Regulatory effect of kainic acid on microglia in different inflammatory states

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Research

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Abstract

Background

Nerve injury is a crucial factor in the development of various neurodegenerative diseases in the central nervous system (CNS). It was demonstrated that neuroinflammatory response mediated by reactive microglia with different activated phenotypes plays an important role in different stages of nerve injury. Nerve injury leads to glutamate spillover, which also causes damage surrounding neurons, neurotoxic reactions, and neuroinflammatory so that glutamate spillover is one of the important mechanisms of nerve injury, among which the kainic acid (KA) signal pathway of glutamate ionotropic receptor induces neurotoxicity most significantly. In addition to inducing nerve damage, KA has been found to regulate microglia, further to induce the functional response of microglia.

Methods

In the present study, we constructed the different phenotypes of microglia stimulated with KA to activate the KA receptor signal pathway of microglia. The activity of microglia, Nitric oxide (NO) levels, lactate dehydrogenase (LDH) production, and cytokine levels were further investigated to explore the different immune response effects of different phenotypes of microglia during the KA stimulation and their role in disease development.

Results

Our results show that after 24 hours (h) of cell culture, KA activation causes a large consumption of pro-inflammatory microglia phenotype, the release of NO and pro-inflammatory cytokines decreased, and the anti-inflammatory cytokines increased. The resting microglia and anti-inflammatory microglia phenotype stimulated with KA are well tolerated after activation. After 48 h of culture, during the KA stimulation, the cell survival rate increased after activation of the pro-inflammatory microglia, and the secretion of NO, pro-inflammatory, and anti-inflammatory cytokines increased. The activation of resting microglia and anti-inflammatory microglia leads to decreased cell survival and decreased secretion of pro-inflammatory and anti-inflammatory cytokines. After 72 h of culture, the cell survival rate and NO release decreased after KA stimulation of the pro-inflammatory microglia. The anti-inflammatory microglia treated by KA increased cell activity and survival rate after activation.

Conclusions

Our results may reveal the situation for the massive consumption of microglia after the KA stimulation in the early phase of nerve injury, at the same time, it was revealed that anti-inflammatory microglia not only had a high survival rate in the high concentration of glutamate but also could produce anti-inflammatory cytokines and play a protective role. It may provide a novel strategy for the treatment and recovery, suggest a scheme for the protection of microglia and resistance to secondary injury.
Introduction

Nerve injury is a common injury; although the fibers retain a considerable regeneration potential in the adult, recovery is usually rather poor, especially in cases of large nerve defects. Gaining increased attention as they impose a considerable socioeconomic impact\textsuperscript{[1, 2]}. Despite different aetiologies, a common feature of nerve injury is the chronic activation of innate immune cells within the CNS\textsuperscript{[3]}. The innate immune system is a rapid and coordinated cellular defense response that can identify pathogenic agents mediated by pattern recognition receptors (PRRs) primarily expressed by microglia, astrocytes, and macrophages to secrete the inflammatory cytokines and contribute to the innate immune response\textsuperscript{[4–8]}. Inflammatory cytokines also initiate inflammatory signaling cascades that may contribute to neuronal injury and cell death\textsuperscript{[9, 10]}. Hence, increased levels of inflammatory cytokines are often observed upon CNS infection, nerve injury, and neurodegenerative diseases\textsuperscript{[10–12]}. However, the current study of activation of the inflammatory response in microglia and its role in the inflammation and nerve injury is still fragmentary.

