Images were captured using DP Controller and DP Manager with a DP71 digital camera (Olympus, Orangeburg, NY, USA) attached to a Nikon Eclipse TE300 inverted microscope (Nikon, Melville, NY, USA). For confocal microscopy, PFA-fixed microglia were immunostained with Abs to A β (6E10, 1:1000, Covance, Emeryville, CA, USA), Rab5, Rab7 (rabbit polyclonal, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Lamp1 (rabbit polyclonal, ab24170, 1:500, Abcam, Cambridge, MA, USA). Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG were used as secondary, followed by 30-min counterstaining with DAPI (Life Technologies, Carlsbad, CA, USA). Images were captured using a 63X oil lens on a LSM 710 confocal

microscope (Carl Zeiss Microimaging Inc., Thornwood, NY, USA). Images were quantified using ImageJ software with a co-localization plugin (<https://imagej.nih.gov/ij/plugins/colocalization.html>) (NIH, Bethesda, MD, USA).

RNA extraction and transcript analyses

Total RNA was extracted from microglia using TRIzol (Life Technologies, Carlsbad, CA, USA). For PCR-based gene expression analyses, cDNA was synthesized with 1 μ g of total RNA as a template using a Verso cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA), and quantitative real-time RT-PCR (RT2-qPCR) was performed on a thermocycler (Mastercycler Gradient, Eppendorf Scientific Inc., Westbury, NY, USA) using 2x SYBR Green qPCR Master Mix (Biotool.com, Houston, TX, USA) and gene specific primer sets (Table 1). All primer sequences were obtained from PrimerBank (<https://pga.mgh.harvard.edu/primerbank/>) [35]. Thermal cycler conditions were as follows: 10 min at 95 °C for activation of polymerase, followed by 40 cycles of a two-step PCR (95 °C for 15 s and 60 °C for 1 min). Relative expression for target genes was determined by the $\Delta\Delta$ Ct method and normalized with glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) gene expression as an internal control. Each Δ Ct value was determined by subtracting *Gapdh* Ct value from the target gene Ct value. The $\Delta\Delta$ Ct was calculated by subtracting the Δ Ct value of the control from the Δ Ct value of other groups. $2^{-\Delta\Delta$ Ct represented the average relative amount of mRNA to control for each target gene.

Statistical analyses

All data were normally distributed and presented as mean values \pm standard errors of the mean (SEM). In the case of single mean comparison, data were analyzed by Student's *t* test. In case of multiple mean comparisons, the data were analyzed by one-way ANOVA. When there were significant differences between ≥ 3 sample means, post hoc comparisons with the Newman-Keuls method was performed using statistics software (Prism 4.0, Graphpad Software, San Diego, CA, USA). A value of $p < 0.05$ was regarded as a significant difference.

Table 1 Primers for qPCR analysis

Genes	Forward (5'-3')	Reverse (5'-3')
IL-1 β	GTGTCITTTCCCGTGGACCTTC	CGGAGCCTGTAGTGCAGTTG
IL-6	TAGTCCTTCTACCCCAATTTCC	TTGGTCCTAGCCACTCCTTC
TNF- α	ACTCCAGGCGGTGCCTATG	CCCTGCCACAAGCAGGAAT
IL-4	GGTCTCAACCCCAAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
IL-13	CCTGGCTCTTGCTTGCCCT	GGTCTTGTGTGATGTTGCTCA

