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An Aβ3-10-KLH vaccine decreases Aβ plaques and astrocytes and microglia activation in the brain of APP/PS1 transgenic mice

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This study aimed to investigate β-amyloid peptide (Aβ) plaques and changes of astroglia and microglia in mice with Alzheimer's disease (AD). In this study, 18 transgenic mice with amyloid precursor protein (APP) /presenilin-1 (PS1) were randomized into the Aβ3-10-KLH vaccine, Aβ1-42 vaccine, and phosphate-buffered saline (PBS) groups. The mice were injected at different time points. The Morris water maze test was used to identify the spatial learning and memory abilities of the mice. Immunohistochemistry was done to examine the Aβ, glial fibrillary acidic protein, and transmembrane protein 119 (TMEM119). Correspondingly, enzyme-linked immunosorbent assay (ELISA) was done to measure interleukin (IL) -1β and tumor necrosis factor (TNF) -α in the brain of transgenic mice. The Morris water maze results showed that both the Aβ3-10-KLH vaccine and the Aβ1-42 peptide vaccine could improve the cognitive function of APP/PS1 transgenic mice significantly. Aβ3-10-KLH and Aβ1-42 inoculations reduced Aβ load and suppressed astrocytes and microglia proliferation in the cortex compared with the PBS group. While there was no significant difference between the two groups, Aβ3-10-KLH and Aβ1-42 vaccines decreased the brain levels of IL-1β and TNF-α as compared with the PBS group, but without difference between the two vaccines. In conclusion, early immunotherapy with an Aβ vaccine reduces the activation of glial cells and deposition of Aβ plaque in the brain of transgenic mice.

Key words: Alzheimer's disease, Aβ3-10-KLH vaccine, Aβ1-42 vaccine, amyloid-beta, astrocytes, microglia, APP/PS1 transgenic mouse

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder and causes progressive deterioration in memory, daily behavioral function, and learning ability (Anand et al., 2014). One of the pathological changes in AD is the presence of deposition of amyloid plaques, the formation of neurofibrillary tangles (NFTs), and gliosis in the brain (Beach et al., 1989). The amyloid cascade hypothesis points out that the β-amyloid peptide (Aβ) accumulation triggers a series of reactions that include NFTs formation, neuron apoptosis, neuroinflammation, and gliosis (Selkoe and Hardy, 2016).

Astrocyte is the main type of glia in the central nervous system (CNS) and works to maintain the brain homoeostasis and support metabolism (Giaume et al., 2007). With the progression of AD, the function of the astrocytes is gradually lost, and some toxic neurotransmitters such as glutamate are released continuously, leading to the death of the surrounding neurons (Simpson et al., 2010). The Aβ plaques also disrupt the gliotransmission system by enhancing the calcium signal pathway (Lee et al., 2014). In addition, Aβ has been found to interact with several erythropoietin acceptors such as nicotinic receptors (a7-nAChRs), purinergic receptor P2Y1, and the glutamate metabotropic receptor mGluR5 (Delekate et al., 2014; Lee et al., 2014; Ronco et

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al., 2014). The aberrant aggregated Aβ plaques induce the production of various proinflammatory mediators, including interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and reactive oxygen species (ROS) that lead to neuroinflammation and neuron death (Heneka et al., 2015a; 2015b; Flores et al., 2018). The selective inhibition of the chemokine receptor CX3CR1 in the brain can modulate the phagocytosis of microglia and the degradation of Aβ plaques (Liu et al., 2010). Plaque-associated microglia express known myeloid markers shared among macrophage progeny, microglia, and peripheral monocytes, including CD45, Iba1, and CD11b (Bennett et al., 2018). TMEM119 is another specific and stable cell-surface marker of human microglia that could be a possible therapeutic target (Bennett et al., 2016). Hence, activating the degradation of Aβ plaques by the microglia could be a way to manage AD.

Over the last few decades, the therapeutic strategies for AD are based on the "amyloid hypothesis" and researchers spent immense efforts in reducing the levels of Aβ (Cline et al., 2018). Some strategies for AD treatment aim to consolidate the clearance of Aβ plaque and elimination of neuroinflammation reactions (Lemere, 2013), and immunotherapy against Aβ and phosphorylated tau (p-Tau) has been suggested for more than 20 years. Pre-clinical studies have shown some efficacy of vaccines and active immunotherapies against Aβ and p-Tau, but the translation to humans proved ineffective and with serious safety issues (Song et al., 2020). Among others, a vaccine against Aβ1-42 (Elan Pharmaceuticals, CA, USA) could remove amyloid plaques and improve perceptive function in transgenic animal models and AD patients (Janus et al., 2000; Vellas et al., 2009), but the clinical trial of the Aβ1-42 vaccine was suspended because of severe side effects like acute meningoencephalitis and cerebral hemorrhage (Rosenberg, 2005).

The aim of this study was to investigate Aβ plaques and changes of astroglia and microglia in mice injected with a new vaccine based on Aβ3-10 as the antigen. The cognitive and behavioral abilities of vaccinated mice were also observed. Due to the molecular and structural feature of the Aβ3-10 peptide, a carrier protein keyhole limpet hemocyanin (KLH), a highly immunogenic protein macromolecule, was synthesized with the Aβ3-10 peptide into an Aβ3-10 polypeptide vaccine (Aβ3-10-KLH). This new vaccine has a smaller fragment and has enhanced immunogenicity due to the coupling of KLH. It can effectively increase the concentration of anti-Aβ antibodies in experimental mice and reduce the deposition of Aβ in the brain. The immune response is mainly a Th2 type inflammatory reaction, thereby avoiding the side effects caused by Th1 type inflammatory reaction. Therefore, this new vaccine could solve the problem presented in the previous vaccine, such as ineffective and serious side effects, and may provide a better understanding of the mechanisms of AD and its treatment.

METHODS

Experimental design

This research was an experimental study which the AD mouse model (APPswe/PS1dE9 double transgenic mice) was used as the study object. This study was done to explore the preventive treatment of vaccines to transgenic mice and further explore the influence on Aβ plaques and glial cells in the cortex. Initially, the mice were subjected to 5 times immunotherapy with Aβ3-10-KLH and Aβ1-42 peptide vaccines at the age of 2.5, 3, 4, 5, 6 months. Then, the effects of the two vaccines on Aβ plaque deposition, astrocyte and microglia expression in the brain of mice, and their relationship with each other were explored further at the age of 10 months. Afterward, the vaccine's effect on the inflammatory response in the brain of mice was explored by detecting the inflammatory factors IL-1β and TNF-α.

Vaccine synthesis

The Aβ3-10-KLH vaccine was prepared by coupling the sulfydryl group of cysteine (Cys) with KLH. The amino acid sequence of Aβ3-10 was H-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-COOH. A Cys was added to the C-terminal of Aβ3-10 to couple with KLH. According to its amino acid sequence, the peptide (EFRHDSGYC) was synthesized by solid-phase synthesis from C-terminal to N-terminal. The peptide was purified by high-performance liquid chromatography with purity >95%. The relative molecular weight was 1113.17 by mass spectrometry. The above synthesis process was completed by Kingston Technology Co., Ltd. (Nanjing, China). The synthetic human Aβ1-42 peptide was obtained from AnaSpec Inc. (Fremont, CA, USA).