Microglia, known as the smallest glial cells, accounts for approximately 15% of the total number of cells in the CNS and is found throughout the brain and spinal cord, which are the principal resident innate immune cells of the CNS, detect adjacent glia, blood vessels, and neurons through their branching processes, and monitor the entire brain environment\textsuperscript{[10, 13]}. In the CNS, microglia, as resident phagocytic cells, play a significant role in the injury response and pathogen defense, which can act as phagocytosis to remove pathogens or damaged neurons\textsuperscript{[10, 14]}. In addition, microglia are also a CNS antigen-presenting cell and immune effector cells and have the ability to phagocytose bacteria, antigen presentation, and produce cytokines and complements\textsuperscript{[15]}. Once the brain environment is disturbed by the physiological or pathological damage, microglia are immediately activated. It will clear excess and apoptotic neurons and repair the damage, which plays a key role in the remodeling of the brain environment\textsuperscript{[16]}. The activation of microglial is a complex process that can result in several phenotypes that correspond to their different responses to the environment, as well as the physical interaction with other cells and their physiological activities in the brain\textsuperscript{[10]}. Differential activation of microglia is often classified as being either pro-inflammatory phenotype (historically termed pro-inflammatory) or anti-inflammatory phenotype (historically classified as anti-inflammatory), based on chemokine and cytokine expression in vivo\textsuperscript{[17–20]}. The pro-inflammatory phenotype is related to the pro-inflammatory cytotoxic gene expression, which is characterized by the secretion of pro-inflammatory cytokines, including tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), Interleukin-1 (IL-1), IL-6, IL-12, and IL-18, with impaired phagocytosis and reactive oxygen species (ROS) and NO, which cause dysfunction of the neural network in the CNS\textsuperscript{[10, 21, 22]}. On the contrary, the anti-inflammatory phenotype is related to the induction of specific proteins to secret the anti-inflammatory cytokines, including IL-4, IL-10, IL-13, and transforming growth factor-\(\beta\) (TGF-\(\beta\)), involved in inhibiting inflammation and restoring homeostasis\textsuperscript{[23, 24]}. However, microglial activation does not refer to a single phenotype, and a continuum of microglia activation is rather considered. They are able to acquire numerous phenotypes upon activation ranging from a phagocytic to an antigen-presenting phenotype.
that mainly depends on the type of stimuli provided in their environment\textsuperscript{[25–28]}. Hence, activated microglia as important immunological factors have multiple functions, which depend on the stimulation circumstance and then exert different effects on neurodegenerative diseases.

Glutamate is the most common neurotransmitter as the soluble factors mediating neuron-microglia communication in the brain, which is also the chemotactic neurotransmitter for the microglia\textsuperscript{29,30}. Glutamate receptors can be divided into two broad categories: metabotropic receptors (mGluRs) and ionotropic receptors (iGluRs). The latter can be further divided into three categories: N-methyl D-aspartate receptor (NMDAR), \(\alpha\)-amino-3 hydroxy-5 methyl-4-isoxazole receptor (AMPAR), and KA\textsuperscript{[10,31]}. Recent studies have found that when the concentration of extracellular glutamate increases, glycoproteins on the microglia membrane can rapidly transport glutamate in synaptic space, which is of great significance to prevent excitatory toxicity of glutamate, which therefore may be therapeutic targets for neurodegenerative diseases\textsuperscript{[32–34]}. However, it is not clear what the reaction mechanisms of microglia regular by KA during the secondary nerve injury of diseases. In addition, studies have shown that KA can enhance the level of inflammatory factors. Activated microglia and astrocytes after KA treatment can release a large number of inflammatory mediators such as NO, IL-6, IL-12, TGF-\(\beta\), TNF-\(\alpha\), IL-18, and IL-1\(\beta\)\textsuperscript{[35,36]}. However, the changes in activation of microglia subtypes and in the release of various cytokines over time are not well clear.

Therefore, in this experiment, we constructed a pro-inflammatory phenotype and an anti-inflammatory phenotype of microglia to stimulate with KA in different observation time points of 24, 48, and 72 h. It was simulated that a series of pathological reactions caused by KA receptor activation of microglia in the extracellular high concentration of glutamate after nerve injury and explore the role in the pathogenesis and development of nerve injury, which may provide a basis for the treatment.

