

Effect of MALT1 inhibition by MI-2 on the microglial phenotype switch, inflammatory cytokine secretion, neuronal loss and oxidative stress in Alzheimer's disease

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Abstract. Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) is a key paracaspase enzyme regulating immune responses, inflammation and oxidative stress. The present study aimed to investigate the effect of MALT1 inhibition on neuroinflammation, neuronal loss and oxidative stress in Alzheimer's disease (AD). A co-culture system involving microglia and neuron cells under β -amyloid ($A\beta$) intervention was used to establish AD cellular models using human microglia HMC3 cells and neuroblastoma SH-SY5Y cells and mouse microglia BV-2 and hippocampal neuron HT-22 cells. The inhibition of MALT1 proteolytic activity was achieved by MALT1 inhibitor 2 (MI-2) treatment, and the NF- κ B pathway was activated by phorbol 12-myristate 13-acetate (PMA) treatment in HMC3 and BV-2 cells. Western blotting, ELISA, Cell Counting Kit-8, EdU staining, reactive oxygen species (ROS) detection and reduced glutathione (GSH) assays were performed to evaluate molecular changes, inflammatory responses, neuronal viability and oxidative stress. MALT1 expression was upregulated following $A\beta$ treatment in HMC3 and BV-2 cells. MALT1 inhibition by MI-2 suppressed the microglial M1 phenotype but enhanced the M2 phenotype, reduced the levels of the proinflammatory cytokines TNF- α

and IL-1 β and inactivated the NF- κ B pathway in HMC3 and BV-2 cells. Moreover, microglial MALT1 inhibition elevated cell viability (verified by Cell Counting Kit-8 and EdU assays), increased the level of reduced glutathione and decreased the levels of reactive oxygen species in SH-SY5Y and HT-22 cells. NF- κ B activation by PMA attenuated the effects of MALT1 inhibition on the microglial phenotype switch and proinflammatory cytokine secretion in HMC3 and BV-2 cells, as well as cell viability and oxidative stress in SH-SY5Y and HT-22 cells. The present study reveals that MALT1 inhibition may suppress microglial M1 phenotype, neuroinflammation, neuronal loss and oxidative stress by inactivating the NF- κ B pathway in AD.

Introduction

Alzheimer's disease (AD) is a common neurodegenerative disease characterized by the deposition of β -amyloid ($A\beta$) and hyperphosphorylated τ protein-induced neurofibrillary tangles, presenting with progressive cognitive dysfunction and memory loss (1,2). There were approximately 54.9 million individuals worldwide who are diagnosed Alzheimer's disease and other dementias in 2021 (3). Its pathogenesis primarily involves neuroinflammation, synaptic damage and neuronal loss, and there is no curative treatment at present (4,5). In an aim to improve the prognosis of patients with AD, in-depth investigations on the molecular mechanisms of AD and novel targets have attracted increasing attention (6,7).

Imbalance in microglial M1/M2 polarization serves as a key mechanism driving neuroinflammation and disease progression of AD (8-12). Upon pathological stimuli such as $A\beta$ plaques, microglia primarily polarize into either the classically activated pro-inflammatory M1 phenotype or the alternatively activated anti-inflammatory/repair-oriented M2

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phenotype (8). M1-type microglia release tumor necrosis factor- α (TNF- α), IL-1 β and nitric oxide (NO), thereby exacerbating neurotoxicity and τ pathology; conversely, M2-type microglia highly express arginase-1 (ARG1) and IL-10, enhance A β phagocytosis and clearance and secrete neurotrophic factors to exert neuroprotection (9,10). In early AD, the M2-type response predominates to clear pathological proteins, however, as the disease advances, microenvironmental changes drive microglial transformation toward the M1 phenotype, leading to chronic inflammation and neuronal injury (8,11). Thus, promoting microglial polarization from M1 toward M2 represents a promising therapeutic strategy to alleviate neuroinflammation in AD (12).

Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) is a paracaspase enzyme serving as a critical intracellular signaling molecule in both immune responses and inflammation (13,14). Its primary role involves mediating NF- κ B activation downstream of immune receptors, including the T and B cell receptor and Toll-like receptors, thereby regulating immune cell activation and promoting the expression of pro-inflammatory genes, such as TNF- α (14,15). MALT1 has been reported to be aberrantly expressed and to participate in the pathogenesis of various diseases, such as cancer, immune disease and infection (16-18). Accordingly, targeting MALT1 has been proposed as a potent treatment of diseases, although further validation is required (19,20).

In neurological disease, MALT1 is upregulated and is associated with higher levels of IFN- γ and IL-17 in patients who have had an acute stroke (21), and is one of the genes most commonly associated with the initiation and progression of multiple sclerosis (22). Moreover, the overexpression of MALT1 promotes NLRP3 inflammasome-mediated inflammation and neural impairment in diabetes (23) and the inhibition of MALT1 proteolytic activity by MALT1 inhibitor 2 (MI-2) mitigates spinal ischemia-reperfusion neural injury and neuroinflammation by regulating glial endoplasmic reticulum stress (24). MALT1 is able to promote myocardial oxidative stress in doxorubicin-induced mice (25), and its degradation suppresses reactive oxygen species (ROS) in the mitochondria of Jurkat cells (26). As MALT1 promotes NF- κ B pathway activation (14), and NF- κ B signaling serves a crucial role in regulating inflammatory responses and oxidative stress, MALT1 may contribute to disease progression by modulating NF- κ B-mediated pathological processes (27). MALT1 may induce oxidative stress via the activation of NF- κ B pathway. A previous study validated this hypothesis, revealing that MALT1 facilitated oxidative stress, reflected by levels of NO, superoxide dismutase and malondialdehyde in sepsis (28). In addition, the analysis based on the public dataset GSE28146 reveals that MALT1 is upregulated in AD hippocampus compared with control tissues and public dataset GSE67835 reveals that MALT1 is abundantly expressed in microglia, followed by neurons, endothelial and oligodendrocyte precursor cells, and seldom expressed in other types of cell in human brain (29,30). Based on the involvement of MALT1 in neurological disease (21-24), its modulation of NF- κ B signaling and inflammation (13-15,31) and the association of NF- κ B and inflammation with AD (32,33), it was hypothesized that targeting MALT1 may attenuate AD microglial M1/M2 imbalance-mediated neuroinflammation,

neural injury and oxidative stress dependent of the NF- κ B pathway. Therefore, the present study aimed to investigate the effects of MALT1 inhibition on neuroinflammation, neuronal loss and oxidative stress based on *in vitro* AD models.

Materials and methods

Cells and culture. Human microglia HMC3 (cat. no. iCell-h301) and neuroblastoma SH-SY5Y (cat. no. iCell-h187) and mouse microglial BV-2 (cat. no. iCell-m011) and mouse hippocampal neuronal HT-22 (cat. no. iCell-m020) cells were obtained from iCell Bioscience Inc. HMC3 and SHSY5Y cells were maintained in MEM with Ham's F12 (MEM F12; Wuhan Servicebio Technology Co., Ltd.). BV-2 and HT-22 cells were grown in DMEM (Wuhan Servicebio Technology Co., Ltd.). All cells were maintained in medium containing 10% FBS (MilliporeSigma) and penicillin/streptomycin reagent (Wuhan Servicebio Technology Co., Ltd.) at 37°C and 5% CO₂. The authentication for SH-SY5Y cell line was performed by short tandem repeat profile.