Animals

Eighteen 2.5-month-old APP/PS1 mice (half male and half female) with the genetic background B6C3-Tg (APPswe/PSEN1dE9) were obtained from the Guangdong Medical Laboratory Animal Center. These transgenic mice typically develop Aβ plaques at approximately 3 months (Zhong et al., 2014). Six age-matched

C57BL/6 mice (three males and three females) were used as control. All mice were kept at the China Medical University Laboratory Animal Center at 23±2°C, with humidity 50%±10%, and under a 12-h light/dark cycle. All the experiments were performed in accordance with National Institutes of Health (NIH; Bethesda, MD, USA) guidelines on the use of laboratory animals and approved by the Animal Ethics Committee of China Medical University (approval No. 103-316) on April 2, 2016.

Immunization protocol

The APP/PS1 mice were randomized to the Aβ3-10-KLH, Aβ1-42, and PBS groups (n=6/group). Six 2.5-month C57BL/6 wild type mice were used as the control group. The Aβ3-10-KLH and Aβ1-42 peptides were dissolved in PBS to a final concentration of 2 mg/ml and then emulsified 1:1 (v/v) with Freund's complete adjuvant (Sigma, St. Louis, MO, USA) for the first inoculation and emulsified 1:1 (v/v) with Freund's incomplete adjuvant (Sigma, St. Louis, MO, USA) for the next vaccinations. The mice in the Aβ3-10-KLH and Aβ1-42 groups were hypodermically injected with 100 μg of the peptide, and the mice in the PBS cluster and control cluster were injected with 100 μl of PBS. The immunization was performed five times (at 2.5, 3, 4, 5, and 6 months). After immunization, the mice were kept at the Experimental Animal Center of China Medical University until they were 10 months old. The animal experiments were approved by the Animal Ethics Committee of China Medical University and performed according to the guidelines of the Animal Care and Use Committee of China Medical University.

Morris water maze test

The spatial learning and memory abilities of the APP/PS1 transgenic mice and WT mice were detected by the Morris water maze test at the age of 10 months. In this experiment, the Morris water maze test system (Huaibei Zhenghua Biologic Apparatus Facilities Co., Ltd., Huaibei, China) was used. It consists of a circular pool with a diameter of 100 cm and a height of 40 cm. The inner wall of the pool was white, the water depth was 30 cm, and the water temperature was maintained at 22±2°C. In the room, the light was constant, and there was no direct light on the pool. The pool was divided into four quadrants by four equidistant points on the pool wall. In the target quadrant (set as the third quadrant), there was a round black platform with a diameter of 9 cm and a height of 28 cm, which was 2 cm below the water surface. During the experiment, the pool and the surrounding environment remained unchanged.

To evaluate the spatial learning ability of mice, the hidden platform positioning experiment was applied. The classic Morris water maze test was used for 5 consecutive days. The platform was set in the third quadrant, and the opposite side was the first quadrant. The quadrant setting sequence was clockwise. Before the experiment, the mice were placed on the platform to adapt for 20 s, and then they were placed into the first quadrant. The recording was terminated after the mice boarded the platform again for 5 s. The longest recording time was 60 s. If the mice could not get on the platform within 60 s, they would be guided to board the platform to adapt for 10 s. The mice were dried and put into the cage after the test. The test was carried out from the four quadrants sequentially for 5 days. The average latencies of the four quadrants per day were recorded.

To evaluate the spatial memory ability of the mice, the space search test was performed on the sixth day. The environment and water temperature were the same as the positioning navigation test. The platform under the water was removed, and then the mice were placed into the first quadrant. The times of crossing the platform in the third quadrant (target quadrant) were recorded.

Tissue preparation

After the Morris water maze test, the mice were anesthetized intraperitoneally deeply with 2% sodium pentobarbital, and the heart was perfused. The thoracic cavity was cut open to expose the cardiopulmonary region fully. The perfusion needle was quickly inserted into the apex of the heart to the ascending part of the aorta. At the same time, the right atrial appendage was cut to make the perfusate flow out after circulation until the limbs and liver became white and the perfusion fluid was clear. The skull was exposed and cut from the middle and both sides. The skull was gently lifted off with tweezers to expose the brain tissue. The left brain was fixed in 4% paraformaldehyde PBS solution, and the right brain was stored in a sterilized centrifuge tube at -80°C.

Detection of IL-1β and TNF-α levels in APP/PS1 transgenic mice by ELISA

The frozen brain tissue stored at -80°C was taken out and thawed on ice. The brain tissue was cut into

small pieces with sterile ophthalmic scissors, and the tissue lysate solution (50 mM Tris buffer + 250 mM NaCl + 5 mM ethylenediaminetetraacetic acid + 2 mM Na3VO4 + 1 mM NaF + 20m M Na4P2O7 + 0.02% NaN3, and pH 7.4) was added. An electric homogenizer was used to grind the brain tissue (10 s/time, 30-s interval, 3-5 times) until there was no obvious solid tissue in the solution, and it was kept at 4°C for 30 min. The homogenate was centrifuged at 4°C at 3000 rpm for 15 min, and the supernatant was kept for enzyme-linked immunosorbent assay (ELISA).

ELISA was performed using the mouse IL-1β ELISA kit (ab100704, Abcam, Cambridge, UK) and TNF-α ELISA Kit (ab100747, Abcam, Cambridge, UK). Both the ELISA kits were removed 20 min earlier from the refrigerator. A blank hole was added and specimen diluent; remaining concentrations were added in the appropriate hole (100 µl/hole), incubated at room temperature for 2.5 h. Prepared 20 min in advance of biotinylated antibody working solution and then washed four times. Blank hole added with biotinylated antibody diluent added remaining blank hole with biotinylated antibody working solution (100 µl/hole). A new cover plate of plastic tapes was placed in reaction holes and incubated at room temperature for 60 min, prepared 20 min in advance of conjugate working solution, protected from light at room temperature, and washed five times. Blank hole added with conjugate diluent added remaining with a conjugate working solution (100 µl/hole). A new cover plate of plastic tapes was placed in reaction holes and incubated at 36°C for 45 min. Powered on the microplate for preheating equipment and to set up testing procedures. Washed five times and joined the chromogenic substrate 100 µl/hole, incubated at 36°C for 30 min without the presence of light. Adding stop solution 100 µl/hole, and the plates were read at 450 nm immediately after mixing (Karim et al., 2019; Wang et al., 2020).