\textbf{Materials And Methods}

\textbf{Cell cultures}

N13 microglia cell line was prepared in the frozen liquid nitrogen and then put into the water with a temperature of 37°C for shaking 30 seconds (s). The whole cell suspension was placed into the centrifuge tube that contained 30 ml Dulbecco's modified Eagle's medium (DMEM), penicillin (100 IU/mL), streptomycin (100 IU/mL) and 10% fetal bovine serum (FBS). After centrifugation at 1000 rpm for 5 minutes (min) at room temperature (RT), microglia suspension was plated in one T75-cell culture flask (Invitrogen, Carlsbad, CA) in the DMEM medium and incubated at 37°C and 5% carbon dioxide (CO\textsubscript{2}) in a humidified incubator. The medium was changed every 2 days until the cells became confluent. When the cell layer reached full confluence, microglia were harvested using pre-warmed trypsin (Sigma) to prepare for the experiment.

For stimulation experiments, microglia were harvested from the remaining cells, seeded in 6 well plates in DMEM with 10% FBS, and set up three different time point groups for 24, 48, 72 h, and the cell count were
2×10^5, 10^5, 5×10^4 cells/well respectively and then stimulated for 12 h. Microglia were divided into treatment groups: (a) unstimulated state (resting microglia phenotype); (b) polarized to pro-inflammatory phenotype induced by 500 ng/ml lipopolysaccharide (LPS; Sigma) and 20 ng/ml recombinant mouse interferon (IFN)-γ (ImmunoTools GmbH, Friesoythe, Germany); (c) polarized to anti-inflammatory phenotype produced by stimulation with 20 ng/ml recombinant mouse IL-4 and IL-10, as well as recombinant human TGF-β (R&D systems, USA) for 24 h. And further stimulated with or without 200 μM KA for 24, 48, 72 h. After stimulating for 24, 48, 72 h, the supernatants were aspirated for LDH activity test, as well as the detection of NO and cytokine levels, and the microglia were analyzed by flow cytometric to check the polarization of resting microglia to pro-inflammatory or anti-inflammatory phenotypes according to standard protocols.

**Detection of polarization of resting microglia to pro-inflammatory or anti-inflammatory phenotypes analyzed with Fluorescence-activated cell sorting (FACS)**

The microglia were washed with phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA; Sigma) twice and stained with combinations of cell surface antibodies for 30 min at 4 °C. The used antibodies included: APC-labeled rat anti-mouse CD11b (Caltag, Burlingame, CA), rat anti-mouse Arg-1 (BD Transduction, USA), FITC-labeled rat anti-mouse CD206 (Biolegend, USA) and PE-labeled rat anti-mouse CD40 (Caltag, Burlingame, CA), FITC-labeled rat anti-mouse inducible NO synthase (iNOS) (BD Transduction). For intracellular staining, 5000 cells/tube with good conditions were first incubated with the surface antibodies, fix with 2% buffered formaldehyde for 20 min at 4 °C, and permeabilized with 0.5% saponin in PBS/BSA. FITC-, PE-, APC-labeled mouse isotype IgG (Serotec) were used as negative controls. The percentages of CD11b, Arg-1, CD206, CD40 and iNOS positive microglia were detected using BC CytoflexS flow cytometer and the data were analyzed using CytExpert 2.2 software (Beckman Coulter, USA).

**Detection of cell activation with Cell Counting Kit-8 (CCK-8) Test**

The culture supematants were transferred into a new 6-well culture plate. Fresh medium with 10% CCK8 was added and incubated for 1-4 hours at 37°C. The absorbance was measured at 570 nm with a reference filter of 570 nm.

**Detection of LDH Activity with LDH Kit**

The total protein concentrations were measured by using the bicinchoninic acid (BCA) protein assay kit (Bio-Rad, Sweden). The standard curve was obtained by using BSA solutions at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, and 0 mg/ml respectively. The concentrations of proteins were quantified from the standard curve.

LDH production was detected in the culture supematants by using Cytotoxicity Detection Kit and following the kit directions. Briefly, 100 μl assay medium was added into triplicate wells for background control, while those wells with the same amount of supematant from untreated common cells or Triton X-
100 treated cells included were for low control or high control. 100 μl samples were added to each well. 100 μl of the freshly prepared reaction mixture was added into each well and incubated for 30 min at RT away from light. The absorbance of the samples was measured at 490 or 492 nm.