Results

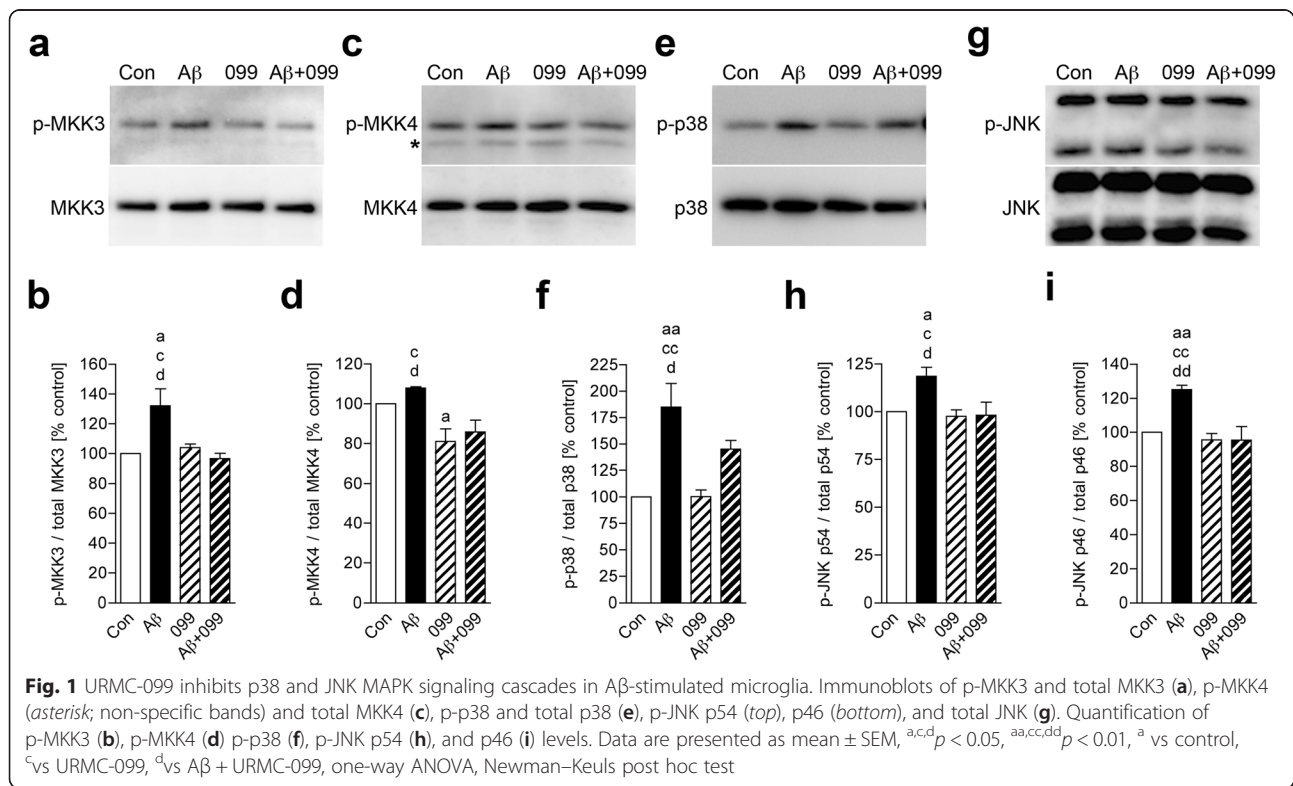
URMC-099 inhibits microglial p38 and JNK MAPK signaling cascades

Aβ activates p38 and JNK MAPK cascades to produce pro-inflammatory mediators in microglia [36–38], and has previously been shown to activate MLK3 in cortical neurons [39], but not microglia. Thus, we tested whether URMC-099 could affect the MAPK cascades in Aβ42-stimulated microglia (Fig. 1). As MKK3/6 and MKK4/7 regulate p38 and JNK phosphorylation downstream MLK3 [40], we examined the phosphorylation patterns of MKK3 and MKK4 (Fig. 1a, c). A 30-min Aβ42 treatment increased MKK3 phosphorylation 32.1 % as compared to untreated controls (Fig. 1b). Moreover, trends in MKK4 phosphorylation were also seen with an 8.0 % increase when compared to untreated controls (Fig. 1d). However, URMC-099 treatment significantly reduced both MKK3 and MKK4 phosphorylation in the Aβ-stimulated microglia with decreases of 26.9 and 20.5 % in MKK3 and MKK4 phosphorylation, respectively (Fig. 1b, d). The phosphorylation of p38 and p46/p54-JNK downstream MKK3 and MKK4 was also assayed (Fig. 1e, g). While a 30-min exposure to Aβ42 showed a rapid induction of both p38 and p46/p54-JNK phosphorylation (increases of 84.8, 18.7, and 25.1 % in p38, p46-JNK, and p54-JNK, respectively, as compared to untreated controls, Fig. 1f, h, i), URMC-099 treatment resulted in significant reduction in p38 and JNK

phosphorylation in the Aβ-stimulated microglia. Here, decreases were seen at 21.5, 17.3, and 23.7 % in p38, p46-JNK, and p54-JNK, respectively (Fig. 1f, h, i). To assess the effects of URMC-099 on Aβ42-mediated microglial cytotoxicity, we measured cell viability using the MTT assay. Aβ42-treatment reduced cell viability (Additional file 2: Figure S2) as observed previously [31]. Although URMC-099 treatment did not ameliorate Aβ42-mediated cytotoxicity, trends in protection of cell viability were observed with 9.4 and 16.0 % increase in 3- and 48-h incubation, respectively, when compared to Aβ42 treatment (Additional file 2: Figure S2). These results support the notion that URMC-099 inhibits MLK3-MKK3/4-mediated activation of p38 and JNK MAPK cascades in Aβ-activated microglia.