Immunohistochemistry

The fixed left-brain tissue samples were cut coronally into 10-µm-thick slices on a microtome and collected for six consecutive sections. The slides were laid to dry in the air at 37°C overnight. The sections were washed with 1× PBS for 15 min and blocked with 5% bovine serum albumin for 1 h at room temperature. The diluted primary antibody was added and incubated for 48 h at 4°C. The fluorescence-labeled secondary antibody was diluted and incubated at room temperature for 2 h in the dark. The sections were washed with 1× PBS for 5 min and sealed with a blocker containing DAPI. The primary antibodies were mouse monoclonal anti-amyloid beta (anti-Aβ)/anti-6E10 antibody (1:100, BioLegend, San Diego, CA, USA, 803015), rabbit polyclonal anti-GFAP antibody (1:500, Abcam, Cambridge, UK, ab7260), and rabbit monoclonal anti-TMEM119 antibody (1:100, Abcam, Cambridge, UK, ab209064). The secondary antibodies were goat anti-mouse Alexa Fluor 488 (1:200, Thermo Fisher Scientific, Waltham, MA, USA, A-11001) and goat anti-rabbit Alexa Fluor 555 (1:200, Thermo Fisher Scientific, Waltham, MA, USA, A-21428). Six representative fields of view were selected for observation using a Nikon C2 laser confocal microscope at 60× and analyzed the representative images with Image J software (Vision 1.8.0, NIH, USA).

Statistical analysis

The data are presented as means ± standard deviations (SD) and analyzed using ANOVA with Tukey's *post hoc* test. SPSS 23.0 (IBM, Armonk, NY, USA) was used for analysis. Two-sided P-values <0.05 were considered statistically significant.

RESULTS

Aβ3-10-KLH immunization improves the behavioral performance of the mice

To determine the effect of the Aβ3-10-KLH vaccine on the cognitive and behavioral abilities of mice, the Morris water maze test was carried out of the mice in each group at the age of 10 months after five vaccine injections. As shown in Fig. 1, in the visual platform training, there were no significant differences in the latency of the Aβ 3-10-KLH vaccine, Aβ1-42 peptide, PBS control, and wild type control groups (P>0.05), indicating that the Aβ3-10-KLH and Aβ1-42 peptide vaccines did not affect the visual and motor functions of the mice. In the hidden platform training, the latency of the Aβ3-10-KLH and Aβ1-42 vaccine groups was significantly shorter than that of the PBS control group on the 3rd to 5th day (***P<0.001), but there were no significant differences between the Aβ3-10-KLH and Aβ1-42 vaccine groups (P>0.05). In the space exploration experiment, compared with the PBS group, the Aβ3-10-KLH and Aβ1-42 vaccine groups significantly increased, and the times of crossing the platform significantly increased (***P<0.001). There were no significant differences between the Aβ3-10-KLH and Aβ1-42 vaccine groups (P>0.05). Therefore, the Morris water maze results showed that both the Aβ3-10-KLH and Aβ1-42 vaccines could significantly improve the cognitive function of APP/PS1 transgenic mice.

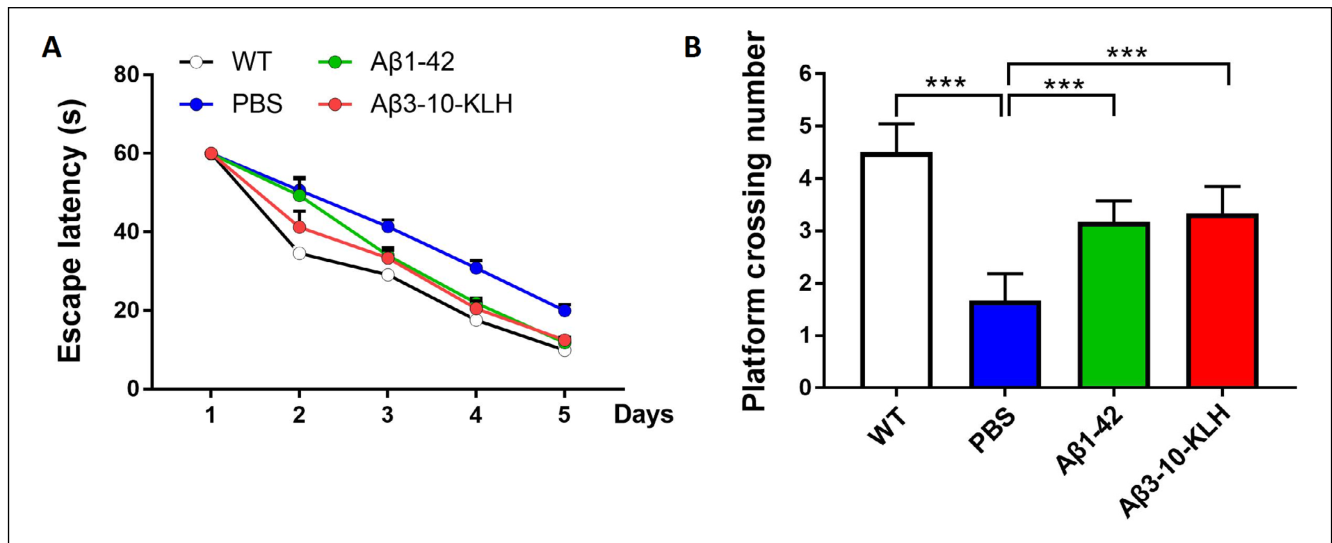

Fig. 1. The cognitive function of the mice was detected by the Morris water maze. (A) On the first day of visual platform training, there was no significant difference in the latency of the Aβ3-10-KLH vaccine, Aβ1-42 peptide, PBS, and wild type control groups ($P>0.05$). The latency of the Aβ3-10-KLH and Aβ1-42 vaccine groups was significantly shorter than that of the PBS group on the 3rd to 5th days (***$P<0.001$). (B) In the space exploration experiment, the mice in the Aβ3-10-KLH vaccine, Aβ1-42 peptide, PBS, and WT groups crossed the platform. Compared with the control group, the number of times of passing through the platform of the Aβ3-10-KLH and Aβ1-42 vaccine groups was significantly higher (***$P<0.001$).

Aβ3-10-KLH immunization reduces Aβ plaques in the cortex

We then evaluated whether the effect of Aβ3-10-KLH and Aβ1-42 vaccines on cognitive function was related to Aβ deposition. As showed in Fig. 2, compared with PBS control mice, Aβ3-10-KLH and Aβ1-42 significantly reduced Aβ deposition in the cerebral cortex, as detected by immunofluorescence. Significant Aβ deposition was not observed in wild-type control mice. Compared with the PBS group, Aβ3-10-KLH and Aβ1-42 inoculation reduced the total fluorescence density of $6E10^+$ Aβ plaques in the cortex (***$P<0.001$), respectively. Aβ3-10-KLH and Aβ1-42 inoculated mice showed a similar decrease in $6E10^+$ Aβ load ($P>0.05$).

Aβ3-10-KLH or Aβ1-42 alleviates the activation of astrocytes

To test the effect of Aβ3-10-KLH or Aβ1-42 on astrocytes, we detected its hallmark protein GFAP expression with immunohistochemistry. Here we demonstrated that $GFAP^+$ astrocytes in experimental clusters were all activated at various degrees compared with the control group. The $GFAP^+$ astrocytes in the PBS group were significantly higher compared with the Aβ3-10-KLH and Aβ1-42 mice (Fig. 3A). The total fluorescence density analysis confirmed that $GFAP^+$ astrocytes were significantly reduced (***$P<0.001$) in the cortex of the Aβ3-10-KLH and Aβ1-42 mice respectively compared with the PBS mice (Fig. 3B). Furthermore, double-immunofluorescence staining was used to detect the relationship between activated astrocytes and Aβ plaque. Fig. 3C showed that in the Aβ3-10-KLH and Aβ1-42 groups, the astrocytes (GFAP in red) around the Aβ plaques (6E10 in green) were relatively increased. In the PBS group, the area of Aβ plaque was higher, while interestingly, the astrocytes around the Aβ plaque were lower. In the control group (WT), there was almost no deposition of Aβ plaques.