Detection of NO Production

NO production was measured by the supernatant levels of nitrite, while the stable biological oxidation product of NO was determined by using the modified Griess reagent. The concentrations of nitrite were quantified by the extrapolation from the standard curve, which was obtained by using sodium nitrite solutions at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0 μM. The samples and Griess reagents were added each 100 μl to each well. After being incubated 15 min at RT away from light, the absorbance of the samples was measured at 540 nm.

Detection of cytokine levels with Enzyme-Linked Immunosorbent Assay (ELISA)

The cytokines levels (TNF-α, IFN-γ, IL-4, IL-10, and IL-6) in the supernatant of microglial cultures before and after KA insult were assessed using commercially available kits (eBioscience) on an ELISA reader at 450 nm following the standard manufacturer's protocols. 100 μl/well of capture antibody (2 μg/ml TNF-α, 1 μg/ml IFN-γ, 2 μg/ml IL-4, 1 μg/ml IL-6, and 2 μg/ml IL-10) was used to coat the plates at 4°C overnight. After being washed with Wash Buffer, uncoated sites were blocked by 200 μl/well of 1X ELISA Diluent for 1 h at RT. After being incubated at room temperature for 1 h, wells were aspirated and then washed 3 times with Wash Buffer of at least 300 μl/well. Duplicates of samples or of recombinant standards were added and the plates were incubated overnight at 4°C. The plates were incubated with detection antibody (1 μg/ml TNF-α, 1 μg/ml IFN-γ, 1 μg/ml IL-4, 1 μg/ml IL-6, and 1 μg/ml IL-10) at RT for 1 h after being washed. Then avidin-conjugated horseradish peroxidase (HRP) was added for 30 min at RT. 100 μl of 3,3',5,5'-Tetramethylbenzidine (TMB) was added to each well and incubated at RT for 10-15 min. The color reaction was stopped by the addition of 100 μl of 2 M sulfuric acid and read at 450 nm.

Data Presentation and Statistics

Each data was presented as mean value ± standard deviation (SD). The statistical program for social sciences 15.0 (SPSS 15.0, USA) was used to analyze the experimental results. Analysis of Variance was used to compare values among groups. For all tests, the level of significance was set to $P < 0.05$ which is shown on the graphs as *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. Statistics used for each data are indicated in the figure legends.

Results

The phenotypes of microglia were confirmed by flow cytometry

Microglia were harvested and their purity was analyzed by flow cytometric analysis. The positive rates of CD11b, the major microglial marker, were up to 99.0% ± 0.6%, 98.0 ± 1.1% and 99.8% ± 0.4% in resting
microglia, pro-inflammatory and anti-inflammatory phenotype respectively (Fig. 1).

The surface markers of pro-inflammatory microglia (iNOS and CD40) and anti-inflammatory phenotype (Arg-1 and CD206) were analyzed by the flow cytometry. As shown in Fig. 1, the results showed that LPS plus IFN-γ stimulation could induce resting microglia to transform into pro-inflammatory phenotype and increase the expression of markers of pro-inflammatory phenotype, iNOS and CD40, which were significantly different from those in the control group. Although iNOS and CD40 were markers of pro-inflammatory phenotype, the anti-inflammatory phenotype also could express these markers at lower levels than that on the pro-inflammatory phenotype. Actually, there was a low-level tendency of iNOS and CD40 on the anti-inflammatory phenotype compared with the levels on the pro-inflammatory phenotype. To sum up, these results indicated that LPS plus IFN-γ can strongly promote resting microglia activation and polarization into the pro-inflammatory microglial phenotype. Besides, it increased iNOS and CD40 on the anti-inflammatory phenotype when compared with resting microglia only.