URMC-099 inhibits activated microglial pro-inflammatory cytokines

URMC-099 exerts anti-inflammatory effects on microglia exposed to the HIV-1 Tat [28]. To determine whether URMC-099 has similar effects for β-amyloidosis, cultured murine microglia were treated with URMC-099 and exposed to soluble Aβ42 for 30 min. While Aβ42 stimulation induced IL-1β, IL-6, and TNF-α gene expression, URMC-099 reversed this induction (decreases of 84.8, 85.9, and 97.4 % to Aβ42 stimulation for IL-1β, IL-6, and TNF-α, respectively, Fig. 2a). The qPCR results were validated by measuring protein production of pro-



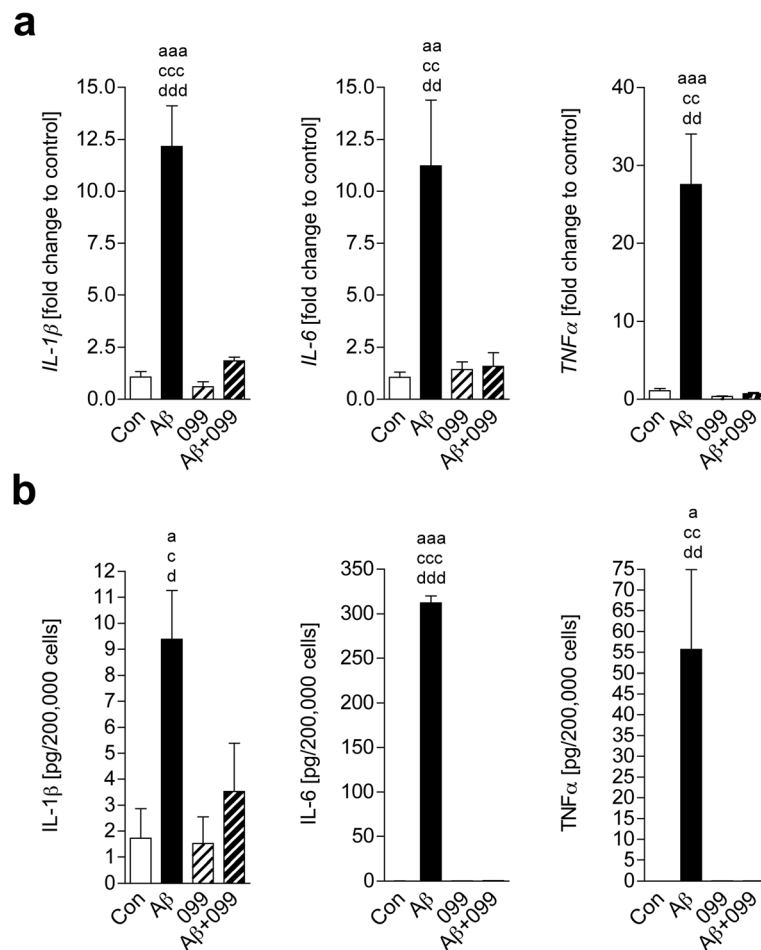


Fig. 2 URMC-099 inhibits A β -activated microglial pro-inflammatory cytokines. **a** A conventional RT2-qPCR was performed to measure IL-1 β , IL-6, or TNF- α expression using primer sets (Table 1) and synthesized cDNA with total RNA isolated from murine microglia ($n = 3$ per group). **b** Quantification of IL-1 β , IL-6, or TNF- α protein secretion from murine microglia ($n = 3$ per group). Data are presented as mean \pm SEM, ^{a,c,d} $p < 0.05$, ^{aa,cc,ddd} $p < 0.01$, ^{aaa} $p < 0.001$, ^avs control, ^cvs URMC-099, ^dvs A β + URMC-099, one-way ANOVA, Newman-Keuls post hoc test

inflammatory cytokines in media. After 48-h A β 42 stimulation, URMC-099 treatment inhibited IL-1 β , IL-6, and TNF- α (decreases of 62.3, 99.8, and 99.9 % to A β 42 stimulation for IL-1 β , IL-6, and TNF- α , respectively, Fig. 2b). These data strongly support the anti-inflammatory effects of URMC-099 on A β -stimulated microglia.