Aβ3-10-KLH or Aβ1-42 vaccination depresses microglial activation in the cortex

On the other hand, considering the important role of microglia in uptake and clearance of different forms of Aβ, we checked the effect of the vaccination on microglia. Here we showed that $TMEM119^+$ microglia of the Aβ3-10-KLH and Aβ1-42 mice were slightly increased compared with the wild type mice, which reached highest in the PBS group (Fig. 4A). The fluorescence density analysis confirmed that the $TMEM119^+$ microglia were significantly fewer in the cortex of the Aβ3-10-KLH and Aβ1-42 groups (***$P<0.001$) than that of the PBS group (Fig. 4B). And there were no significant differences between the Aβ3-10-KLH and Aβ1-42 groups.

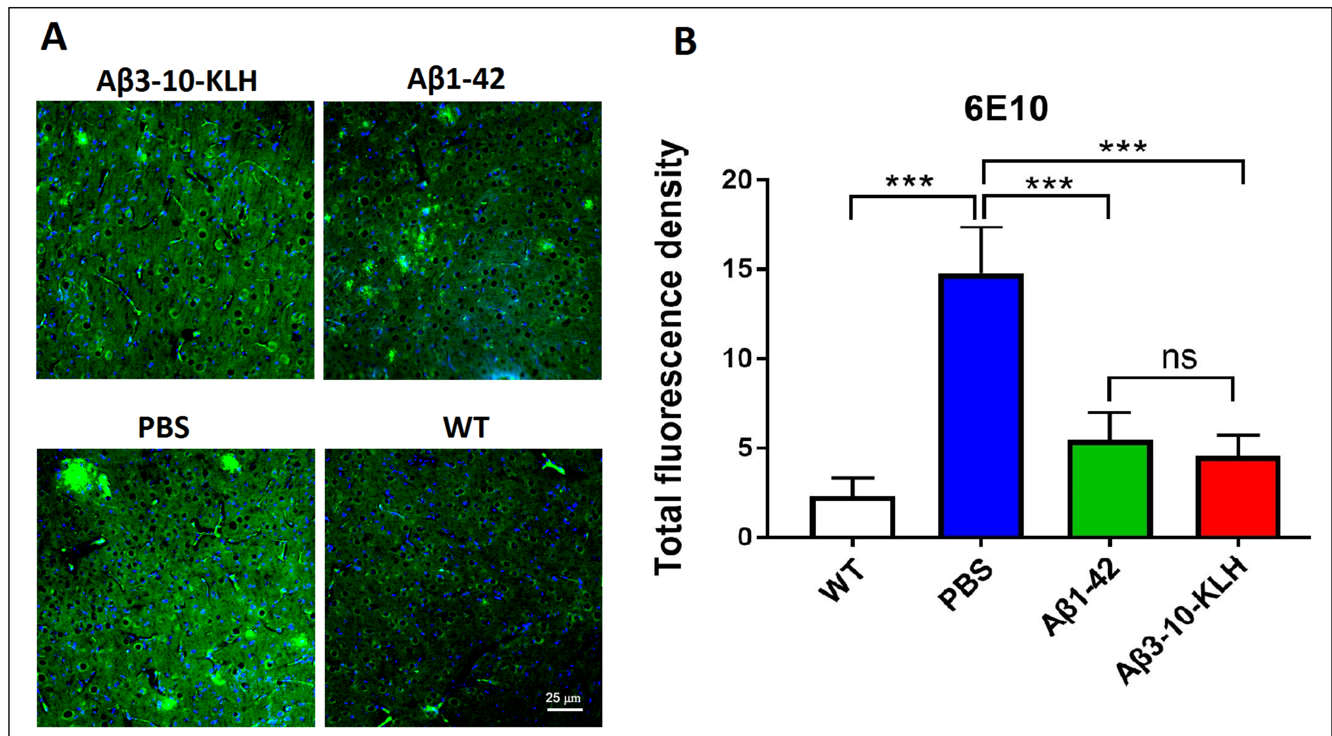

Fig. 2. Aβ3-10-KLH immunization reduces Aβ plaques in the cortex. Mouse monoclonal anti-amyloid beta (anti-Aβ)/anti-6E10 antibody was used to stain plaques. (A) The deposition of Aβ plaques in the cortex of immunized and PBS groups was obvious. Compared with the PBS group, the Aβ plaque burdens were significantly reduced in the Aβ3-10-KLH and Aβ1-42 mice. There were almost no amyloid plaques in wild type mice. Scale bar=25 μm. (B) Compared with the PBS group, the total fluorescence density of $6E10^+$ plaques in the cortex of the Aβ3-10-KLH and Aβ1-42 groups was lower (***P<0.001), but there were no differences between the Aβ3-10-KLH and Aβ1-42 mice (ns: no significance).

Aβ3-10-KLH or Aβ1-42 vaccination reduces the contents of IL-1β and TNF-α in the brain of the mice

In the CNS, activated astrocytes and microglia are the main sources of inflammatory factors such as cytokines, chemokines, and neurotransmitters. The released cytokines, especially IL-1β and TNF-α, are the main effectors of neuroinflammatory signals, affecting the neurophysiological mechanism of cognition and memory (Gemma et al., 2007). Cytokines can establish a feedback loop to activate more astrocytes and microglia, leading to further inflammatory molecule production that will also recruit monocytes and lymphocytes, and other cells to cross the blood-brain barrier (BBB), thereby enhancing the inflammatory response of the CNS (Das et al., 2008). Therefore, we used Aβ3-10-KLH and Aβ1-42 vaccine to detect IL-1β and TNF-α levels in the brain after immunotherapy to show the inflammatory response in the after-treatment and further to verify the effect of active immunotherapy on inflammation.

The contents of IL-1β and TNF-α detected by ELISA in the brain of transgenic mice treated with the Aβ3-10-KLH or Aβ1-42 vaccine were decreased significantly than the PBS group. As shown in Fig. 5A and 5B, the contents of IL-1β and TNF-α in the brain of transgenic mice immunized with Aβ3-10-KLH vaccine and Aβ1-42 vaccine group were significantly reduced (***P<0.001) in comparison with the PBS group. There were no significant differences in the content of IL-1β and TNF-α in the brain of the Aβ3-10-KLH and Aβ1-42 vaccine groups (P>0.05). Taken together, the reduction of proinflammatory mediators IL-1β and TNF-α may be one of Aβ3-10-KLH and Aβ1-42 vaccines' mechanisms in decreasing aberrant aggregated Aβ plaques.