Besides, the combined stimulation of IL-4, IL-10, and TGF-β could induce resting microglia to transform into the anti-inflammatory phenotypes, and the expression of markers of anti-inflammatory phenotype, such as Arg-1 and CD206, was significantly different from that in the control group. Arg-1 and CD206, which were anti-inflammatory phenotype markers, were significantly higher in anti-inflammatory phenotype compared to other phenotypes of microglia (Fig. 1). The expression of Arg-1 and CD206 was non-significantly elevated in the pro-inflammatory phenotype microglia compared to the resting microglia. These data suggested that IL-4, IL-10, and TGF-β could shift M0 towards anti-inflammatory phenotype microglia. Therefore, the cell model was successfully established which can be used for subsequent experiments.

The pro-inflammatory phenotype enhanced KA-induced microglial cytotoxicity while anti-inflammatory microglia protected the microglia injury

To explore the effects of the three microglial phenotypes on the KA-induced microglia injury, we cultured the three microglial phenotypes with 200 μM KA for 24 h, 48 h, 72 h, respectively. Then we examined microglia activation via the CCK-8 assay, analyzed the microglia damage by LDH test and detected cytokine production such as IFN-γ, TNF-α, IL-6, IL-4, and IL-10 by ELISA.

Cultured for 24 h, the activity of pro-inflammatory phenotype decreased sharply after adding KA, while M0 and anti-inflammatory phenotypes were well tolerated (Fig. 2). After 48 h of culture, under the influence of KA, the survival rate of each phenotype was still decreased, but it tended to recover especially the anti-inflammatory phenotype with KA (Fig. 2). After 72 h of culture, as can be seen in Fig. 2, the cell survival rate decreased after activation of KA of pro-inflammatory phenotype, indicating that the cell was inhibited after activation of KA of pro-inflammatory phenotype. However, the activation and survival rate of the anti-inflammatory phenotype has increased.

The level of LDH was measured to detect the degree of microglia injury
We detected the degree of microglia damage by examining the levels of LDH. LDH is an indicator for the loss of cell membrane integrity, and high levels of LDH in the culture supernatants implies an increased number of dead cells or cells with damaged plasma membranes. Generally, the levels of Intracellular LDH (ILDH) were higher than extracellular LDH (ELDH) as shown in Figs. 3.

After 24 h of KA treatment, the pro-inflammatory phenotype began to apoptosis ($P < 0.05$). At 48 h, the level of ELDH of the pro-inflammatory phenotype increased sharply and decreased after 72 h ($P < 0.05$). Interestingly, the anti-inflammatory phenotype showed resistance to KA insult again, since similar levels of ILDH were observed in the anti-inflammatory phenotype both before and after KA treatment (Fig. 3). This is consistent with the trend of CCK8 results (Fig. 2).

The pro-inflammatory phenotype increased pro-inflammatory cytokines production after KA insult

NO has been involved in many pathological processes within the CNS and can be formed enzymatically from L-arginine via iNOS in neuroglia. When KA was added for 24 h, the production of NO in the pro-inflammatory phenotype increased sharply. Over time, the level of glutamate spillover gradually decreased, and the production of NO began to decline (Fig. 4, $P < 0.05$).

Our results also demonstrated that KA treatment significantly reduced the IFN-γ, TNF-α, and IL-6 production and produced higher levels of IL-4 and IL-10 in the pro-inflammatory phenotype, while the anti-inflammatory phenotype was on the contrary at 24 h (Fig. 5). However, the expression of proinflammatory cytokines in the pro-inflammatory phenotype increased when KA was treated after 48 h and 72 h. Taken together, our observations indicated that the activation of KA causes a large consumption of pro-inflammatory phenotype at 24 h, and with time elapsing, the activity of the pro-inflammatory phenotype is regulated by KA decreased, while the activity of the anti-inflammatory phenotype recovered due to the activation of KA. Therefore, it is indicated that the pro-inflammatory phenotype could enhance the inflammatory response in KA-induced microglial cytotoxicity while the anti-inflammatory phenotype enhanced the microglial protective pathway.