Preparation of A β . Synthetic A β (25-35) peptide fragment (MedChemExpress) was dissolved in sterile distilled water to prepare a 1 mM stock solution. The peptide solution was incubated at 37°C for 7 days to allow aggregation prior to use. The aggregated peptide was aliquoted and stored at -20°C until use. For experiments, the stock solution was diluted in culture medium immediately before treatment.

Cell treatment. A co-culture system involving neuronal cells and conditioned medium (CM) derived from microglia was conducted to mimic AD pathology *in vitro* (Fig. 1) (34,35).

HMC3 and BV-2 cells were plated and divided into blank, A β and A β + MI-2 groups: The cells in the blank group were cultured without treatment; cells in the A β group were treated with 1 μ M A β ; cells in the A β + MI-2 group were treated with 1 μ M A β and 200 nM MI-2 (MALT1 inhibitor; MedChemExpress), simultaneously. Following 24 h treatment at 37°C, the supernatant was harvested as the CM and adopted for ELISA. The HMC3 and BV-2 cells were collected for western blot analysis. For co-culture, the SH-SY5Y and HT-22 cells were seeded and treated with the CM of HMC3 and BV-2 cells for 24 h at 37°C, respectively. The SH-SY5Y and HT-22 cells were collected for Cell Counting kit-8 (CCK-8), EdU, ROS and reduced glutathione (GSH) assay.

Phorbol 12-myristate 13-acetate (PMA, NF- κ B activator; MedChemExpress) was administered to explore whether the effects of MI-2 were mediated by the NF- κ B pathway. The HMC3 and BV-2 cells were assigned to A β , A β + MI-2, A β + PMA and A β + MI-2 + PMA groups: The cells in the A β group were treated with 1 μ M A β ; cells in the A β + MI-2 group were treated with 1 μ M A β and 200 nM MI-2, simultaneously; cells in the A β + PMA group were exposed to 1 μ M A β and 1 μ M PMA, simultaneously; cells in the A β + MI-2 + PMA group were treated with 1 μ M A β , 200 nM MI-2 and 1 μ M PMA, simultaneously. Following 24 h treatment at 37°C, the cells and CM were collected for further experiments.

The concentrations of A β , MI-2 and PMA were determined with reference to previous studies (36-38), together with preliminary dose-response experiments.

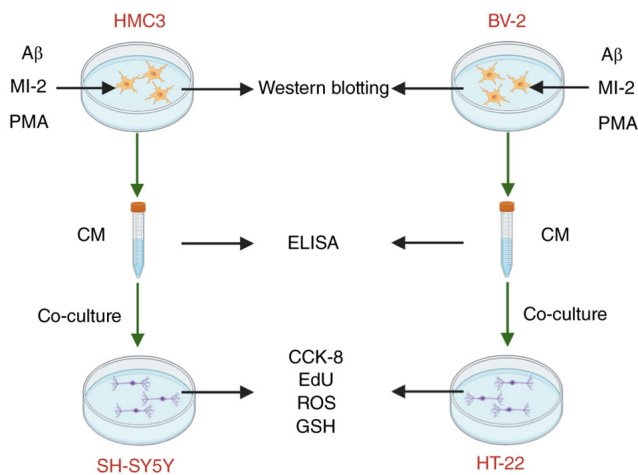


Figure 1. Co-culture system design. A β , amyloid- β ; MI-2, MALT1 inhibitor-2; PMA, phorbol 12-myristate 13-acetate; CM, culture medium; CCK, Cell Counting Kit; ROS, reactive oxygen species; GSH, glutathione.

Small interfering RNA (siRNA)-mediated MALT1 knockdown.

The HMC3 and BV-2 cells were transfected with 50 pmol siRNA targeting MALT1 or negative control siRNA (siNC) using siRNA mate plus transfection reagent (all Shanghai GenePharma Co., Ltd.) according to the manufacturer's instructions at 37°C for 24 h. As HMC3 and BV-2 cells were of human and murine origin, respectively, species-specific siRNA sequences were required. The siMALT1 sequences for HMC3 cells were as follows: Forward), 5' GAUCAUCUAUAC AAGUAUATT 3' and anti-sense (reverse), 5' UAUACUUGU AUAGAUGAUCTT 3'. The siMALT1 sequences for BV-2 cells were sense (forward), 5' GCAGCAUGUUGUUGUAC ATT 3' and reverse), 5' UGUACAACAACAUGCUGCTT 3'. siNC sequences for both cell lines were as follows: 5' UUC UCCGAACGUGUCACGUTT 3' (forward) and 5' ACGUGA CACGUUCGGAGAATT 3' (reverse).

Following incubation for 24 h at 37°C, the cells were collected and divided into four groups: The cells in the blank group received neither siRNA transfection nor A β treatment; cells in the A β group were exposed to 1 μ M A β without siRNA transfection; cells in the A β + siNC group were transfected with siNC and treated with 1 μ M A β ; cells in the A β + siMALT1 group were transfected with siMALT1 and treated with 1 μ M A β . Following 24 h treatment at 37°C, the supernatant was harvested for ELISA and the cells were collected for western blot analysis.

Western blot analysis. The HMC3 and BV-2 cells were washed and lysed using the RIPA Lysis kit (Wuhan Servicebio Technology Co., Ltd.) for 30 min. Protein was collected via centrifugation (10,000 $\times$ g) at 4°C for 15 min and detected using the Protein Quantification kit (Wuhan Servicebio Technology Co., Ltd.). Equal amounts of protein (30 μ g/lane) were separated by 4–20% electrophoresis and transferred onto PVDF membranes (Wuhan Servicebio Technology Co., Ltd.). Membranes were blocked via 5% non-fat milk (Wuhan Servicebio Technology Co., Ltd.) for 1.5 h at room temperature and exposed to primary antibodies for 1 h at 37°C, including anti-MALT1 (1:500; cat no. 11660-1-AP), anti-cylindromatosis protein (CYLD)

(1:1,000, cat no. 11110-1-AP), anti-iNOS (both 1:1,000; cat no. 22226-1-AP), anti-ARG1 (1:5,000; cat no. 16001-1-AP; all Proteintech Group, Inc.), anti-phosphorylated (p-)p65 (cat no. GB15997), anti-p65 (both 1:1,000; both Wuhan Servicebio Technology Co., Ltd.) (cat no. GB153882) and anti-GAPDH (1:10,000; cat no. AF7021; Affinity Biosciences). The HRP conjugated secondary antibodies (1:10,000; cat no. GB23303; Wuhan Servicebio Technology Co., Ltd.) were incubated for 0.5 h at room temperature. The blots were visualized with Super Sensitive ECL Luminescence kit (Meilunbio). Band intensities were quantified using ImageJ software (Ver1.8; National Institutes of Health). The integrated density of each target protein band was normalized to GAPDH.

ELISA. The CM of HMC3 and BV-2 cells was collected and centrifuged (10,000 $\times$ g) at 4°C for 15 min. The supernatant was employed for ELISA detection using the human and mouse TNF- α (cat no. ml106471 for human; cat no. ml002095 for mouse) and IL-1 β ELISA kits (cat no. ml058059 for human; cat no. ml106733 for mouse; Shanghai Enzyme-linked Biotechnology Co., Ltd. according to the manufacturer's protocols. In brief, the test samples and standard samples were added to the pre-coated plates for 0.5 h. The plates were then exposed to enzyme-labeled reagent for 0.5 h. Following washing, chromogenic solutions were adopted for 15 min. The stop solution was used and the optical density (OD) values at 450 nm were read using a microplate reader. Finally, the concentrations of TNF- α and IL-1 β were calculated using the OD values according to standard curves.