DISCUSSION

The pathogenesis of AD is complex which is characterized by a progressive loss of neurons and involves Aβ plaques formation, activation of astrocytes and microglia (Guo et al., 2020; Tiwari et al., 2019). Vaccines and active immunotherapy are being studied against AD (Bittar et al., 2018; Song et al., 2020). The aim of this study was to investigate Aβ plaques and changes of astroglia and microglia in mice injected with a new

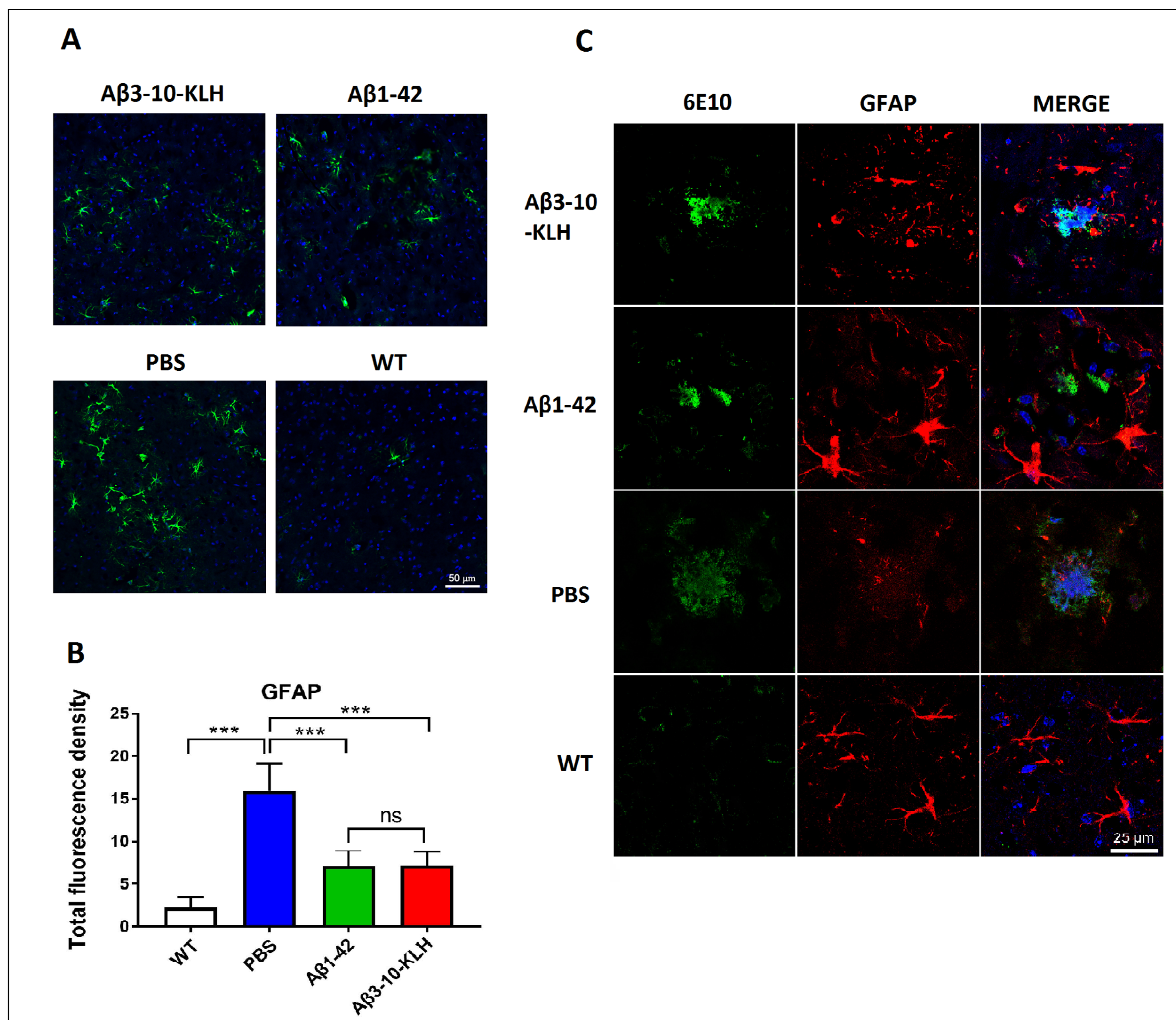

Fig. 3. Aβ3-10-KLH and Aβ1-42 alleviate the activation of astrocytes. (A) The activation of the astrocytes in the cortex of the Aβ3-10-KLH and Aβ1-42 mice was higher in control group (WT) and lower than in the PBS group. Scale bar=50 μm. (B) The total fluorescence density of GFAP+ astrocytes in Aβ3-10-KLH and Aβ1-42 group were lower than PBS group (***P<0.001). (C) Double-immunofluorescence staining showing that the Aβ plaques (green) in the Aβ3-10-KLH and Aβ1-42 mice were smaller than in the PBS mice, while the astrocytes (red) surrounding the Aβ plaques were increased. Scale bar=25 μm.

vaccine based on Aβ3-10 as the antigen. The results strongly suggest that early immunotherapy with an Aβ vaccine reduces the activation of glial cells and deposition of Aβ plaque in the brain of transgenic mice.

Although there are many theories on the pathophysiological process of AD (Guo et al., 2020; Tiwari et al., 2019), the amyloid hypothesis is still the main accepted theory (Cline et al., 2018). The amyloid hypothesis states that the secretion and accumulation of Aβ lead to the formation of Aβ plaques, which contribute to molecular and cellular alterations through pathological changes. (Selkoe and Hardy, 2016). Currently, the US Food and Drug Administration has approved only five drugs for clinical use (Yiannopoulou and Papageorgiou, 2020), but unfortunately, none of these drugs can stop the development of AD and can only improve the clinical symptoms of AD (Liu et al., 2010; Lyman et al., 2014) partially. The AN-1792 clinical trial of anti-Aβ immunotherapy, significantly improved the clinical manifestations, but it was halted due to significant adverse events (Patton et al., 2006). One of our previous study showed that Aβ3-10-KLH vaccination induces serum anti-Aβ antibodies and improves the memory function in transgenic mice, and induced Th2-polarized im-

