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Short Communication

Inhibition of phagocytosis reduced the classical activation of BV2 microglia induced by amyloidogenic fragments of beta-amyloid and prion proteins

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The inflammatory responses in Alzheimer's disease and prion diseases are dominated by microglia activation. Three different phenotypes of microglial activation, namely classical activation, alternative activation, and acquired deactivation, have been described. In this study, we investigated the effect of amyloidogenic fragments of amyloid β and prion proteins ($A\beta_{1-42}$ and $PrP_{106-126}$) on various forms of microglial activation. We first examined the effect of $A\beta_{1-42}$ and $PrP_{106-126}$ stimulation on the mRNA expression levels of several markers of microglial activation, as well as the effect of cytochalasin D, a phagocytosis inhibitor, on microglial activation in $A\beta_{1-42}$- and $PrP_{106-126}$-stimulated BV2 microglia. Results showed that $A\beta_{1-42}$ and $PrP_{106-126}$ induced the classical activation of BV2 microglia, decreased the expression level of alternative expression markers, and had no effect on the expression of acquired deactivation markers. Cytochalasin D treatment significantly reduced $A\beta_{1-42}$- and $PrP_{106-126}$-induced up-regulation of proinflammatory factors, but did not change the expression profile of the markers of alternative activation or acquired deactivation in BV2 cells which were exposed to $A\beta_{1-42}$ and $PrP_{106-126}$. Our results suggested that microglia interact with amyloidogenic peptides in the extracellular milieu-stimulated microglial classical activation and reduce its alternative activation, and that the uptake of amyloidogenic peptides from the extracellular milieu amplifies the classical microglial activation.

Keywords Alzheimer's disease; prion disease; microglial activation

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Introduction

The abnormal assembly and deposition of amyloid fibrils in brain characterizes a diverse group of neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, and prion diseases [1] and many of them involving the degeneration of neurons and microglial activation [2]. Prion and AD are neurodegenerative disorders characterized neuropathologically by the presence of extracellular amyloid deposits, extensive neuronal loss, and gliosis. In AD, the amyloid deposits are composed of amyloid-beta (Aβ) peptides, which result from the abnormal proteolytic cleavage of the amyloid precursor protein [3], whereas, in prion diseases, the amyloid deposits are constituted mainly by the scrapie isoform of the prion protein (PrP^{Sc}), a conformational variant of the cellular prion protein (PrP^{C}) [4]. $PrP_{106-126}$ is a peptide fragment that mimics PrP^{Sc} toxicity and forms fibrils *in vitro* and is commonly used as a model for investigating prion disease [5–8].

Microglia are the principal immune effector cells in the central nervous system, and play an important role in receiving and propagating inflammatory signals in response to the activation of peripheral immune system. In the absence of inflammatory stimuli, microglia are quiescent even though they are actively involved in immune surveillance [9, 10]. In response to inflammatory triggers, microglia are activated and display macrophage-like capabilities including phagocytosis, antigen presentation, and inflammatory cytokines production [11, 12]; this proinflammatory state is known as classical activation and the main identifying features of this state include the over-expression of tumor necrosis factor-alpha (TNF-α) and nitric oxide synthase 2 (NOS2).

Microglia have been demonstrated to show an anti-inflammatory alternative activation phenotype when stimulated with IL-4 or IL-13 [13]. These alternatively activated cells produce several components associated with tissue repair and reconstruction after injury [14–17]. The over-expression of arginase 1 (Arg1) and mannose receptor 1 (Mrc1) characterizes this state of activation.

Recently, a third subtype of microglial activation, termed acquired deactivation, has been identified [13]. This phenotype is induced by IL-10 and incorporates a mixed-phenotype population that exhibits immunosuppression and is associated with the uptake of apoptotic cells. This type of activation is characterized by the up-regulation of suppressor of cytokine signaling 3 (SOCS3) and interleukin 4 receptor α (IL-4Rα).