A β , MI-2, and PMA direct treatment of the HT-22 and SH-SY5Y cells. The HT22 and SH-SY5Y cells were seeded and divided into blank, A β , A β + MI-2 and A β + PMA groups: The cells in the blank group were cultured without treatment; cells in the A β group were treated with 1 μ M A β ; cells in the A β + MI-2 group were treated with 1 μ M A β and 200 nM MI-2; cells in the A β + PMA group were exposed to 1 μ M A β and 1 μ M PMA. Following 24 h treatment at 4°C, the cell viability, ROS and GSH levels were measured.

CCK-8 assay. The SH-SY5Y and HT-22 cells were harvested following co-culture and re-plated to 96-well plates (3 $\times$ 10³ cells/well). Following treatment for 24 h as aforementioned, the cells were stained with CCK-8 reagent (Wuhan Servicebio Technology Co., Ltd.) for 2 h. The OD values at 450 nm were measured via the microplate reader.

EdU staining. The SH-SY5Y and HT-22 cells were washed by PBS following co-culture, incubated with EdU incubation solution (Wuhan Servicebio Technology Co., Ltd.) for 2 h at 37°C, blocked with 4% paraformaldehyde at room temperature for 15 min and incubated with Click-iT solution (Wuhan Servicebio Technology Co., Ltd.) at room temperature for 30 min. DAPI (Beyotime Biotechnology) was employed to stain the nuclei. The fluorescent images were captured using an inverted fluorescence microscope (Motic Incorporation, Ltd.). The EdU positive rate was calculated as the number of EdU-positive cells divided by that of DAPI-positive cells.

ROS staining. The SH-SY5Y and HT-22 cells were washed by PBS following co-culture and 10 μ M DCFH-DA (Beyotime Biotechnology) was added for 20 min at 37°C. The cells were then washed with serum-free MEM F12 or DMEM (Wuhan Servicebio Technology Co., Ltd.). The fluorescent images were captured using an inverted fluorescence microscope (Motic Incorporation, Ltd.) under identical acquisition settings, and the fluorescence intensity values were assessed using ImageJ software (Ver1.8; National Institutes of Health). The relative ROS levels were calculated as the fluorescence intensity of experimental groups divided by that of the blank (MI-2 treatment) or A β group (PMA treatment).

GSH. The GSH levels were determined using the GSH Assay kit (cat. No. E-BC-K030-M; Elabscience Bionovation Inc.). Briefly, the SH-SY5Y and HT-22 cells were collected and 1×10^5 cells were lysed in PBS (Wuhan Servicebio Technology Co., Ltd.). Total GSH detection reagent was added for 25 min at 25°C and OD values were read at 412 nm. For oxidized GSH (GSSG) detection, GSH scavenger auxiliary reagents were added before the reaction with total GSH detection reagent at 25°C for 60 min. GSH levels were determined via the formula: GSH = total GSH - 2 $\times$ GSSG. The relative GSH levels were calculated as the GSH concentrations of experimental groups divided by that of the blank (MI-2 treatment) or A β group (PMA treatment).

Statistical analysis. The experiments were performed with three independent biological replicates. Data distribution normality was assessed using the Shapiro-Wilk test and homogeneity of variances was evaluated using the Brown-Forsythe test. Data are presented as the mean $\pm$ SD. One-way ANOVA followed by Tukey's test was conducted to compare differences using GraphPad 9 software (Dotmatics). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

MALT1 inhibition by MI-2 regulates the microglial phenotype, neuroinflammation and NF- κ B pathway. MALT1 expression was higher in the A β group compared with the blank group [fold-change (FC)=2.3, $P < 0.01$ in the HMC3 cells and FC=2.1, $P < 0.05$ in the BV-2 cells], however, there was no significant difference between the A β + MI-2 and A β groups ($P > 0.05$ in both the HMC3 and BV-2 cells); conversely, CYLD expression was lower in the A β compared with the blank group (FC=0.5, $P < 0.01$ in the HMC3 cells and FC=0.4, $P < 0.05$ in the BV-2 cells), but higher in the A β + MI-2 vs. the A β group (FC=3.1, $P < 0.001$ in the HMC3 cells and FC=4.6, $P < 0.01$ in the BV-2 cells; Fig. 2A), indicating that MALT1 expression was elevated in microglia of AD cell models and MI-2 treatment suppressed MALT1 proteinase activity.

iNOS expression was increased in the A β compared with the blank group (FC=2.6, $P < 0.01$ in the HMC3 and FC=2.0, $P < 0.001$ in the BV-2 cells), but decreased in the A β + MI-2 group compared with the A β group (FC=0.7, $P < 0.05$ in both the HMC3 and BV-2 cells); moreover, ARG1 expression was decreased in the A β group compared with the blank group (both FC=0.3, $P < 0.01$) in the HMC3 and BV-2 cells; no significant difference was observed between the A β + MI-2 and A β

group in the HMC3 cells ($P > 0.05$) and ARG1 expression was elevated in the A β + MI-2 compared with A β group in the BV-2 cells (FC=2.1, $P < 0.05$; Fig. 2B); these findings suggested that MALT1 inhibition suppressed the microglial M1 phenotype and might enhance the M2 phenotype in AD cell models.

The TNF- α expression was higher in the A β group compared with the blank group (FC=2.2, $P < 0.01$ in the HMC3 and FC=3.3, $P < 0.001$ in the BV-2 cells), whereas it was lower in the A β + MI-2 compared with the A β group (FC=0.6 in the HMC3 and 0.7 in the BV-2 cells; both $P < 0.05$). IL-1 β expression was elevated in the A β group vs. the blank group in the HMC3 and BV-2 cells (FC=2.1, $P < 0.05$ in the HMC3 and FC=2.7, $P < 0.001$ in BV-2 cells), while it was only decreased in the A β + MI-2 group vs. the A β group in BV-2 cells (FC=0.7, $P < 0.01$), with no significant difference in the HMC3 cells (FC=0.7, $P > 0.05$; Fig. 2C), suggesting that MALT1 inhibition suppressed neuroinflammation in AD cell models.

p-p65 expression was elevated in the A β compared with the blank group (FC=2.5 in the HMC3 and FC=3.7 in the BV-2 cells; both $P < 0.01$), but was reduced in the A β + MI-2 group compared with the A β group (FC=0.5, $P < 0.05$ in the HMC3 cells, and FC=0.4, $P < 0.01$ in the BV-2 cells; Fig. 3A and B), indicating that MALT1 inhibition blocked the NF- κ B pathway in AD cell models.

Microglial MALT1 inhibition by MI-2 regulates neuronal viability and oxidative stress. Cell viability was determined by both the CCK-8 assay OD value and EdU-positive cells; these were lower in the A β compared with the blank group in the SH-SY5Y and HT-22 cells (CCK-8 assay: FC=0.8, $P < 0.05$ in the SH-SY5Y cells, and FC=0.7, $P < 0.01$ in the HT-22 cells; EdU assay: both FC=0.5, $P < 0.01$), however, these values were higher in the A β + MI-2 group vs. the A β group in the HT-22 cells (CCK-8 assay: FC=1.2; EdU assay: FC=1.6; both $P < 0.05$), but were not altered in the SH-SY5Y cells (CCK-8 assay: FC=1.1; EdU assay: FC=1.5; both $P > 0.05$; Fig. 4A and B). These findings suggested that microglial MALT1 inhibition may alleviate neuronal loss in AD cell models.