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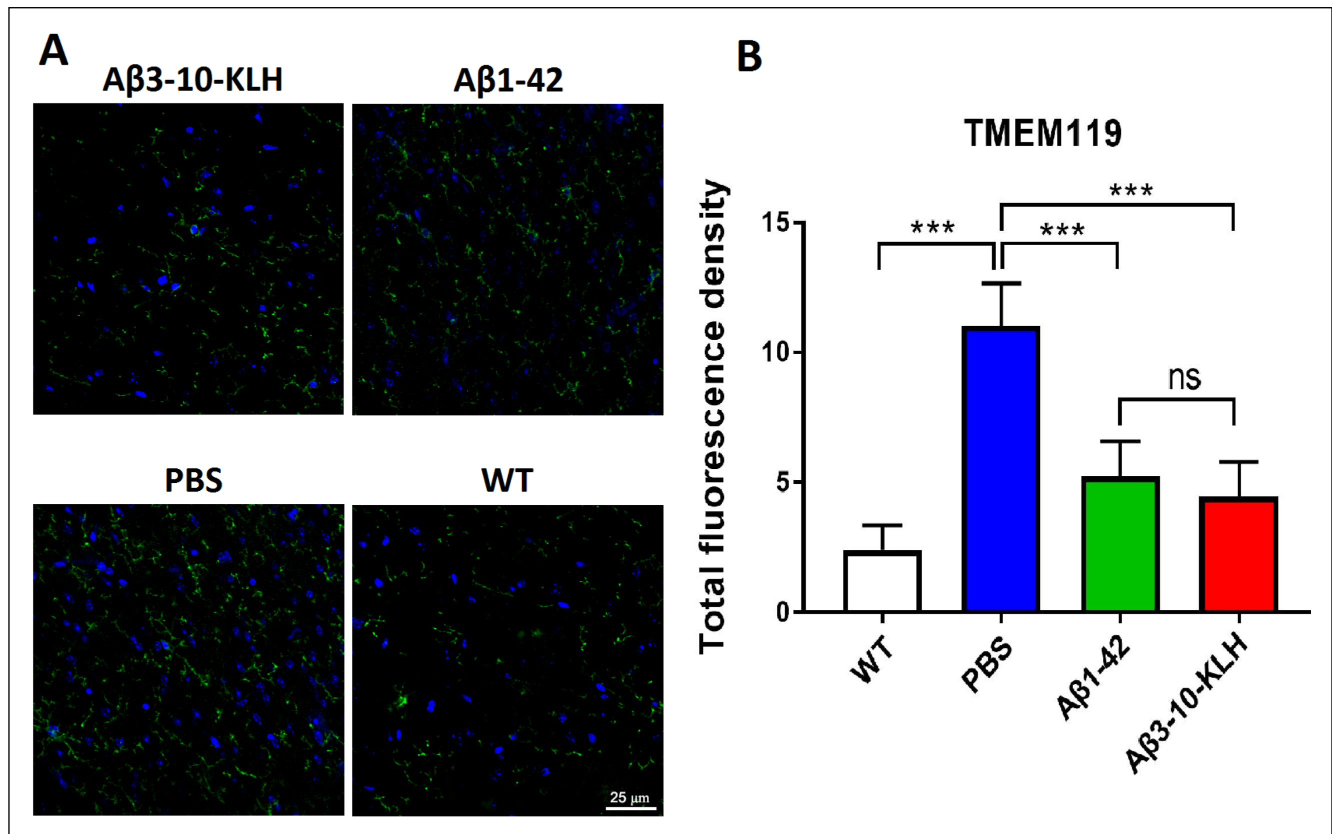

Fig. 4. Aβ3-10-KLH or Aβ1-42 vaccination depresses microglial activation in the cortex. (A) Administration of Aβ3-10-KLH and Aβ1-42 reduced the activation of microglial cells in the cortex of inoculated mice as showed with lower TMEM119+ compared with the PBS group. Scale bar=25μm. (B) The total fluorescence density of the TMEM119+ microglia in the cortex was determined. (ns: no significance, ***P<0.001).

mune responses, which suggested that it could effectively protect the transgenic AD mice without severe adverse events such as meningoencephalitis induced by Th1 immune responses (Ding et al., 2016). Furthermore, it is also suggested that Aβ3-10-KLH vaccination could significantly improve behavioral outcomes due to high antibody production and cognitive function improvement, (Wang et al., 2020) also revealed that after vaccine injection, mice produced high levels of Aβ antibody, and cognitive function was significantly improved, which resembles our study findings. In this study, both Aβ3-10-KLH and Aβ1-42 vaccination could reduce Aβ deposition in APP/PS1 transgenic mice. This study strongly suggests that the use of the Aβ3-10-KLH vaccine could be a treatment method for AD. Nevertheless, additional studies are necessary to ensure the higher safety of Aβ3-10-KLH vaccination compared with Aβ1-42 vaccination.

The astrocytes play the role of supporting and separating nerve cells and are involved in the BBB and the regulation of synaptic activity. They also produce and secrete some neurotransmitters and express some neurotransmitter receptors (Iglesias et al., 2017; Vasile et al., 2017). In AD, reactive astrocytes also influence the clearance and deposition of Aβ plaques through the β-binding receptors CD36 and CD47 (Acosta et al., 2017). In addition, astrocytic dysfunction enhances the deterioration of neurons (Acosta et al., 2017). Astrocytes also express some metalloendopeptidases and matrix metalloproteinases, including NEP, IDE, MMP-2, and MMP-9, which promote the degradation of Aβ species in APP/PS1 transgenic rats (Yan et al., 2006; Mulder et al., 2012; Ries and Sastre, 2016). On the other hand, activated astrocytes produce lots of α1-antichymotrypsin in controlling Aβ degradation and triggering hyperphosphorylation of tau (Avila-Munoz and Arias, 2014). The reactive astrocytes may help remove dysfunctional synapses or synaptic fragments and enhance the inflammatory effects of damaged neurons (Gomez-Arboledas et al., 2018). In this study, Aβ3-10-KLH and Aβ1-42 immunization significantly alleviated astrogliosis in APP/PS1 transgenic mice, but, interestingly, it was observed that the astrocytes around Aβ plaques showed apoptosis in the PBS group. A possible reason might be that Aβ plaques are alleviated after immunotherapy. Activated astrocytes participated in the process of