In this study, we investigated the effect of the exposure to amyloidogenic fragments $A\beta_{1-42}$ and $PrP_{106-126}$ on microglial activation. Results showed that the exposure led to classical activation, reduced alternative activation and had no effect on acquired deactivation. Cytochalasin D, a phagocytosis inhibitor, reduced classical activation of BV2, but had no effect on the other two types of activation.

Materials and methods

$A\beta_{1-42}$ and $PrP_{106-126}$ treatment

$A\beta_{1-42}$ and reverse control Rev_{42-1} peptides were purchased from UNIV-bio (Beijing, China). For fibril formation, Aβ peptides were resuspended in sterile phosphate buffer solution (PBS) (final concentration, 1 mM) and incubated at 37°C for 1 week. Experiments were conducted with final peptide concentrations of 5 μM [18]. $PrP_{106-126}$ (KTNMKHMAGAAA AGAVVGGLG) and scramble PrP (Scr, AVGMHAGKG LANTAKAGAMVG) were purchased from Sangon BioTech (Shanghai, China) and dissolved in sterile PBS to a concentration of 1 mM and left to aggregate at 37°C for 12 h. The experiments were conducted with final peptide concentrations of 50 μM [18, 19].

Cell culture and treatment conditions

BV2 microglial cells, a murine microglia cell line, were obtained from Union Medical University (Beijing, China). Cells were cultured in DMEM and F12 medium (Hyclone, Logan, USA) supplemented with 10% heat-inactivated FBS (Gibco, Grand Island, USA), 100 μg/ml streptomycin, 100 U/ml penicillin (Gibco), and 2 mM glutamine at 37°C with 5% CO_2. For each experiment, 2×10^5 cells were plated into 12-well plates, cultured for 12 h, and then stimulated with $A\beta_{1-42}$ (5 μM) or $PrP_{106-126}$ (50 mM) for 1, 12, 24, 36, 48, and 60 h, respectively, at 37°C in a 5% CO_2 humidified atmosphere. For cytochalasin D treatment, BV2 cells were co-treated with $PrP_{106-126}$ and 2 μM cytochalasin D (Fermentek, Israel) or $A\beta_{1-42}$ and 2 μM cytochalasin D for 12, 24, and 36 h, respectively. Then cells were collected for RNA extraction. All experiments were performed for three times.

Enzyme-linked immunosorbent assay (ELISA) for TNF-α and iNOS secretion

Cell culture supernatants were assayed for TNF-α and iNOS with ELISA kit from Boster Biotech (Wuhan, China) according to manufacturer's instructions.

Thioflavine-T fluoreometric assay

The fibril formation of $A\beta_{1-42}$ and $PrP_{106-126}$ was measured by using a thioflavine-T (ThT) fluorometric assay according to published reports [20].

RNA isolation and complementary DNA synthesis

Total RNA of BV2 cells was extracted using SV Total RNA Isolation System (Promega, Madison, USA), and reverse transcribed into cDNA by using cDNA synthesis kit (Fermentas, Glen Burnie, USA) and oligo(dT) 18 primers. In accordance with manufacturer's instructions, a final volume of 20 μl was used and incubated for 60 min at 42°C, containing RNA template (1 μg), dNTPs (0.5 mM), oligo(dT) primers (1 μM), RT buffer, and nuclease-free water and enzyme was inactivated at 70°C for 5 min.

Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) was performed by using SYBR Green I Master Mix (Bio-Rad, Hercules, USA) and a thermal cycler (DNA Engine Opticon™ 2 system; MJ Research, Waltham, USA) with primers shown in **Table 1**. The qPCR amplification reaction was performed in a final volume of 20 μl, containing 10 μl Master Mix, 0.5 μl of each primer (10 μM), 30 ng of cDNA and water. The amplification procedure was as follows: denaturation at 95°C for 2 min; 40 PCR cycles of 95°C for 5 s, 55°C for 20 s, 72°C for 30 s; 1 cycle at 84°C for 1; 10 s at 70°C and slow heating at a rate of 0.2 per second up to 100°C with continuous fluorescence measurement. Quantification was performed by using comparative C_T method ($2^{-\Delta\Delta CT}$) [21]. All samples were analyzed in triplicate.