The ROS levels increased in the A β group compared with the blank group (FC=5.5, $P < 0.001$ in the SH-SY5Y and FC=4.0, $P < 0.01$ in the HT-22 cells), but decreased in the A β + MI-2 group compared with the A β group (both FC=0.4, $P < 0.01$; Fig. 4C); by contrast, the GSH level decreased in the A β compared with the blank group (FC=0.46, $P < 0.01$ in the SH-SY5Y and FC=0.71, $P < 0.05$ in HT-22 cells), but increased in the A β + MI-2 group compared with the A β group (FC=1.90, $P < 0.05$ in the SH-SY5Y cells, and FC=1.30, $P < 0.05$ in the HT-22 cells; Fig. 4D), suggesting that microglial MALT1 inhibition relieved neuronal oxidative stress in AD cell models.

NF- κ B activation by PMA attenuates the effects of MALT1 inhibition in microglia. p-p65 expression was elevated in the A β + PMA compared with the A β group (FC=2.8 in the HMC3 and FC=2.5 in the BV-2 cells; both $P < 0.001$), and was increased in the A β + MI-2 + PMA compared with the A β + MI-2 group (FC=5.3, $P < 0.001$ in the HMC3 cells, and FC=5.1, $P < 0.01$ in the BV-2 cells; Fig. 5A and B), indicating that PMA effectively activated the NF- κ B pathway and attenuated the effects of MALT1 inhibition on the NF- κ B pathway in AD cell models.

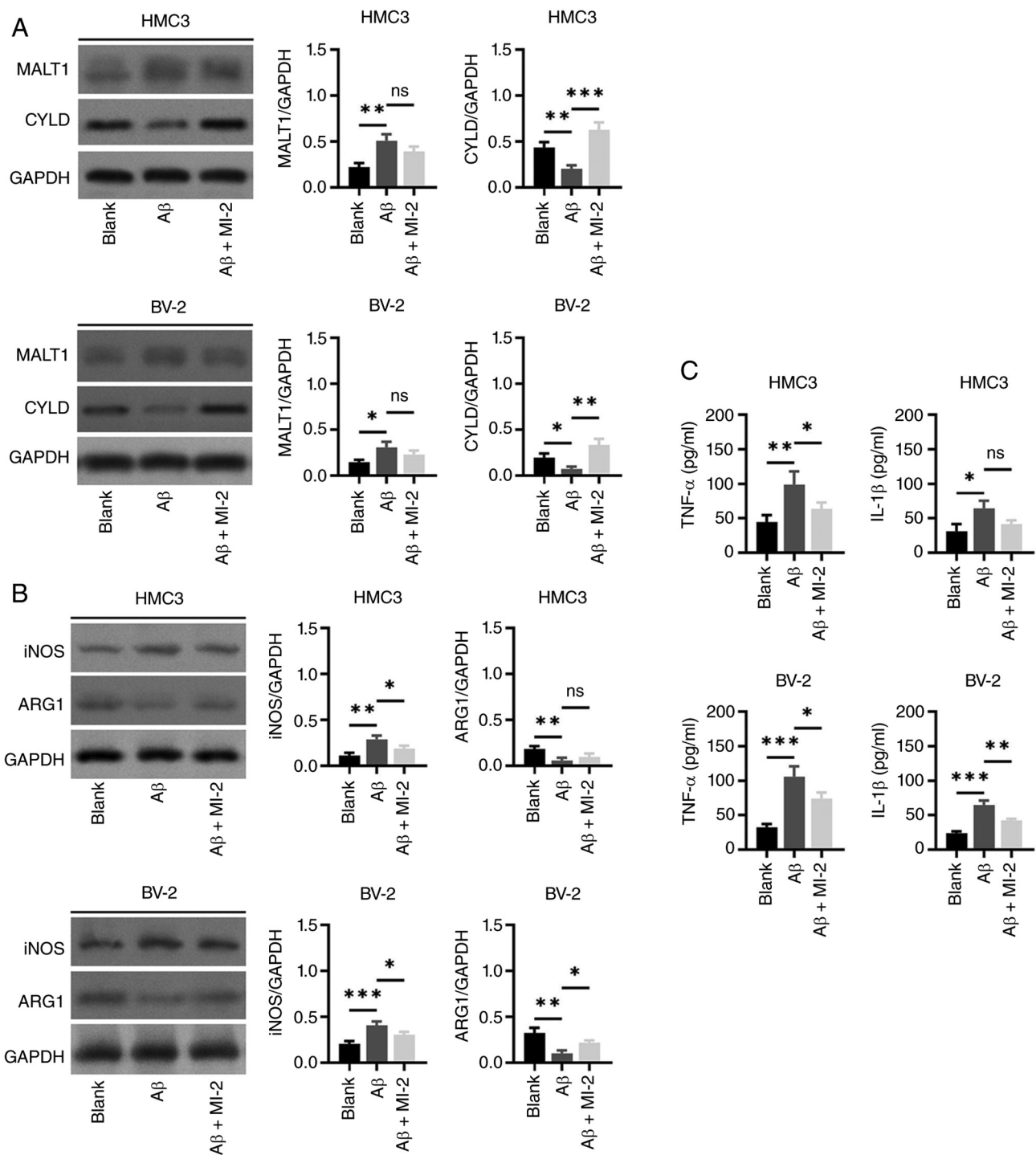


Figure 2. Effect of MALT1 inhibition on the microglial phenotype switch and inflammation. Expression levels of (A) MALT1 and CYLD, (B) microglial M1 phenotype marker iNOS and the M2 phenotype marker ARG1 and (C) inflammatory cytokines TNF- α and IL-1 β in HMC3 and BV-2 cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ns, not significant; MALT, mucosa-associated lymphoid tissue lymphoma translocation protein; CYLD, CYLD lysine 63 deubiquitinase; iNOS, inducible nitric oxide synthase; ARG, arginase; A β , amyloid- β ; MI-2, MALT1 inhibitor-2.

iNOS expression was higher in the A β + PMA compared with the A β group (FC=1.6, $P < 0.05$ in the HMC3 and FC=1.6, $P < 0.01$ in the BV-2 cells), and was also higher in the A β + MI-2 + PMA compared with the A β + MI-2 group (FC=3.0 in the HMC3 cells, and FC=1.9 in BV-2 cells; both $P < 0.05$); on the contrary, ARG1 expression was lower in the A β + PMA compared with the A β group (FC=0.3 in SH-SY5Y cells, and FC=0.4 in the HT-22 cells, both $P < 0.05$), and was also lower in the A β + MI-2 + PMA compared with the A β + MI-2 group

(both FC=0.5, both $P < 0.01$ in the HMC3 cells and BV-2 cells, Fig. 6A); these findings suggested that NF- κ B activation by PMA facilitated the microglial M1 phenotype, suppressed the M2 phenotype and attenuated the effects of MALT1 inhibition on the microglial phenotype in AD cell models.