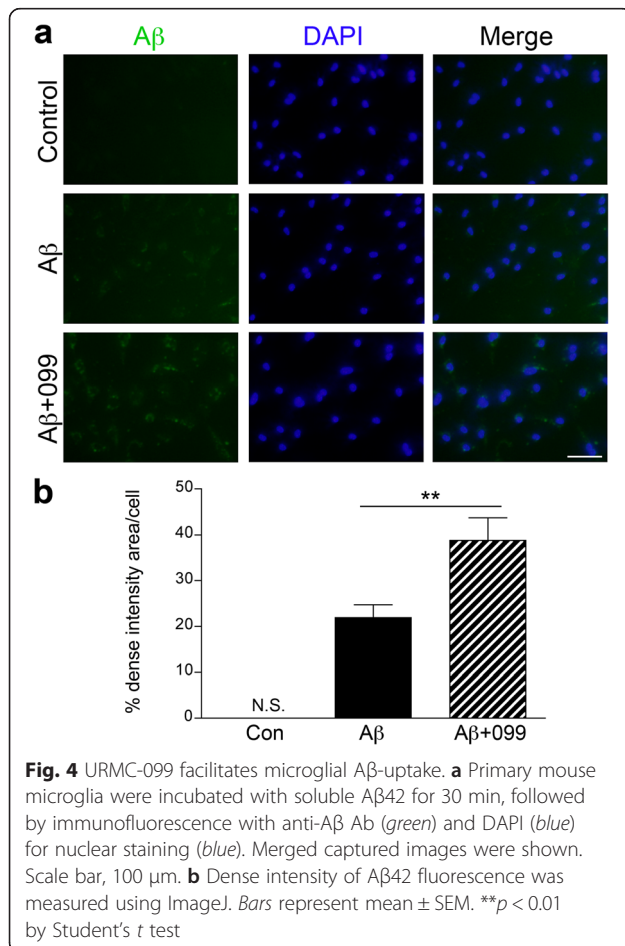
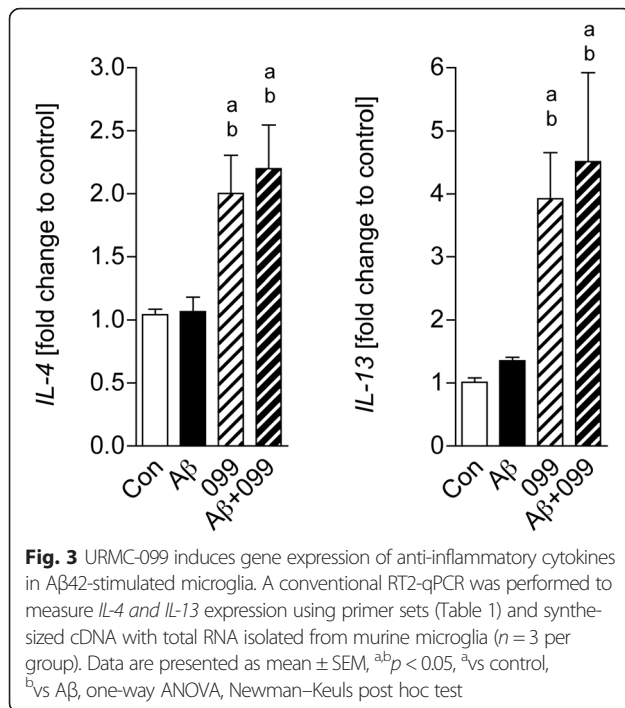
Anti-inflammatory effects of URMC-099 in A β 42-stimulated microglia

To determine the neuroinflammatory phenotype in URMC-099-treated microglia, total RNA was isolated and RT2-qPCR was performed for genes specific to IL-4 and IL-13. Data were shown as fold change compared to control (Fig. 3). While 30-min A β 42 stimulation did not alter gene expression of IL-4 and IL-13, URMC-099 significantly induced this expression pattern (increases of 111.1 and 345.7 % in IL-4 and IL-13, respectively, Fig. 3), further supporting the

anti-inflammatory effects of URMC-099 on A β -stimulated microglia.

URMC-099 facilitates microglial A β uptake

Since URMC-099 has anti-inflammatory effects in A β -stimulated microglia, we hypothesized that contemporaneous administration of URMC-099 to microglia could accelerate A β phagocytosis and subsequent degradation [41–44]. To this end, we tested if URMC-099 could facilitate A β 42 internalization. URMC-099-treated microglia were incubated with A β 42 for 30 min, then immunostained using pan-A β primary and Alexa 488-secondary antibodies (Fig. 4a). Quantification of immunofluorescent intensity showed that relative immunofluorescence was increased in URMC-099-treated microglia, as compared to A β 42 treatment only (76.9 % increase, Fig. 4b), suggesting that URMC-099 promotes A β phagocytosis.