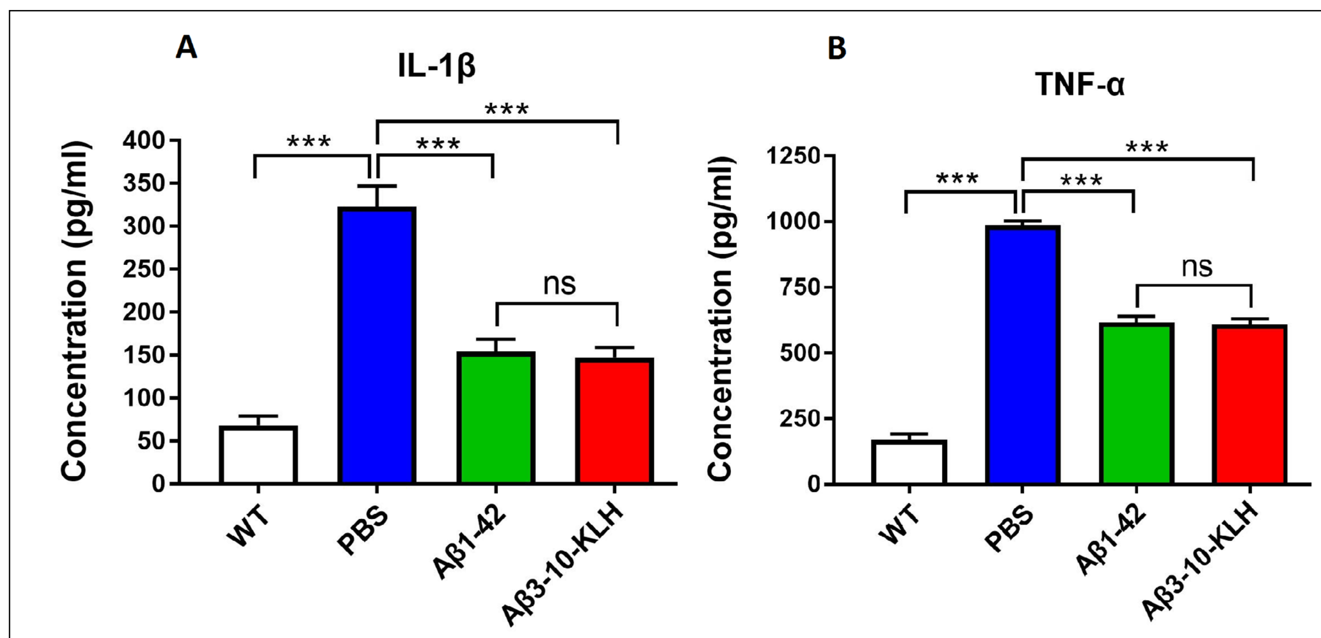

Fig. 5. Aβ3-10-KLH or Aβ1-42 vaccination reduces the contents of IL-1β and TNF-α in the brain of the mice. Compared with the PBS group, the contents of IL-1β (A) and TNF-α (B) in the brain of the transgenic mice were decreased significantly in the Aβ3-10-KLH and Aβ1-42 vaccine group at 10 months of age (***$P<0.001$). There was no significant difference between Aβ3-10-KLH and Aβ1-42 vaccine group (ns: no significance).

Aβ plaque clearance, but astrocytes around severe Aβ plaques were gradually necrotic. It suggests that Aβ plaques have obvious toxicity on astrocytes and that the elimination of Aβ plaques would be essential for the maintenance of astrocytes homeostasis.

In the CNS, the microglia are the available and intrinsic phagocytes that take part in the uptake and clearance of different forms of Aβ (Lee and Landreth, 2010; Villacampa and Heneka, 2020). Some studies showed that the accumulation and deposits of Aβ induced the mobilization of an innate immune response, then caused the pathogenic cascades of AD, suggesting the critical link between the immune system (especially microglia) and Aβ plaques (Heneka et al., 2015a; 2015b; Weitz and Town, 2012). With the formation of Aβ plaques and NTFs, the brain of AD patients presents an obvious chronic neuroinflammatory response characterized by increased reactive microglia and increased levels of proinflammatory cytokines (Scheltens et al., 2016). Furthermore, free irons also participate in increasing oxidative stress in microglia, causing a series of biochemical changes in the CNS (Chiziane et al., 2018), but whether the activation of microglia is beneficial or harmful to the neurons is still obscure (Swanson et al., 2020; Villacampa and Heneka, 2020). Recent studies suggest that different phenotypes of microglia could have different effects on AD progression (Cunningham, 2013; Lyman et al., 2014). Activated microglia not only strongly induces the activation of astrocytes by secreting neurotoxins and various complement components but also promotes synaptic degeneration and neuronal death together with active astrocytes (Liddelow et al., 2017). In this study, the involvement of the microglia in the AD-associated changes was assessed after Aβ3-10-KLH and Aβ1-42 vaccination. There was a general decrease of the TMEM119 labeling intensity in vaccinated APP/PS1 mice compared with the control group. This might suggest that reducing the formation of Aβ plaques through immunotherapy reduced the activation of microglia cells in the brain at the early stage of AD. Nevertheless, because the response of microglia in mouse models seems more intense than in humans (Navarro et al., 2018), the effect of immunotherapy on microglia and neuroinflammation in late AD brains needs further study.

Although immunotherapy has been observed to have a good effect in transgenic animals, the limitation of the study is due to the small sample size. Adverse effects and possible mouse death were not observed. Whether the results can be translated to humans is unknown. Therefore, more studies are needed to verify their effectiveness in clinical research.

CONCLUSION

The application of Aβ3-10-KLH and Aβ1-42 vaccines in APP/PS1 transgenic mice reduced the formation of

Aβ plaques and decreased astrocyte and microglia activation. This study suggests a new and safe measure for treating AD. Immunotherapy offers promising therapeutics that could prevent the development of amyloid pathologies and cognitive decline.

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REFERENCES

Acosta C, Anderson HD, Anderson CM (2017) Astrocyte dysfunction in Alzheimer disease. J Neurosci Res 95: 2430–2447.

Anand R, Gill KD, Mahdi AA (2014) Therapeutics of Alzheimer's disease: Past, present and future. Neuropharmacology 76 Pt A: 27–50.

Avila-Munoz E, Arias C (2014) When astrocytes become harmful: functional and inflammatory responses that contribute to Alzheimer's disease. Ageing Res Rev 18: 29–40.

Beach TG, Walker R, McGeer EG (1989) Patterns of gliosis in Alzheimer's disease and aging cerebrum. Glia 2: 420–436.

Bennett FC, Bennett ML, Yaqoob F, Mulinyawe SB, Grant GA, Hayden Gephart M, et al. (2018) A combination of ontogeny and CNS Environment establishes microglial identity. Neuron 98: 1170–1183.

Bennett ML, Bennett FC, Liddelow SA, Ajami B, Zamanian JL, Fernhoff NB, et al. (2016) New tools for studying microglia in the mouse and human CNS. Proc Natl Acad Sci 113: E1738–1746.

Bittar A, Sengupta U, Kayed R (2018) Prospects for strain-specific immunotherapy in Alzheimer's disease and tauopathies. NPJ Vaccines 3: 9.

Chiziane E, Telemann H, Krueger M, Adler J, Arnhold J, Alia A, Flemmig J (2018) Free heme and amyloid-beta: a fatal liaison in Alzheimer's disease. J Alzheimers Dis 61: 963–984.

Cline EN, Bicca MA, Viola KL, Klein WL (2018) The amyloid-beta oligomer hypothesis: beginning of the third decade. J Alzheimers Dis 64: S567–S610.

Cunningham C (2013) Microglia and neurodegeneration: the role of systemic inflammation. Glia 61: 71–90.

Das S, Basu A (2008) Inflammation: a new candidate in modulating adult neurogenesis. J Neurosci Res 86: 1199–1208.

Delekate A, Fuchtemeier M, Schumacher T, Ulbrich C, Foddis M, Petzold GC (2014) Metabotropic P2Y1 receptor signalling mediates astrocytic hyperactivity in vivo in an Alzheimer's disease mouse model. Nat Commun 5: 5422.

Ding L, Meng Y, Zhang HY, Yin WC, Yan Y, Cao YP (2016) Active immunization with the peptide epitope vaccine Abeta3–10-KLH induces a Th2-polarized anti-Abeta antibody response and decreases amyloid plaques in APP/PS1 transgenic mice. Neurosci Lett 634: 1–6.

Flores J, Noel A, Foveau B, Lynham J, Lecrux C, LeBlanc AC (2018) Caspase-1 inhibition alleviates cognitive impairment and neuropathology in an Alzheimer's disease mouse model. Nat Commun 9: 3916.

Gemma C, Bickford PC (2007) Interleukin-1beta and caspase-1: players in the regulation of age-related cognitive dysfunction. Rev Neurosci 18: 137–148.