The levels of TNF- α and IL-1 β were increased in the A β + PMA vs. the A β group (TNF- α : FC=2.3 in the HMC3 and FC=2.0 in the BV-2 cells, both $P < 0.001$; IL-1 β : FC=2.5, $P < 0.001$ in the HMC3 and FC=1.6, $P < 0.01$ in the BV-2 cells),

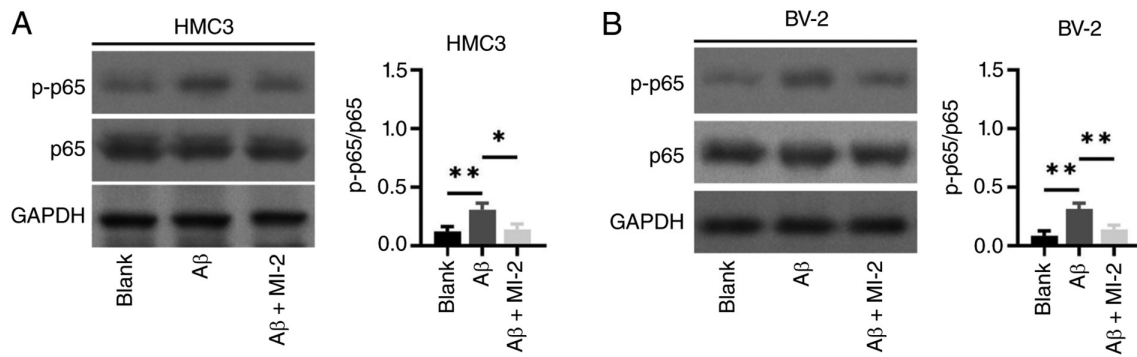


Figure 3. Effect of MALT1 inhibition on the NF- κ B pathway. Expression of p-p65 in (A) HMC3 and (B) BV-2 cells. * $P < 0.05$, ** $P < 0.01$. MALT, mucosa-associated lymphoid tissue lymphoma translocation protein; p-, phosphorylated; A β , amyloid- β ; MI-2, MALT1 inhibitor-2.

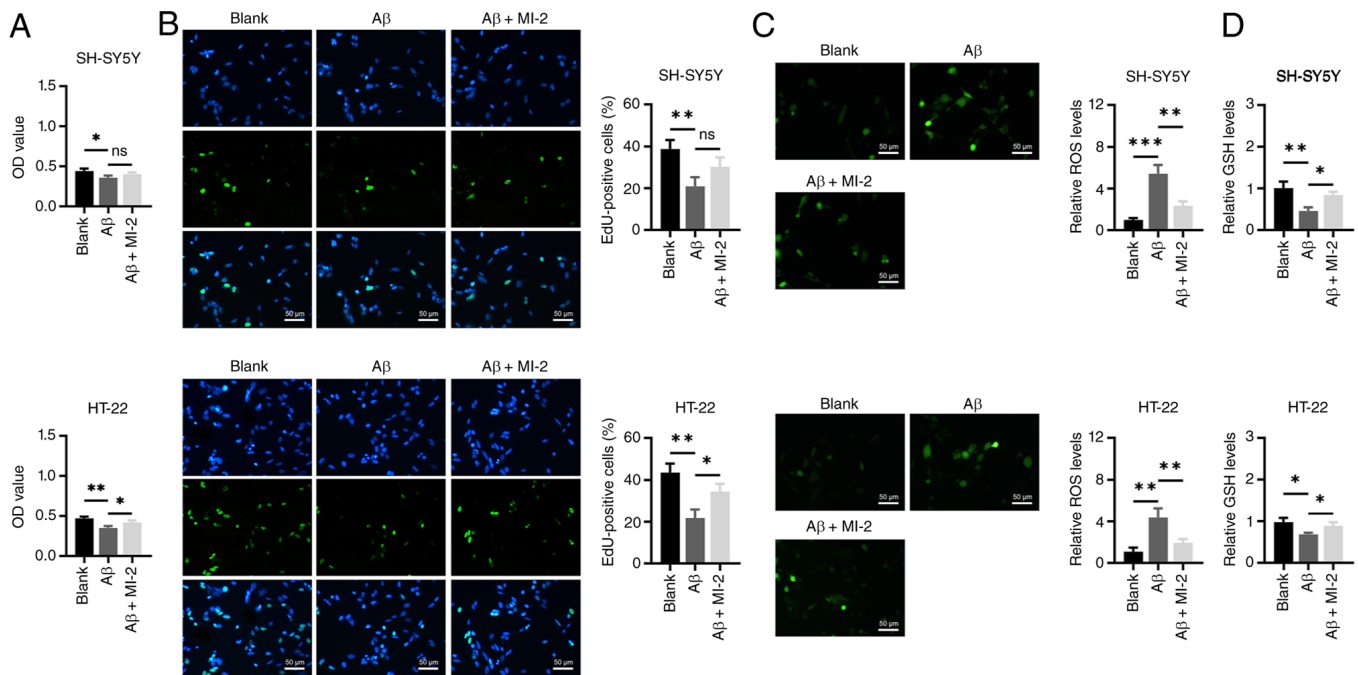


Figure 4. Effect of microglial MALT1 inhibition on neuronal viability and oxidative stress. (A) OD values obtained from Cell Counting Kit-8 assay. (B) EdU-positive cells. Relative (C) ROS and (D) GSH levels in SH-SY5Y and HT-22 cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ns, not significant; MALT, mucosa-associated lymphoid tissue lymphoma translocation protein; OD, optical density; ROS, reactive oxygen species; GSH, glutathione; A β , amyloid- β ; MI-2, MALT1 inhibitor-2.

which were also elevated in the A β + MI-2 + PMA vs. the A β + MI-2 group (TNF- α : FC=3.3, $P < 0.01$ in the HMC3 and FC=2.5, $P < 0.001$ in the BV-2 cells, IL-1 β : FC=3.1, $P < 0.001$ in the HMC3 and FC=2.0, $P < 0.01$ in the BV-2 cells; Fig. 6B), suggesting that the activation of NF- κ B by PMA promoted neuroinflammation and attenuated the effects of MALT1 inhibition on neuroinflammation in AD cell models.

Microglial NF- κ B activation by PMA attenuates the effects of microglial MALT1 inhibition on neuronal viability and oxidative stress. Cell viability was decreased in the A β + PMA group compared with the A β group (CCK-8 assay: FC=0.7, $P < 0.05$ in the SH-SY5Y cells, and FC=0.6, $P < 0.01$ in the HT-22 cells; EdU assay: both FC=0.4, both $P < 0.01$) and was decreased in the A β + MI-2 + PMA compared with the A β + MI-2 group (CCK-8 assay: FC=0.8, $P < 0.05$ in the SH-SY5Y and FC=0.7, $P < 0.01$ in the HT-22 cells, EdU assay:

FC=0.7, $P < 0.05$ in the SH-SY5Y and FC=0.6, $P < 0.01$ in the HT-22 cells; Fig. 7A and B), indicating that microglial NF- κ B activation by PMA induced neuronal loss and attenuated the effects of MALT1 inhibition on neuronal loss in AD cell models.