Discussion

The neuroinflammation after nerve injury is the complex innate immune response of neural tissue. Accumulating evidence suggests that neuroinflammation is one of the most common pathologies in nerve injury[37]. Microglia is the most motile resident innate immune cell in the CNS. And similar to immune cells, microglia play “double-faced” roles in the processes of neuroinflammation, depending on the course of the disease and certain environments of inflammation. Gliotransmitters serve as an important cue for microglia communications, including glutamate and NO, mediate neuron-glia and glia-glia communications, and glial cell responses to the subtle changes of the nerve injury environment[38]. A large number of researches have shown that nerve injury induces glutamate spillover, induces neuroinflammation, neurotoxic reactions, and apoptosis in the peripheral neurons to cause the secondary injury. Besides, KA signaling pathway between microglia and glutamate receptors provides the signals
and substrates for investigating the mechanisms of nerve injury. Therefore, the role of microglia in the regulation of glutamate is crucial in the early stage of nerve injury. In this paper, we will discuss the interactions between microglia and glutamate receptors at different stages of neuroinflammation and the potential areas in understanding the role of microglia in neuroinflammation for future research.

The initial stage of inflammation in the nerve injury

Microglial processes are highly active. They are always activated earlier than other glial cells under hazardous stimulations or pathological states of the CNS\cite{39}. Therefore, they are the first to perceive initial damage in the CNS. Under a homeostatic environment, microglia were in the resting state. Under initial injury stresses of the CNS, dying neurons can release a high concentration of ATP and glutamate, which is then rapidly hydrolyzed to form a gradient. This gradient immediately attracts and activates nearby microglia in response to the insults\cite{40}. It can cause a transient intercellular Ca\textsuperscript{2+} increase and K\textsuperscript{+} spillover and lead to the production of mature pro-inflammation cytokines\cite{10, 41, 42}. When the extracellular glutamic acid concentration increases, the glycoprotein in the microglia membrane can rapidly transport glutamate in the synaptic gap, which is of great significance to prevent excitatory toxicity of glutamate.

Our results showed that after 24 h of culture, with the high extracellular KA concentrations, and the activation of microglia stimulated with KA resulted in a large consumption of pro-inflammatory phenotype, the release of NO and pro-inflammatory cytokines decreased, and the number of anti-inflammatory cytokines increased. The above results may reveal the situation of the massive consumption of microglia in the early stage of nerve injury and anti-KA neuro-excitotoxicity of the pro-inflammatory phenotype. When KA stimulated the anti-inflammatory phenotype, it caused a significant decrease in IL-4 and IL-10. These two cytokines were anti-inflammatory cytokines that not only inhibited the expression of the pro-inflammatory cytokines TNF-\alpha and IL-6 but also protected neurons by different regulatory mechanisms. However, KA with resting microglia and anti-inflammatory phenotypes were well tolerated after activation, but both expressed low levels of cytokines, especially anti-inflammatory cytokines in the anti-inflammatory phenotype, which indicated that the neuroprotective effect of the anti-inflammatory phenotype was inhibited by KA activation during the early stage of inflammation.

Therefore, it is speculated that the neuroprotective effect of microglia can be enhanced by promoting the transformation of microglia to the anti-inflammatory phenotype and inhibiting its KA in the early stage of diseases.

The progression of inflammation in the nerve injury

The activated microglia are highly plastic cells and are divided into classic (pro-inflammatory) and alternative (anti-inflammatory) activation phenotypes, and the polarization of the cells is dependent on disease development. In neurological disorders, microglia alter the excitability of the neural network by modulating the neuroinflammatory responses\cite{43, 44}. During the progression of inflammation in the nerve injury, reactive microglia secrete abundant pro-inflammatory mediators and clean up pathogens or damaged tissue debris. At the same time, the responses of microglia and immune cells are coordinated to
protect tissues from secondary damage caused by overactivation of inflammation. Once the cascade is triggered, both pro- and anti-inflammatory responses can be simultaneously activated. In this process, microglia can exhibit different functions to deteriorate or alleviate tissue damage. The “two-sided” character of neuroinflammation is decided by the diverse phenotypes of microglia and their interaction with glutamate. The understanding of the underlying mechanisms will be instrumental to the intervention of nerve injury development.