URMC-099 alters scavenger receptor expression in microglia

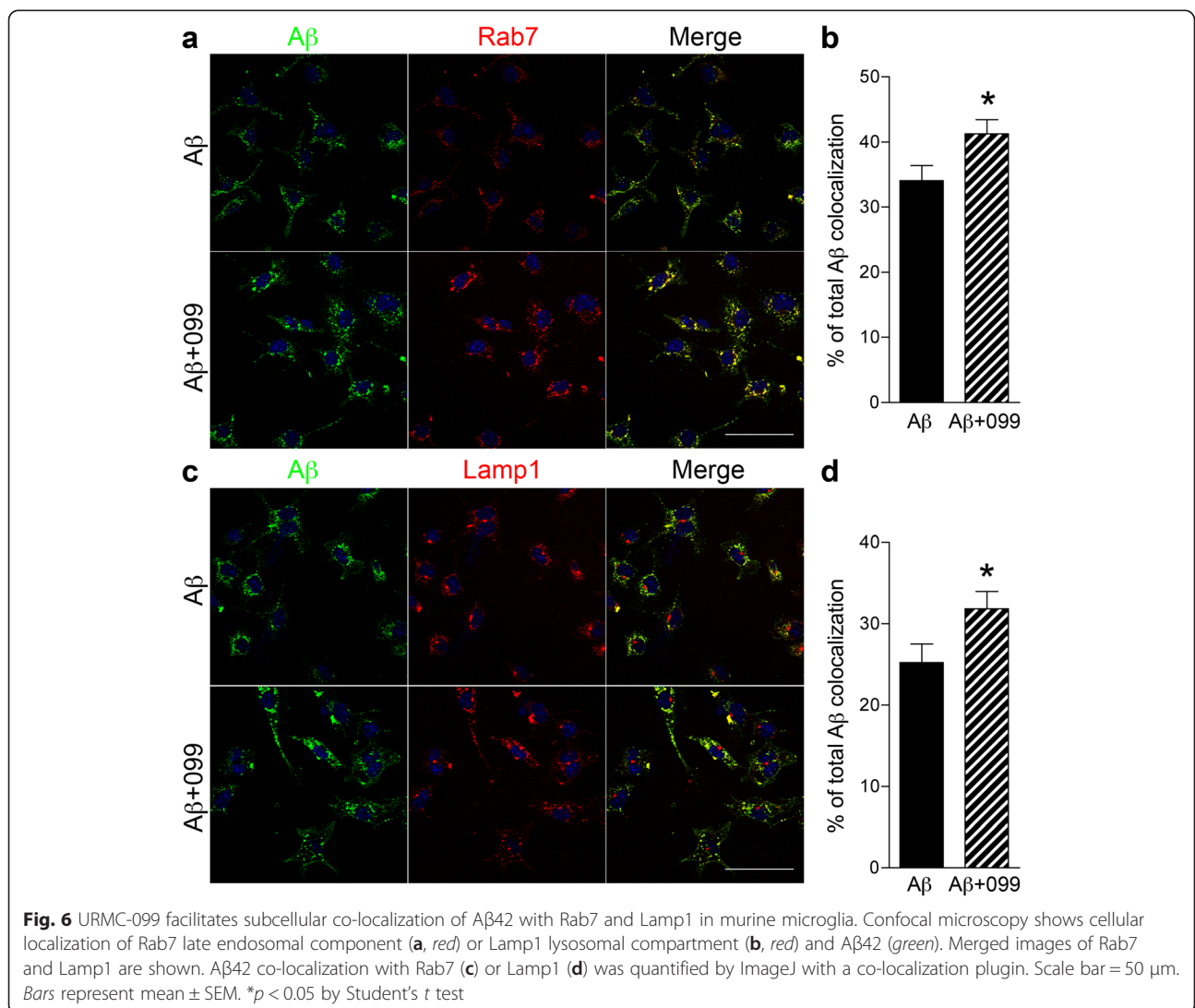
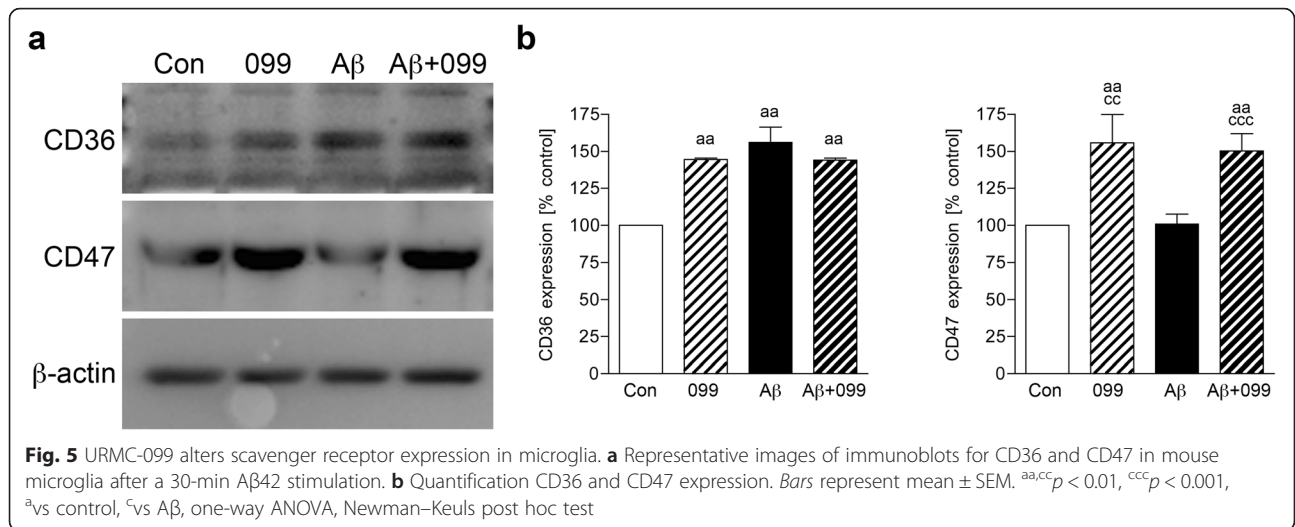
A previous study demonstrated that microglial phagocytic capacity is linked to scavenger receptor (SR) expression levels in microglia in AD mouse models [45]. To pursue the mechanism of how URM-099 facilitates Aβ phagocytosis, we examined the expression of SRs. CD36 and CD47 expression was assessed by immunoblotting (Fig. 5a). CD36 expression was increased in all treated groups (increases of 44.4, 56.1, and 44.1 % in URM-099 only, Aβ only, and Aβ plus URM-099 to control, respectively, Fig. 5b), while CD47 expression was significantly increased with URM-099 treatment (increases of 55.9 and 50.2 % in URM-099 only and Aβ plus URM-099 to control, respectively, Fig. 5b), demonstrating URM-099-specific alteration in SR expression.