The ROS levels were increased in the A β + PMA compared with the A β group (FC=2.3, $P < 0.01$ in the SH-SY5Y and FC=1.5, $P < 0.05$ in the HT-22 cells) and in the A β + MI-2 + PMA compared with the A β + MI-2 group (FC=4.8, $P < 0.001$ in the SH-SY5Y cells, and FC=1.7, $P < 0.05$ in the HT-22 cells; Fig. 7C); conversely, the GSH levels were decreased in the A β + PMA compared with the A β group (FC=0.5 in SH-SY5Y cells, and FC=0.6 in the HT-22 cells, both $P < 0.05$) and in the A β + MI-2 + PMA compared with the A β + MI-2 group (FC=0.5 in the SH-SY5Y cells, and FC=0.6 in the HT-22 cells, both $P < 0.01$, Fig. 7D), implying that microglial NF- κ B activation by PMA induced neuronal oxidative stress and attenuated the

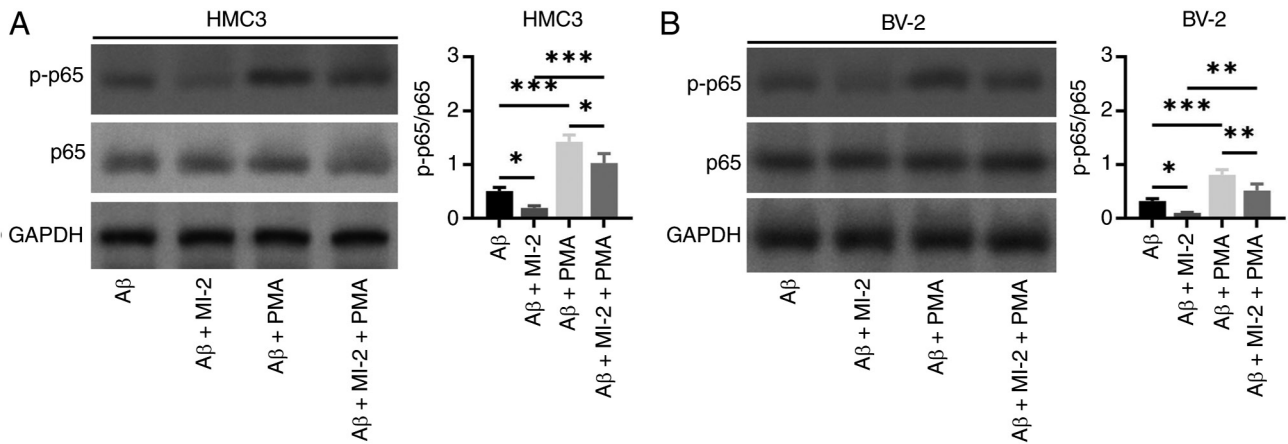


Figure 5. Effect of PMA on the NF- κ B pathway. Expression of p-p65 in (A) HMC3 cells and (B) BV-2 cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. PMA, phorbol 12-myristate 13-acetate; p-, phosphorylated; A β , amyloid- β ; MI-2, MALT1 inhibitor-2.

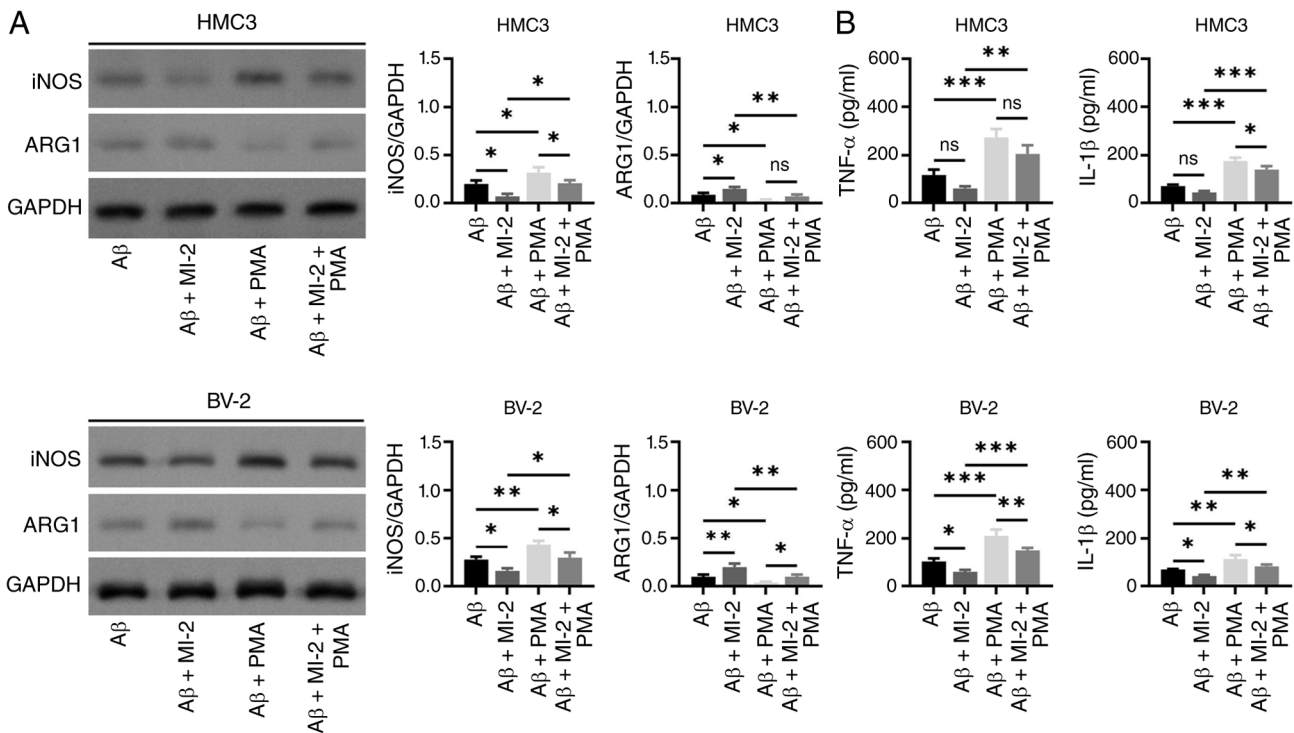


Figure 6. Effect of NF- κ B activation on the microglial phenotype switch and inflammation. Expression levels of (A) microglial M1 phenotype marker iNOS and the M2 phenotype marker ARG1 and (B) inflammatory cytokines TNF- α and IL-1 β in HMC3 and BV-2 cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ns, not significant; iNOS, inducible nitric oxide synthase; ARG, arginase; A β , amyloid- β ; MI-2, MALT1 inhibitor-2.

effects of MALT1 inhibition on neuronal oxidative stress in AD cell models.

Effect of direct treatment of MI-2 and PMA on neuronal viability and oxidative stress. Direct effects of MI-2 and PMA treatments on the SH-SY5Y and HT-22 cells, without CM or microglia, were determined. A β direct treatment decreased viability (FC=0.9, $P < 0.01$ in the SH-SY5Y and FC=0.9, $P < 0.05$ in the HT-22 cells) and elevated oxidative stress (reflected by increased ROS and decreased GSH levels (ROS level: FC=4.0 in the SH-SY5Y cells, and FC=3.0 in the HT-22 cells, both $P < 0.01$; GSH: FC=0.8, $P < 0.05$ in the SH-SY5Y cells, and FC=0.7, $P < 0.01$ in the HT-22 cells), however, neither MI-2 nor

PMA direct treatment altered viability and oxidative stress in the A β -stimulated SH-SY5Y and HT-22 cells ($P > 0.05$; Fig. S1A-C). In summary, MI-2 and PMA treatments affected the SH-SY5Y and HT-22 cell viability and oxidative stress via microglia indirectly.