Table 1 Primers used for quantitative PCR

Factor name	Sequence (5′→3′)
Arg1	GCATATCTGCCAAAGACATCG
	CCATCACCTTGCCAATCCC
Mrc1	GCAGTGGGCTGGAGGAA
	TGCTGAGGGAATGATAAATGG
TNF-α	CCCTTCCTCCGATGGCTAC
	CGCCTCCTTCTTGTTCTGG
NOS2	GAGCGAGTTGTGGATTGTC
	GGCAGCCTCTTGTCTTTG
SOCS3	GAGATTTCGCTTCGGGACT
	GCTGAGCAGCAGGTTCG
IL-4Rα	CCCAGTGGTAATGTGAAGC
	TCCAGGTGCCAGTGAGTA
β-actin	CCTTCTGACCCATTCCCACC
	GCTTCTTTGCAGCTCCTTCG

Statistical analysis

Each experiment was done on three separate occasions. Data were expressed as mean ± SD. All comparisons of data were made using one-way analysis of variance followed by *post hoc* Turkey's test or Student's *t*-test. SPSS software (version 13.0; SPSS Inc., Chicago, USA) was used to analyze data and $P < 0.05$ was considered statistically significant.

Results

$A\beta_{1-42}$ or $PrP_{106-126}$ up-regulates markers of classical activation, down-regulates of markers of alternative activation and has no effect on acquired deactivation of BV2

To investigate the effect of $A\beta_{1-42}$ or $PrP_{106-126}$ on the activation state of microglia, we stimulated BV2 cells with $A\beta_{1-42}$ or $PrP_{106-126}$ and detected the expression level of specific markers of different microglial activation phenotypes at different time points. Results showed that the exposure to $A\beta_{1-42}$ or $PrP_{106-126}$ significantly up-regulated the expression level of these two specific markers of microglial classical activation, TNF-α and NOS2 [**Figs. 1(A), 2(A)**]. The up-regulation started as early as 1 h post exposure, when the amyloidogenic fragment-induced up-regulation of TNF-α and NOS2 expression was already statistically significant. The up-regulation of the expression level of these two proinflammatory factors was most significant between 12 and 36 h post exposure. At 48 and 60 h time points, the expression level of cytokine decreased gradually; it reverted to control level for NOS2, but still significantly higher than control level for TNF-α. Consistent with these results, the exposure to $A\beta_{1-42}$ or $PrP_{106-126}$ significantly increased the production of iNOS-generated nitric oxide and the protein level of TNF-α between 12 and 36 h post exposure [**Figs. 1(B), 2(B)**].

$A\beta_{1-42}$ or $PrP_{106-126}$ treatment reduced the expression level of Arg1 and Mrc1 below control level [**Figs. 1(C), 2(C)**]. The inhibitory effect was more significant at the time interval between 24 and 60 h post exposure. For specific markers of acquired deactivation, $A\beta_{1-42}$ or $PrP_{106-126}$ treatment had no effect on the expression of SOCS3 and IL-4Rα [**Figs. 1(D), 2(D)**].

ThT fluorometric assay showed the presence of amyloid aggregates in $A\beta_{1-42}$ and $PrP_{106-126}$ preparation with the treatment of BV2 microglia [**Figs. 1(E), 2(E)**].

Inhibition of phagocytosis reduced classical activation of BV2 microglia by $A\beta_{1-42}$ or $PrP_{106-126}$

Figure 3 showed that the treatment of BV2 cells with cytochalasin D, an inhibitor of the phagocytosis, during amyloidogenic stimulation significantly reduced $A\beta_{1-42}$- and $PrP_{106-126}$-induced up-regulation of proinflammatory factors at all time points examined. However, the stimulatory effect of amyloidogenic treatment on proinflammatory factors was not completely abrogated by the inhibition of phagocytosis.