Giaume C, Kirchhoff F, Matute C, Reichenbach A, Verkhratsky A (2007) Glia: the fulcrum of brain diseases. Cell Death Differ 14: 1324–1335.

Gomez-Arboledas A, Davila JC, Sanchez-Mejias E, Navarro V, Nunez-Diaz C, Sanchez-Varo R, et al. (2018) Phagocytic clearance of presynaptic dystrophies by reactive astrocytes in Alzheimer's disease. Glia 66: 637–653.

Guo T, Zhang D, Zeng Y, Huang TY, Xu H, Zhao Y (2020) Molecular and cellular mechanisms underlying the pathogenesis of Alzheimer's disease. Mol Neurodegener 15: 40.

Heneka MT, Carson MJ, El Khoury J, Landreth GE, Brosseron F, Feinstein DL, et al. (2015a) Neuroinflammation in Alzheimer's disease. Lancet Neurol 14: 388–405.

Heneka MT, Golenbock DT, Latz E (2015b) Innate immunity in Alzheimer's disease. Nat Immunol 16: 229–236.

Iglesias J, Morales L, Barreto GE (2017) Metabolic and Inflammatory adaptation of reactive astrocytes: role of PPARs. Mol Neurobiol 54: 2518–2538.

Janus C, Pearson J, McLaurin J, Mathews PM, Jiang Y, Schmidt SD, et al. (2000) A beta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease. Nature 408: 979–982.

Karim MR, Wang YF (2019) Phenotypic identification of CD19+CD5+CD1d+ regulatory B cells that produce interleukin 10 and transforming growth factor β1 in human peripheral blood. Arch Med Sci 15: 1176–1183.

Lee CY, Landreth GE (2010) The role of microglia in amyloid clearance from the AD brain. J Neural Transm 117: 949–960.

Lee L, Kosuri P, Arancio O (2014) Picomolar amyloid-beta peptides enhance spontaneous astrocyte calcium transients. J Alzheimers Dis 38: 49–62.

Lemere CA (2013) Immunotherapy for Alzheimer's disease: hoops and hurdles. Mol Neurodegener 8: 36.

Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, et al. (2017) Neurotoxic reactive astrocytes are induced by activated microglia. Nature 541: 481–487.

Liu Z, Condello C, Schain A, Harb R, Grutzendler J (2010) CX3CR1 in microglia regulates brain amyloid deposition through selective protofibrillar amyloid-beta phagocytosis. J Neurosci 30: 17091–17101.

Lyman M, Lloyd DG, Ji X, Vizcaychipi MP, Ma D (2014) Neuroinflammation: the role and consequences. Neurosci Res 79: 1–12.

Mulder SD, Veerhuis R, Blankenstein MA, Nielsen HM (2012) The effect of amyloid associated proteins on the expression of genes involved in amyloid-beta clearance by adult human astrocytes. Exp Neurol 233: 373–379.

Navarro V, Sanchez-Mejias E, Jimenez S, Munoz-Castro C, Sanchez-Varo R, Davila JC, et al. (2018) Microglia in Alzheimer's disease: activated, dysfunctional or degenerative. Front Aging Neurosci 10: 140.

Patton RL, Kalback WM, Esh CL, Kokjohn TA, Van Vickle GD, Luehrs DC, et al. (2006) Amyloid-beta peptide remnants in AN-1792-immunized Alzheimer's disease patients: a biochemical analysis. Am J Pathol 169: 1048–1063.

Ries M, Sastre M (2016) Mechanisms of Aβ clearance and degradation by glial cells. Front Aging Neurosci 8: 160.

Ronco V, Grolla AA, Glasnov TN, Canonico PL, Verkhratsky A, Genazzani AA, Lim D (2014) Differential deregulation of astrocytic calcium signalling by amyloid-beta, TNFalpha, IL-1beta and LPS. Cell Calcium 55: 219–229.

Rosenberg RN (2005) Immunotherapy for Alzheimer disease: the promise and the problem. Arch Neurol 62: 1506–1507.

Scheltens P, Blennow K, Breteler MM, de Strooper B, Frisoni GB, Salloway S, Van der Flier WM (2016) Alzheimer's disease. Lancet 388: 505–517.

Selkoe DJ, Hardy J (2016) The amyloid hypothesis of Alzheimer's disease at 25 years. EMBO Mol Med 8: 595–608.

Simpson JE, Ince PG, Lace G, Forster G, Shaw PJ, Matthews F, Ageing Neuropathology Study Group (2010) Astrocyte phenotype in relation to Alzheimer-type pathology in the ageing brain. Neurobiol Aging 31: 578–590.

Song G, Yang H, Shen N, Pham P, Brown B, Lin X, et al. (2020) An immunomodulatory therapeutic vaccine targeting oligomeric amyloid-beta. J Alzheimers Dis 77: 1639–1653.

Swanson MEV, Scotter EL, Smyth LCD, Murray HC, Ryan B, Turner C, et al. (2020) Identification of a dysfunctional microglial population in human Alzheimer's disease cortex using novel single-cell histology image analysis. Acta Neuropathol Commun 8: 170.

Tiwari S, Atluri V, Kaushik A, Yndart A, Nair M (2019) Alzheimer's disease: pathogenesis, diagnostics, and therapeutics. Int J Nanomedicine 14: 5541–5554.

Vasile F, Dossi E, Rouach N (2017) Human astrocytes: structure and functions in the healthy brain. Brain Struct Funct 222: 2017–2029.

Vellas B, Black R, Thal LJ, Fox NC, Daniels M, McLennan G, Tompkins C, Leibman C, Pomfret M, Grundman M, AN1792 (QS-21)-251 Study Team (2009) Long-term follow-up of patients immunized with AN1792: reduced functional decline in antibody responders. Curr Alzheimer Res 6: 144–151.

Villacampa N, Heneka MT (2020) Microglia in Alzheimer's disease: Local heroes! J Exp Med 217: e20192311.

Wang JC, Zhu K, Zhang HY, Wang GQ, Liu HY, Cao YP (2020) Early active immunization with Aβ3–10-KLH vaccine reduces tau phosphorylation in the hippocampus and protects cognition of mice. Neural Regen Res 15: 519–527.

Weitz TM, Town T (2012) Microglia in Alzheimer's disease: it's all about context. Int J Alzheimers Dis 2012: 314185.

Yan P, Hu X, Song H, Yin K, Bateman RJ, Cirrito JR, et al. (2006) Matrix metalloproteinase-9 degrades amyloid-beta fibrils in vitro and compact plaques in situ. J Biol Chem 281: 24566–24574.

Yiannopoulou KG, Papageorgiou SG (2020) Current and future treatments in Alzheimer disease: an update. J Cent Nerv Syst Dis 12: 1179573520907397.

Zhong Z, Yang L, Wu X, Huang W, Yan J, Liu S, et al. (2014) Evidences for B6C3-Tg (APPswe/PSEN1dE9) double-transgenic mice between 3 and 10 months as an age-related Alzheimer's disease model. J Mol Neurosci 53: 370–376.