MALT1 knockdown by siMALT1 modifies the microglial phenotype, neuroinflammation and NF- κ B pathway. Considering the specificity of MI-2 and potential off-target risk, siMALT1 was used to knockdown MALT1 for validation. MALT1 expression was effectively knocked down following siMALT1 transfection in both the HMC3 and BV-2 cells (both FC=0.2, $P < 0.001$; Fig. S2).

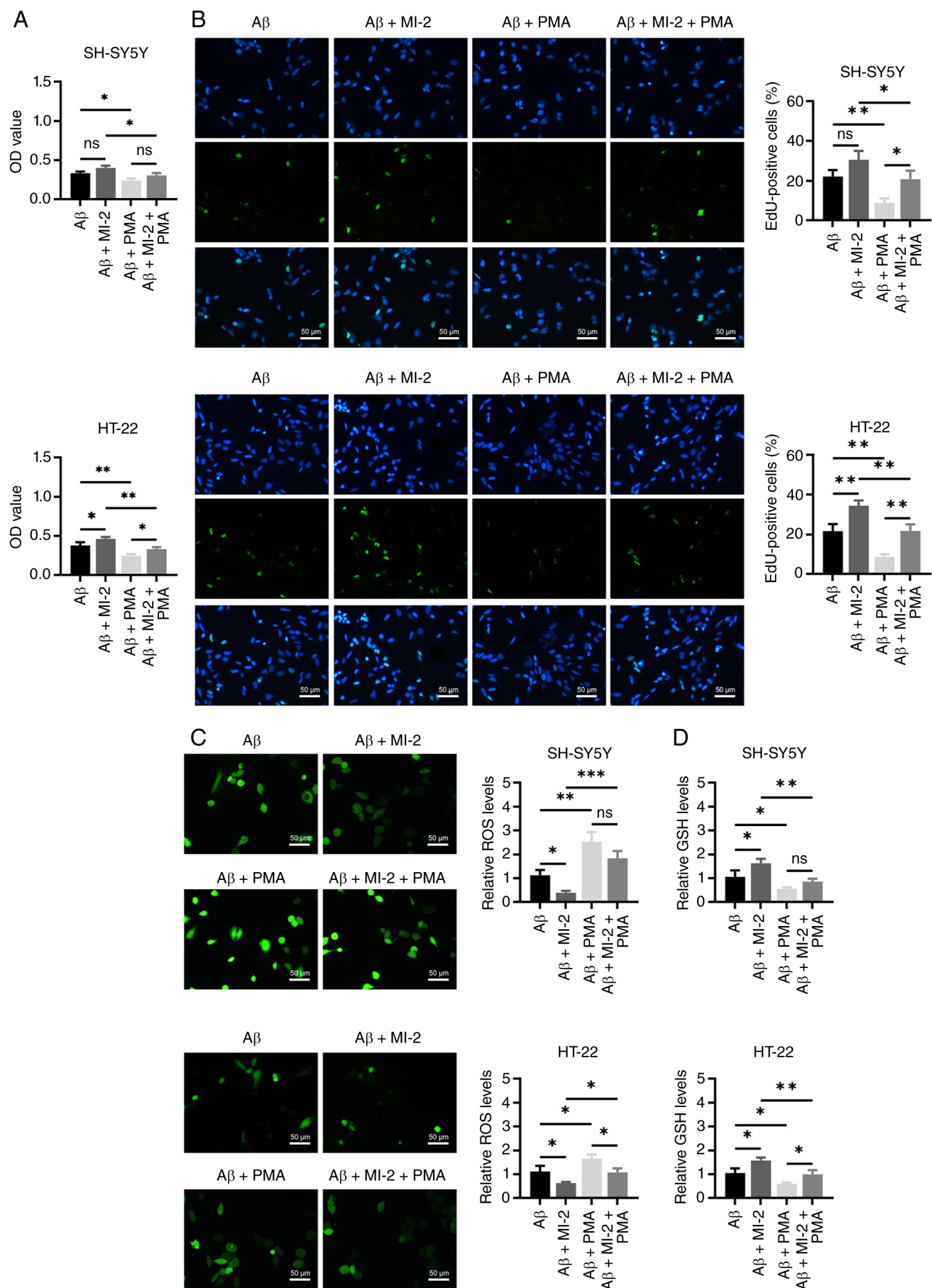


Figure 7. Effect of microglial NF- κ B activation on neuronal viability and oxidative stress. (A) OD values obtained from Cell Counting Kit-8 assay. (B) EdU-positive cells. Relative (C) ROS and (D) GSH levels in SH-SY5Y and HT-22 cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; OD, optical density; ROS, reactive oxygen species; GSH, glutathione; A β , amyloid- β ; MI-2, MALT1 inhibitor-2; PMA, phorbol 12-myristate 13-acetate; ns, not significant.

siMALT1 downregulated MALT1 expression (FC=0.2 in the HMC3 cells, and FC=0.1 in the BV-2 cells; both $P<0.001$) and iNOS expression (FC=0.5, $P<0.01$ in the HMC3 and FC=0.6, $P<0.05$ in the BV-2 cells), while upregulating ARG1 expression (FC=1.9 in the HMC3 cells, and FC=1.7 in the BV-2 cells; both $P<0.05$) in the A β -stimulated HMC3 and BV-2 cells (Fig. S3A). Moreover, siMALT1 decreased the levels of TNF- α (both FC=0.7, $P<0.05$ in the HMC3 and BV-2 cells) and IL-1 β (FC=0.7, $P<0.05$ in the HMC3 and FC=0.6, $P<0.01$ in the BV-2 cells) and decreased p-p65 expression (FC=0.4, $P<0.01$ in the HMC3 cells, and FC=0.5, $P<0.05$ in the BV-2 cells) in the A β -stimulated HMC3 and BV-2 cells (Fig. S3B and C).

Discussion

As a key paracaspase enzyme regulating immune responses and inflammation (13-15), MALT1 is dysregulated in immune- and inflammation-associated diseases (28,39,40). Notably, MALT1 is dysregulated in neurological disease (21,22). However, the expression of MALT1 in AD remains unclear. The present study demonstrated that MALT1 was upregulated in the microglia of AD cell models, indicating its key role in the development of AD. MALT1 regulates immunity and inflammation, which are key contributors to the pathogenesis of AD, therefore, MALT1 was upregulated in AD cellular models.

Targeting MALT1 through allosteric modulation has emerged as the dominant strategy for suppressing its proteolytic activity, with recent medicinal chemistry efforts primarily directed toward optimizing compounds that bind and disrupt function at regulatory sites distant from the catalytic center (20). Pharmacological strategies targeting MALT1 are broadly categorized by their binding modalities: Irreversible covalent inhibitors and reversible non-covalent inhibitors (37,41-43). The prototypical covalent inhibitors MI-2 and JNJ-67856633 exemplify the former class (37,41,42), whereas MLT-827 represents the latter approach through its non-covalent allosteric binding mechanism (43). The first-developed selective MALT1 inhibitor, MI-2 establishes a covalent adduct with the nucleophilic Cys464 residue within the protease catalytic triad, which has been demonstrated to effectively inhibit MALT1 proteolytic activity and achieve a sustained target engagement by previous studies (37,44,45). Therefore, the present study used MI-2 for the inhibition of MALT1 and detected CYLD expression to validate the suppressed MALT1 proteolytic activity; MI-2 effectively inhibited MALT1 proteolytic activity in AD cell models.