Cytochalasin D treatment did not change the expression profile of these markers of alternative activation or acquired deactivation in BV2 cells upon exposure to $A\beta_{1-42}$ and $PrP_{106-126}$ (data not shown).

Discussion

To better understand the nature of interactions between microglia and amyloidogenic fragments during Alzheimer's and prion disease, we investigated the effect of exposure to amyloidogenic fragments on the activation state of microglia. BV2 microglia were therefore exposed to synthetic fragments $A\beta_{1-42}$ or $PrP_{106-126}$, and the expression level of specific markers of different microglial activation phenotypes was

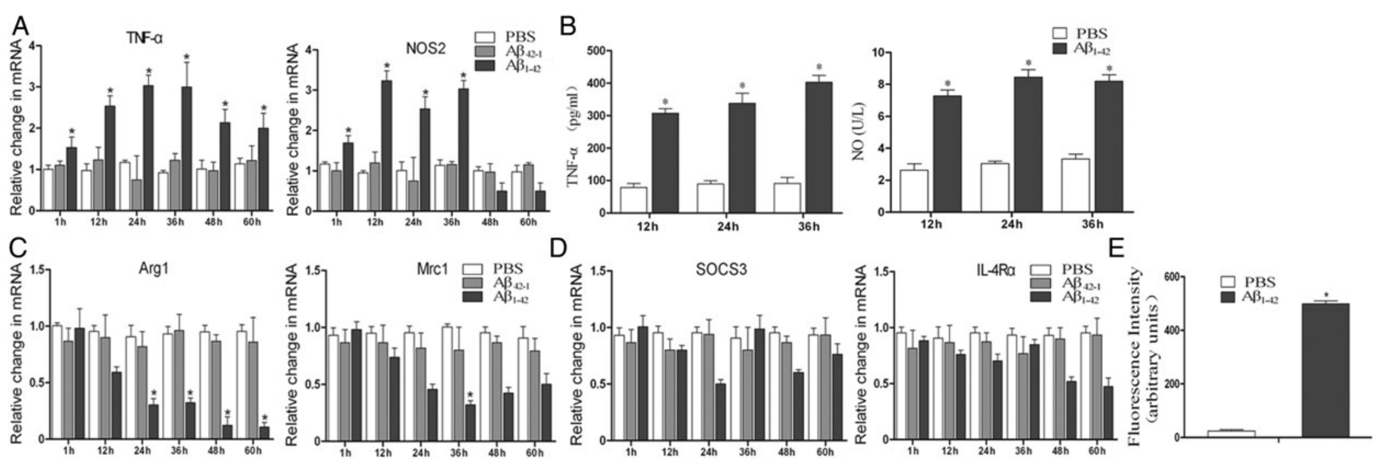

Figure 1 mRNA expression of TNF-α, NOS2, Arg1, Mrc1, SOCS3 and IL-4Rα after treatment with $A\beta_{1-42}$ or $A\beta_{42-1}$ in BV2 microglia BV2 cells were treated with 5 μM $A\beta_{1-42}$ or $A\beta_{42-1}$ for different time periods. The mRNA level changes of TNF-α and NOS2 (A), Arg1 and Mrc1 (C), and SOCS3 and IL-4Rα (D) were measured by quantitative PCR and were expressed as fold changes relative to the mRNA level in control cells which were exposed to PBS only. The secretion of TNF-α and NO was tested by using ELISA (B), and the presence of amyloid aggregates of $A\beta_{1-42}$ using thioflavine-T (ThT) fluorometric assay (E). Data were expressed as mean ± SD of triplicate experiments. $^*P < 0.05$.