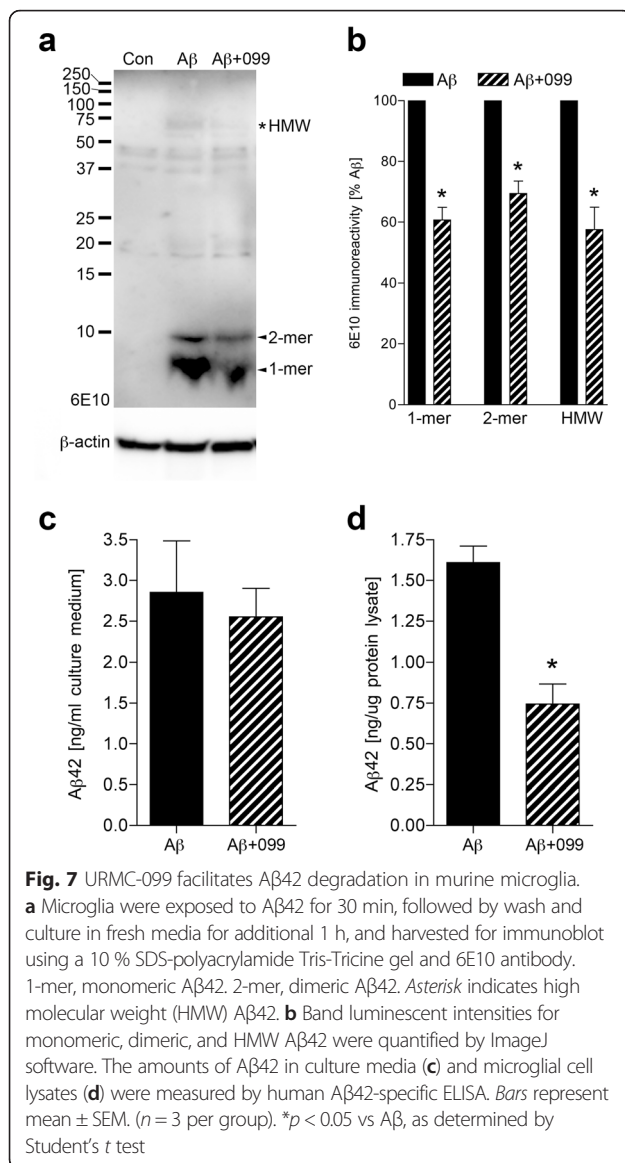
URMC-099 increases Aβ co-localization with Rab7 and Lamp1