As time went on and the concentration of KA decreased, cell activation, the secretion of NO, pro-inflammatory and anti-inflammatory cytokines increased after the activation of pro-inflammatory phenotype KA, indicating that the activation of the pro-inflammatory phenotype KA has a bidirectional regulatory effect on neuroinflammation. However, the activation of KA in resting microglia and anti-inflammatory phenotypes resulted in decreased cell activation and the secretion of pro-inflammatory and anti-inflammatory cytokines, indicating that the activation of KA in anti-inflammatory cells leads to the down-regulation of neuroinflammation regulation function in the nerve injury. Therefore, microglia play an important regulatory role in neuroinflammatory response induced by nerve injury after KA stimulation. It is speculated that the pro-inflammatory phenotype is the main regulation of neuroinflammatory response in the nerve injury, which may be caused by the activation of its KA receptor. If the pro-inflammatory activation is inhibited during the treatment of the nerve injury, the harmful and pro-inflammatory effect of the pro-inflammatory phenotype will be inhibited, so the same method achieves good results.

The prognosis of inflammation in the nerve injury

The CNS homeostatic state is maintained by the cooperation between glial cells and resident immune cells. The disruption of the balance by endogenic or exogenic factors induces the onset of neuroinflammation, originally as a defensive response to resist or clear the harmful factors for tissue repair. The development of inflammation in the CNS is a process to rebuild the balance by neuroglia and immune cells, a counterbalance of pro- and anti-inflammation, and a mixture of destroy and repair actions[45]. In most acute and focal CNS injuries or infections, once the harmful factors have been removed and homeostasis has been restored, the tissue will be repaired. It has also been reported that during neuroinflammation repair, microglia produce IL-10 and TGF-β, which leads to anti-inflammatory signaling (anti-inflammatory) and wound healing (anti-inflammatory)[10, 46]. In addition, microglia can self-control their polarization through autocrine and paracrine means, and this response is protective but is downregulated once the damage or pathogen has been addressed. If the self-regulation is interrupted, unregulated, long-term, or chronic inflammation occurs that exacerbates tissue damage[47]. Therefore, under continuous stimulations or circumstances such as aging or genetic defect, the failures of pathogen clearance and the homeostasis rebuilding would prolong the inflammation progression and lead to a chronic condition. The chronic inflammation in the CNS is a hallmark pathology of neurodegenerative diseases.

In our study, after 72 h of culture, the activation of microglia and NO release were decreased after activation of KA of the pro-inflammatory phenotype, which indicated that the cells were inhibited after
activation of KA of the pro-inflammatory phenotype. However, activation of KA of the anti-inflammatory phenotype increased cell activation. Therefore, it is speculated that the anti-inflammatory phenotype is the main regulation of neuroimmune response during the later period of never injury.

**Conclusions**

In summary, the “two-sided” character of microglia and the balance of pro-/anti-inflammation in the CNS remains elusive due to the complex mechanisms in the pathogenesis of CNS-directed autoimmune diseases. But in the nerve injury, extracellular glutamate regulation of microglia polymorphism is more unknown so that we observed the different phenotypes of microglia at multiple time points after KA stimulation to simulate the regulatory effect of extracellular KA consecutive regulated microglia in nerve injury. The above results may reveal the cause of microglia depletion after the release of glutamate in the early phase of inflammation, and the phenomenon of pro-inflammatory phenotype on neuro-excitotoxicity, and the reason that the neuroinflammatory response is dominated by pro-inflammatory phenotype in the development stage of the nerve injury, and the mechanism of anti-inflammatory phenotype dominance in the convalescent period was also revealed. Hence, the effect of glutamate spillover caused by nerve injury on the KA receptor of microglia plays an important role in the development of the disease, and it is of great value to develop regulatory schemes for specific subtypes of microglia at different stages of glutamate spillover for the treatment of disease.