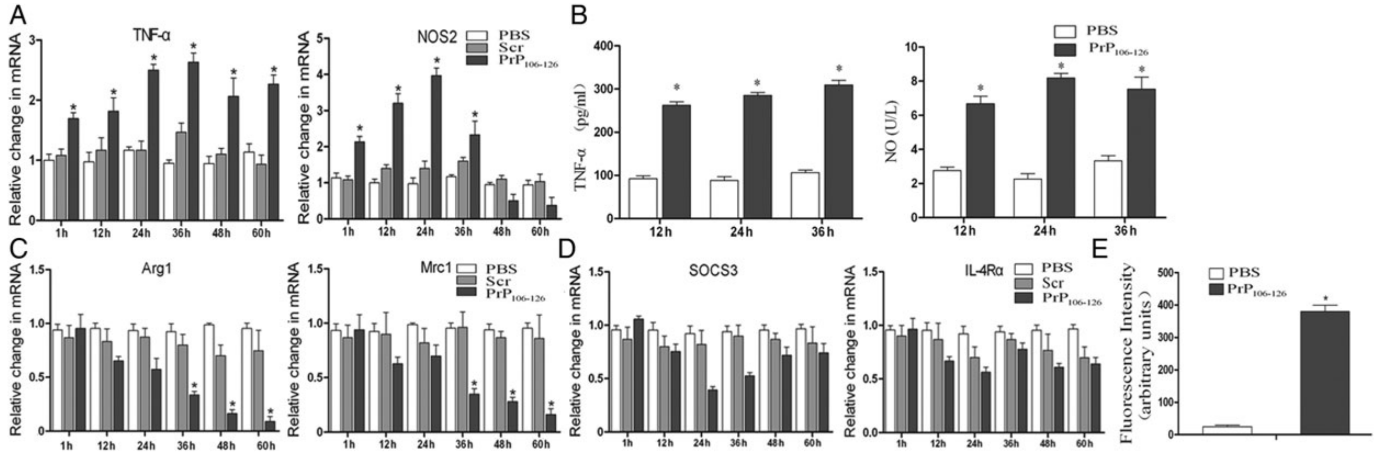

Figure 2 mRNA expression of TNF-α, NOS2, Arg1, Mrc1, SOCS3 and IL-4Rα after treatment with $PrP_{106-126}$ or Scr in BV2 microglia BV2 cells were treated with 50 μM $PrP_{106-126}$ or Scr for different time periods. The mRNA level changes of TNF-α and NOS2 (A), Arg1 and Mrc1 (C), and SOCS3 and IL-4Rα (D) were expressed as fold changes relative to the mRNA level in control cells which were exposed to PBS only. The secretions of TNF-α and NO were measured by using ELISA (B), and the presence of amyloid aggregates of $PrP_{106-126}$ using thioflavine-T (ThT) fluorometric assay (E). Data were expressed as mean ± SD of triplicate experiments. *$P < 0.05$.