In response to Aβ binding to SRs, microglia start to engulf Aβ by phagocytosis, and then Aβ enters into the endolysosomal pathway. Thus, we investigated how URM-099 affects the endolysosomal trafficking underlying Aβ phagocytosis. Microglia treated as described above were immunostained with antibodies to Rab7 (for late endosomes, Fig. 6a) and Lamp1 (for lysosomes, Fig. 6c) at 1-h post-incubation with Aβ. Confocal microscopy demonstrated that co-localization of Rab7 and Lamp1 with Aβ42 was increased in URM-099-treated microglia, compared to untreated microglia (21.2 and 26.3 % increases in Rab7 and Lamp1, respectively, Fig. 6c, d). To investigate Aβ metabolism, microglia were exposed to Aβ42 for 30 min, washed and cultured in fresh media for additional 1 h, then harvested for immunoblotting (Fig. 7a). Co-treatment with URM-099 significantly reduced immunoreactivity of monomeric, dimeric, and high molecular weight (HMW) Aβ42, as compared to Aβ42 treatment only (39.3, 30.6, and 42.3 % decreases in monomeric, dimeric, and HMW Aβ42, respectively, Fig. 7b). To validate these results, microglia were exposed to Aβ42 for 30 min, followed by wash and culture in fresh media for additional 1 h, and then Aβ42 release and retention in microglia were quantified using ELISA. While URM-099 had no effect on Aβ42 release (Fig. 7c), co-treatment with URM-099 significantly reduced Aβ42 with a 50.2 % reduction (Fig. 7d). These data suggest that URM-099 promotes microglial endolysosome-mediated degradation.

Discussion

Microglia are the resident immune cells that serve as the first line of immune defense against invading pathogens in the CNS. The immune response is accelerated by the cell's abilities to recognize pathogen-associated microbial





patterns, induce key co-stimulatory molecules, and secrete cytokines that facilitate induction of the adaptive immune response in disease [46–48]. A principal microglial function is to act as scavenger cells to first phagocytose then clear substances such as A β [48–50]. Notably, A β is considered to be the initiating factor in AD inducing neuroinflammation, subsequent synaptic and axonal injuries, tau hyperphosphorylation, and ultimately neuronal death [15–17, 51]. With these in mind, a number of therapeutic strategies have been developed for facilitating A β removal with the goal of improving disease outcomes. Indeed, anti-inflammatory drugs, amyloid degradation enzymes, and active/passive immunizations have been evaluated as treatment approaches [52–55]. In particular, NSAIDs and antioxidants are effective not only in

attenuating the neurotoxins secreted by A β -stimulated microglia but also in facilitating microglial phagocytic activity for A β clearance in animal models of human disease [1, 25, 26]. To date, there has been no clear validation of any of these approaches for disease-modifying outcomes in patients with AD, thus further studies for alternative disease-modifying strategies are warranted. Hence, we investigated whether URM-099 could ameliorate A β -mediated microglial activation.

URM-099 was discovered as a result of a large screening for MLK3 inhibitors structurally containing a pyrrolopyridine scaffold with an aryl piperazine side chain [28, 29]; it can modify the production of pro-inflammatory mediators by inhibiting MAPK signaling cascades [36–38]. MLKs are MAPK kinase kinases (MAPKKKs) that function as bonafide serine/threonine kinases, although their catalytic domains have features of both serine/threonine and tyrosine kinases. MLKs regulate the p38/JNK signaling cascades that coordinate and orchestrate numerous immune processes [40]. URM-099 is an inhibitor specific to MLK3 [28, 29] that is one of the most widely expressed members of the MLK family [56]. MLK3 is expressed in immune effector cells including microglia in the CNS and is activated by cellular and metabolic stress [28, 57–60]. Based on these findings, we posit that URM-099 is a potential candidate for AD therapeutics. Although it was previously unclear whether A β induces classical microglial activation through MLK-MAPK pathways, we showed that URM-099 reduces phosphorylation of MKK3/MKK4 and p38/JNK and partially protected A β -mediated microglial cytotoxicity, suggesting its therapeutic effect targeting MLK-MKK-MAPK pathways in A β -mediated neuroinflammation in microglia.