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Study concept and design: ZB and ZJ. Acquisition of data: ZZL, WD, and ZB. Analysis and interpretation of data: ZZL, WD, and ZB. Drafting of the manuscript: ZZL, WD, and ZB. Statistical analysis: ZZL and ZB. Critical revision of the manuscript for important intellectual content: ZB, WD and ZJ. The author(s) read and approved the final manuscript.

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Ethics declarations

Consent for publication

Not applicable.

Competing interests
The authors declare that they have no competing interests.

**Additional information**

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**Abbreviations**

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole receptor

BCA: Bicinchoninic acid

BSA: Bovine serum albumin

CCK-8: Cell Counting Kit-8

CNS: Central nervous system

CO2: Carbon dioxide

DMEM: Dulbecco’s modified Eagle’s medium

ELISA: Enzyme-Linked Immunosorbent Assay

FACS: Fluorescence-activated cell sorting

FBS: Fetal bovine serum

IFN-γ: Interferon-γ

iGluRs: Ionotropic receptors

IL-1: Interleukin-1

iNOS: Inducible NO synthase

KA: Kainic acid

LDH: Lactate dehydrogenase

LPS: Lipopolysaccharide

mGluRs: Metabotropic receptors
NMDAR: N-methyl D-aspartate receptor

NO: Nitric oxide

PBS: Phosphate buffered saline

PRRs: Pattern recognition receptors

ROS: Reactive oxygen species

RT: Room temperature

SD: Standard deviation

SPSS: Statistical program for social sciences

TGF-β: Transforming growth factor-β

TMB: Tetramethylbenzidine

TNF-α: Tumor necrosis factor-α

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Figures

Figure 1

Microglial phenotypes were identified by flow cytometric analysis. Positive rate of CD11b. After subculture of microglia, flow cytometry was used to detect the positive rate of CD11b in microglia. Up to 99.0% ± 0.6% of the cells were CD11b positive in the M cultures (M0). And inflammatory-M (M1) and anti-inflammatory-M (M2) were 98.0 ± 1.1% and 99.8% ± 0.4% respectively. Identification of inflammatory-M. Microglial cells from three groups (M0, M1 and M2) were stained and analyzed by the flow cytometry for the detection of markers in M1 phenotype: iNOS, CD40. It was shown that the higher levels of iNOS and
CD40 were observed in M1 phenotype. Identification of anti-inflammatory-M. Microglial cells from three groups (M0, M1 and M2) were stained and analyzed by flow cytometry for the detection of markers in M2 phenotype: Arg-1, CD206. Whereas, higher levels of Arg-1 and CD206 were seen in M2 phenotype. The results were expressed as positive percentages.

Figure 2

M1 phenotype enhanced KA-induced microglial cytotoxicity, while M2 phenotype protected against the microglia injury from KA insult. CCk8 assay was used to measure microglial viability. Cell viability of anti-inflammatory-M was obviously high, whether or not treated with KA insults at 24-72hs. Also, the anti-inflammatory-M not only inhibited inflammation, also prevented them from KA induced-cytotoxicity for a relative long time. Data were presented as mean ± SD. n = 3 in each group. ns P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 3

ILDH and ELDH levels. Before treatment with KA at 24 h, both levels of ILDH and ELDH in inflammatory-M group were significantly increased compared to other groups, indicating that inflammatory-M was associated with more loss of cell membrane integrity. And anti-inflammatory-M showed resistance to KA insult again. Data were presented as mean ± SD. n = 3 in each group. ns P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 4

NO production. An obvious higher level of NO production was found in inflammatory-M than both anti-inflammatory-M and M groups (P < 0.05). Data were presented as mean ± SD. n = 3 in each group. ns P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 5

Cytokine production before and after KA treatment. Detail descriptions were seen in the text. Data were presented as mean ± SD. n = 3 in each group. ns P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001.