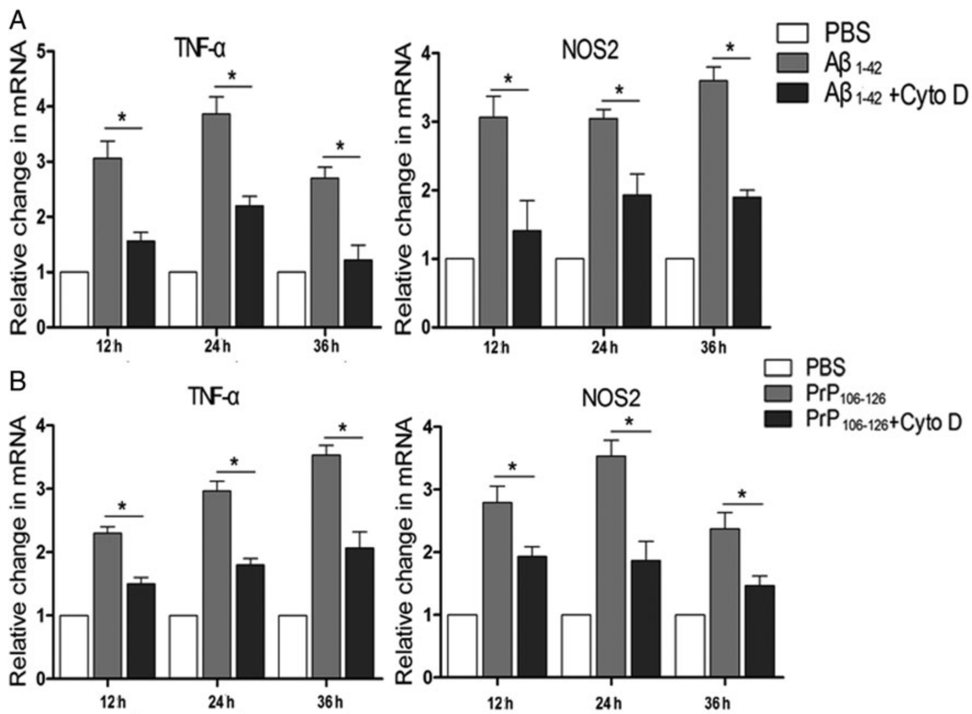

Figure 3 Inhibition of phagocytosis reduced classical activation of BV2 microglia by amyloidogenic fragments BV2 cells were co-incubated with cytochalasin D and amyloidogenic peptides. Data were expressed as mean ± SD of triplicate experiments. *$P < 0.05$, vs. control (treated with PBS only).

determined at various post-exposure time points. Results showed that the expression profile of all examined genes upon exposure to either $A\beta_{1-42}$ or $PrP_{106-126}$ was basically identical, which supported the close similarity in microglia-associated pathogenic processes during Alzheimer's and prion diseases.

$A\beta_{1-42}$ or $PrP_{106-126}$ induces the proinflammatory classical activation of microglia, which is consistent with the well-established fact of microglia-associated proinflammatory process during Alzheimer and prion diseases [22, 23].

The analysis of the effect of amyloidogenic fragments treatment on genes associated with alternative activation of microglia revealed that $A\beta_{1-42}$ or $PrP_{106-126}$ treatment reduced the expression level of Arg1 and Mrc1 below control level. These results suggested that the exposure to amyloidogenic peptides might increase the threshold for microglial alternative activation. However, these findings were not consistent with those from previous reports which showed that neurodegenerative diseases, including AD, are characterized by co-expression of alternative activation and classical activation of the microglia in the brain of affected animals or patients [24]. In light of the findings we reported here, a possible explanation of the alternative microglial activation observed in previous reports was that other cerebral cells such as neuron and astrocytes were stimulated to release anti-inflammatory factors such as IL-4, which might be an indirect result of the amyloidogenic peptides-induced proinflammatory process. These factors in turn stimulated alternative activation of microglia and permitted the establishment of a balance between the opposite poles of proinflammation and increased self-toxicity and anti-inflammation and longer tissue survival in activated microglia in neurodegenerative diseases.

When compared with control, exposure to $A\beta_{1-42}$ or $PrP_{106-126}$ had no significant effect on the expression level of SOCS3 and IL-4Rα, two genes associated with the third phenotype of microglial activation, also known as acquired deactivation, suggesting that this activation phenotype did not play a relevant role in the pathogenesis of Alzheimer's and prion diseases.

Cytochalasin D treatment significantly reduced $A\beta_{1-42}$- and $PrP_{106-126}$-induced up-regulation of proinflammatory factors, suggesting that the most important part of the stimulatory effect of amyloidogenic peptides took place only after their uptake inside BV2 cells, and that this stimulatory effect started as early as microglia interact with amyloidogenic peptides in extracellular milieu. This result also highlighted the importance of phagocytosis in the pathogenesis of amyloidogenic neurodegenerative diseases and confirms previous reports of the involvement of phagocytosis in the establishement of the neuro-inflammatory process associated with AD [25].

Taken together, our results suggest that the interactions of microglia with amyloidogenic peptides in the extracellular milieu stimulate microglial classical activation and reduce its alternative activation, and that the uptake of amyloidogenic peptides from the extracellular milieu amplifies classical microglial activation.

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