In AD, microglia contribute to tissue injury through eliciting neuroinflammation and changing the CNS microenvironment. Like peripheral macrophages, microglia can be polarized into M1 and M2 phenotypes based on their functional properties [25, 26, 61–63]. A β activation of microglia is associated with production of pro-inflammatory cytokines (IL-1 β /IL-6/TNF- α), traits associated closely with the M1 phenotype [7, 8, 11, 25, 47, 64]. Alternatively, polarization to an M2 phenotype can initiate an anti-inflammatory and repair phase [1, 25, 26]. The normal homeostatic balance between M1 and M2 phenotypes seems to be disturbed during disease progression in AD with the presence of more M1 microglia appearing coincident with aging and disease [26]. Herein, URM-099 treatment was shown to inhibit IL-1 β /IL-6/TNF- α expression and coordinate with the up-regulation of IL-4 and IL-13 genes. These data suggest that URM-099 treatment may initiate phenotypic changes by inducing anti-inflammatory cytokines, thus has neuroprotective activities [42–44].

Additionally, microglia in the M2 phase speed A β phagocytosis and degradation without neurotoxin production [41–44]. An increase in phagocytosis of A β was observed with URM-099 treatment. Microglial phagocytic capacity is linked to SR expression levels [45]. CD36 is one of the SRs for A β and regulates A β clearance as well as brain inflammation [65]. While A β exposure increased CD36 expression similar to a previous study with BV-2 microglia [66], co-administration of URM-099 with A β failed to reverse this up-regulation. In contrast, URM-099 increases CD47 expression in microglia both alone and with A β . Since CD47 is an integrin-associated transmembrane protein and participates in A β uptake and microglial pro-inflammatory responses by forming the receptor complex CD36/CD47/ α 6 β 1 integrin, which in turn stimulates microglial phagocytic activity [67–69]. By increasing CD47 activity, our results suggest that URM-099 facilitates CD36/CD47/ α 6 β 1-integrin-mediated microglial phagocytic activity of A β . Moreover, URM-099 facilitates colocalization of Rab7 and Lamp1 with A β 42, which may promote endolysosomal-mediated A β degradation and metabolism. Our data in aggregate suggests that these pathologic events can be altered with the challenge of how and when to optimally use disease-modifying agents such as URM-099 to restore homeostasis to the brain's microenvironment.

Conclusions

URM-099 inhibits A β -mediated phosphorylation of MKK3/4-p38/JNK and pro-inflammatory responses, and up-regulates phagolysosomal trafficking and degradation of A β . Such inhibition of phosphorylation by URM-099 correlates with inhibition of microglial activation. URM-099 serves as an immune modulatory and neuroprotective agent that may be developed to combat AD.

Additional files

Additional file 1: Figure S1. URM-099 treatment for 3, 24, or 48 h has no effect on microglial viability by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are presented as mean \pm SEM. (TIF 1588 kb)

Additional file 2: Figure S2. MTT assay of microglia treated with A β 42 and URM-099. Data are presented as mean \pm SEM. (TIF 613 kb)

Abbreviations

Ab, antibody; AD, Alzheimer's disease; ANOVA, analysis of variance; A β , amyloid- β ; BCA, bicinchoninic acid assay; CD, cluster of differentiation; cDNA, complementary deoxyribonucleic acid; CNS, central nervous system; DAPI, diamino-2-phenyl-indol; ELISA, enzyme-linked immunosorbent assay; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; IgG, immunoglobulin G; IL, interleukin; JNK, c-Jun amino-terminal kinase; Lamp1, lysosomal-associated membrane protein 1; MAPK, mitogen-activated protein kinase; MCSF, monocyte colony-stimulating factor; MKK, mitogen-activated protein kinase kinase; MLK, mixed-lineage kinase; NSAID, nonsteroidal anti-inflammatory drug; PCR, polymerase chain reaction;

PFA, paraformaldehyde; RIPA, radioimmunoprecipitation assay buffer; RNA, ribonucleic acid; SR, scavenger receptor; TBST, Tris-buffered saline containing 0.1 % Tween-20; TNF- α , tumor necrosis factor- α

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional file.

Authors' contributions

WD, CME, and YL performed the experiments and analyzed the data. SW and BD performed the experiments and assisted in the data analysis. HAG provided the URM-099 for study, provided experimental suggestions, and assisted in writing the manuscript. HEG supervised the research and wrote the manuscript. TK supervised, designed the research, analyzed the data, and wrote the manuscript. All authors discussed the results and conclusions, reviewed, and commented the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors with the exception of HAG and HEG declare that they have no competing interests. HAG and HEG are members of the scientific advisory board of WavoDyne Therapeutics, Inc., which holds the exclusive license for URM-099.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Housing, care, breeding of non-transgenic mice, and microglial isolation were approved by the Institutional Animal Care and Use Committee at the University of Nebraska Medical Center.

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