MALT1 is known for its modification of the immune cell phenotype and inflammation in multiple diseases, such as sepsis, experimental colitis, ankylosing spondylitis and spinal cord injury (28,46-48). However, its role in the modification of the microglia and the progression of AD remains unclear. The present study found that the inhibition of MALT1 by MI-2 suppressed the microglial M1 phenotype and neuroinflammation, whereas it enhanced the microglial M2 phenotype in AD cell models. MALT1 promotes microglial differentiation towards the pro-inflammatory type (M1 phenotype) due to its role in regulating other immune cells, such as macrophages and CD4⁺ T cells (28,46,48); moreover, MALT1 activates the NF- κ B pathway, which is key for the

microglial M1 phenotype switch (14,49), therefore, MALT1 inhibition suppresses the microglial M1 phenotype, whereas it enhances the M2 phenotype in AD cell models. In addition, the microglial M1 phenotype is associated with exacerbated inflammatory cytokine secretion and MALT1 directly modifies several inflammatory pathways, such as NF- κ B, JNK and mTOR (14,23,50), which facilitates inflammation; therefore, MALT1 inhibition suppresses neuroinflammation in AD cell models. Here, inhibition of MALT1 by MI-2 only increased ARG1 expression (the M2 phenotype marker) in the BV-2 cells, but did not alter its expression in the HMC3 cells. This may be due to the differences in response between human and mouse cell lines, or because MI-2 in human cells affects other pathways that support the M2 phenotype besides ARG1. The same phenomenon was observed following MALT1 inhibition of IL-1 β levels in BV-2 cells and HMC3 cells, which might result from the differences in response between human and mouse cell lines.

Dysregulated microglial M1 phenotype polarization and neuroinflammation exacerbate neuronal loss and oxidative stress in AD (51,52). Under AD pathological conditions, the accumulation of A β plaque and hyperphosphorylated τ proteins drive persistent microglial activation toward a pro-inflammatory M1 phenotype, which results in the excessive release of pro-inflammatory cytokines (IL-1 β and TNF- α) and ROS, contributing to synaptic dysfunction and neuronal apoptosis; concurrently, microglial M1 phenotype polarization suppresses the neuroprotective functions of the anti-inflammatory M2 phenotype, including the diminished secretion of neurotrophic factors, further compromising neuronal survival (51). Additionally, neuroinflammation amplifies oxidative stress via NADPH oxidase activation and other pathways, inducing mitochondrial dysfunction and lipid peroxidation, that establishes a self-perpetuating neuroinflammation-oxidative stress loop, ultimately driving widespread neuronal loss (52). The present study further detected the effects of microglial MALT1 inhibition by MI-2 on neuronal viability and oxidative stress, which revealed that microglial MALT1 inhibition attenuated neuronal loss and oxidative stress in AD cell models. The potential reason for this is that MALT1 inhibition suppressed the microglial M1 phenotype and neuroinflammation, and improved neuronal loss and oxidative stress during AD (51,52).

As the resident innate immune cells of the central nervous system, microglia exhibit M1 polarization that is associated with the activation of the NF- κ B pathway (53). Upon stimulation, NF- κ B upregulates pro-inflammatory cytokines, such as TNF- α , IL-1 β , IL-6 and iNOS, while suppressing anti-inflammatory cytokines, such as IL-10 and TGF- β , thereby establishing a self-perpetuating inflammatory loop that exacerbates neuroinflammation during the development and progression of AD (54,55). A β oligomers drive microglial polarization toward the M1 phenotype via the NLRP3/NF- κ B signaling pathway, contributing to synaptic dysfunction and neuronal apoptosis; conversely, NF- κ B inhibition shifts microglial polarization toward the M2 phenotype, characterized by the increased expression of ARG1 and chitinase-like protein 3 and enhances A β phagocytic clearance (56-58). Considering the key function of MALT1 in regulating NF- κ B (13,14), the present study detected whether NF- κ B activation attenuated

the effects of MALT1 inhibition in AD cell models; activation of NF- κ B by PMA attenuated the effects of MALT1 inhibition on microglial phenotype switches and neuroinflammation, as well as neuronal loss and oxidative stress in AD cell models. These findings suggested that the protective effects of MALT1 inhibition were mediated, at least partly, through modulation of the NF- κ B pathway during AD progression.

Several limitations of the present study should be noted. First, the primary findings are from a limited set of assays in immortalized cell lines, lacking primary cell validation, human induced pluripotent stem cell (iPSC)-based models and *in vivo* evidence. Future studies incorporating these models are warranted to validate the proposed mechanism of MALT1 inhibition in AD. Second, PMA is commonly applied to activate the NF- κ B pathway (38,59); moreover, p-p65 is the key component implicated in the NF- κ B pathway (60,61). However, the causal involvement of the NF- κ B pathway remains to be established, as pathway activation was mainly assessed by p-p65 expression and PMA may exert effects beyond NF- κ B activation. Further studies using more specific NF- κ B pathway modulators are required to confirm this mechanism. Third, iNOS and ARG1 are key markers for microglial M1 and M2 types, respectively, and the inflammatory cytokines including TNF- α and IL-1 β also partially reflect the microglial M1 type (62). Nevertheless, the assessment of microglial polarization was based on a limited number of M1/M2 markers, which may not fully represent the complex phenotypic states of microglia. In addition, cell viability assays alone are insufficient to evaluate neurodegeneration or neuronal loss. Fourth, although PMA-mediated NF- κ B activation attenuated the effects of MI-2 in the present study, this does not definitively establish NF- κ B as the exclusive downstream mediator of MALT1. Exploration of alternative pathways (such as JNK, mTOR and inflammasome-related signaling) is necessary to provide a more nuanced mechanistic interpretation. Fifth, the blood-brain barrier permeability, long-term safety and potential immune suppression of MALT1 inhibition were not assessed in the present study, which are important for the evaluation of MALT1 as a target for AD.

Future studies are warranted to translate the present *in vitro* findings into *in vivo* contexts. Specifically, the therapeutic efficacy of MI-2 or genetic MALT1 ablation should be evaluated in amyloid precursor protein/presenilin 1 transgenic mouse models or A β -injected AD mouse models, with assessments encompassing cognitive behavior, microglial M1/M2 polarization profiles, amyloid plaque burden, neuronal survival and oxidative stress markers in brain tissue. Mechanistically, complementary approaches such as NF- κ B-specific inhibitors (BAY 11-7082), co-immunoprecipitation of the MALT1-B cell lymphoma/leukemia 10-containing membrane-associated guanylate kinase protein 1 signaling complex and MALT1 proteolytic activity probes *in vivo* may delineate whether NF- κ B serves as the predominant downstream effector or if parallel pathways (JNK and mTOR) also contribute to phenotypes. Additionally, the incorporation of microglia-neuron 3D cerebral organoid models may offer a more physiologically relevant platform to validate the neuroprotective effects of MALT1 inhibition. These investigations may inform the translational potential of targeting MALT1 as a disease-modifying strategy for AD.

In conclusion, the present study demonstrated that MALT1 inhibition suppressed the microglial M1 phenotype, neuroinflammation, neuronal loss and oxidative stress in AD, which may be associated with inactivation of the NF- κ B pathway. These findings suggested its potential as a candidate therapeutic target of AD.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JS and YL conceived and designed the study. XHou and HX contributed to data analysis. HF, XHan, MN, YS and LZ performed the data interpretation. JS and YL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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