Anti-TNF-α reduces amyloid plaques and tau phosphorylation and induces CD11c-positive dendritic-like cell in the APP/PS1 transgenic mouse brains

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ABSTRACT

Inflammation plays an important role in the pathogenesis of Alzheimer’s disease (AD). Overexpression of tumor necrosis factor-α (TNF-α) occurs in the AD brain. Recent clinical studies have shown that the anti-TNF-α therapy improves cognition function of AD patients rapidly. However, the underlying mechanism remains elusive. The present study investigates the effects of intracerebroventricular injection of the monoclonal TNF-α antibody, Infliximab, on the pathological features of AD in the APP/PS1 double transgenic mice. We found that Infliximab administration reduced the levels of TNF-α, amyloid plaques, and tau phosphorylation as early as three days after daily injection of 150 μg Infliximab for three days. The number of CD11c-positive dendritic-like cells and the expression of CD11c were found to be increased concurrently after Infliximab injection. These data suggested that the CD11c-positive dendritic-like cells might contribute to the Infliximab-induced reduction of AD-like pathology. Furthermore, our results support the use of anti-TNF-α for the treatment of AD.

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1. Introduction

Alzheimer’s disease (AD) is the most common cause of dementia in the elderly, which is characterized clinically by a cognitive decline and pathologically by the formation of amyloid β-peptide (Aβ) and neurofibrillary tangles in the brain. Because of the lack of effective treatment, AD is a huge global burden (Roberson and Mucke, 2006; Brookmeyer et al., 2007). Hence, it is imperative to search for new drugs for AD.

Neuroinflammation is known to play a key role in AD (Frank-Cannon et al., 2009). Epidemiological studies have shown that non-steroidal anti-inflammatory drugs may delay...
or prevent the onset of AD, especially with prolonged use (Zandi et al., 2002; in ’t Veld et al., 2001; Stewart et al., 1997; Szekely et al., 2004). It is clear that NSAIDs show evidence of preventative effects but little evidence of treatment effects. Thus, other types of anti-inflammatory agents for the treatment of AD need to be investigated. It was reported that perispinal administration of etanercept, an anti-TNF fusion protein, improves cognition of patients with AD in a 6-month pilot study (Tobinick et al., 2006). Rapid cognitive improvement began within hours after administration of anti-TNF-α to patients with moderate late-onset AD (Tobinick and Gross, 2008). The further study that was performed by the same group suggested that perispinal administration could deliver the drug into lateral and third cerebral ventricles rapidly (Tobinick et al., 2009). The underlying mechanism by which anti-TNF-α improves cognition in AD remains to be clarified.

Many studies have demonstrated the relationship between excess TNF-α in the brain and the pathological features of AD (Tarkowski et al., 1999; Perry et al., 2001; Tarkowski et al., 2003). A recent study suggested that inhibition of soluble TNF signaling in a mouse model of AD prevents pre-plaque amyloid-associated neuropathology (McAlpine et al., 2009). We hypothesized that the benefits of anti-TNF treatment for AD might attribute to the reduction of amyloid-associated neuropathology. Here, we investigated the protective effects of intracerebroventricular (ICV) injection of TNF-α monoclonal antibody (Infliximab) on the AD pathological features in aged APP/PS1 double transgenic mice. Our results suggest a possible mechanism of action mediated by Infliximab.

2. Results

2.1. Infliximab decreases amyloid plaques in APP/PS1 transgenic mice

We first stained the brain sections of the 12-months old APP/PS1 mice by Thioflavin T, a fluorescent agent widely used for staining of amyloid plaques, and observed numerous amyloid plaques in the cerebral cortex and the hippocampus (Fig. 1a). The same staining of the brain sections from control mice showed only background (data not shown). Significantly less amyloid plaques were seen in the sections of mice 3–14 days after ICV injection of Infliximab (Fig. 1b), suggesting that administration of Infliximab reduces the number of amyloid plaques in the APP/PS1 mouse brains.

To confirm the above results, we investigated the effects of Infliximab on cerebral amyloid plaques by using immunohistochemical staining with antibody against Aβ1-42. Consistent with the observations with Thioflavin T staining, we observed marked reduction in the number of amyloid plaques in the brains of APP/PS1 mice 3–14 days after ICV injection of Infliximab (Fig. 2a). Semi-quantitative analysis revealed that Aβ deposits in the treated mice were reduced to approximately 40%–60% (Fig. 2b). We also determined buffer-soluble and -insoluble (extracted with 70% formic acid) Aβ42 by ELISA and found that ICV injection of Infliximab led to reduction of both soluble and insoluble Aβ42 in the mouse brain (Figs. 2c and d).

Fig. 1 – Thioflavin T staining of brain sections of 12-month old APP/PS1 mice. (a) Brain sections of APP/PS1 mice without any treatment. Magnification was shown by 400× or 200×. (b) Brain sections of APP/PS1 mice after ICV injection of normal human IgG or 150 μg Infliximab per day for three days. Ctrl, normal human IgG-injected group; D3, D7, D14, mice sacrificed 3, 7, and 14 days after the last ICV injection of Infliximab, respectively. Magnification, 200×.
2.2. Infliximab decreases tau phosphorylation in APP/PS1 transgenic mice

Abnormal hyperphosphorylation of tau is a characteristic biochemical change in AD brain and is also seen in the brains of the APP/PS1 transgenic mice (Kurt et al., 2003). By using Western blotting with an antibody specific to phosphorylated tau, we found that ICV injection of Infliximab reduced tau hyperphosphorylation significantly (Fig. 3a). Semi-quantitative analysis indicated a reduction of tau phosphorylation by up to 70% at 3 days after Infliximab injection. The reduction appeared to be less when examined at longer times after Infliximab injection (Fig. 3b).

2.3. Infliximab leads to the increase of local CD11c-positive dendritic-like cells in brain

It has been reported that brain CD11c-positive dendritic-like cells helped clear amyloid plaques in AD mouse model (Butovsky et al., 2006, 2007). Thus, we studied whether ICV
Injection of Infliximab induced CD11c-positive dendritic-like cells by immunohistochemical staining with antibody against CD11c. We found a marked increase in the CD11c-positive dendritic-like cells in the brains of mice 3, 7, and 14 days after Infliximab administration (Figs. 4a and b). Consistent with these morphological observations, we also observed increased CD11c by Western blots in the brain tissue after Infliximab injection (Figs. 4c and d).

To investigate the role of CD11c-positive dendritic-like cells in the brain, we carried out immunofluorescence
staining with anti-\(A\beta_{1-42}\) and anti-CD11c for the consecutive sections. We observed that most of the CD11c-positive dendritic-like cells were located near amyloid plaques (Fig. 5), suggesting a potential role of the dendritic-like cells in clearing the plaques.

2.4. **Infliximab decreases TNF-\(\alpha\) in APP/PS1 transgenic mice**

To investigate the potential mechanism by which ICV injection of Infliximab leads to reduced amyloid deposition and tau phosphorylation in APP/PS1 mice, we investigated the level of TNF-\(\alpha\) before and after the injection of Infliximab. By using Western blot analysis, we found that Infliximab injection decreased TNF-\(\alpha\) in the mouse brain significantly at 3 and 7 days after injection (Fig. 6). The reduction seemed to disappear 14 days after Infliximab injection.

3. **Discussion**

Anti-TNF-\(\alpha\) therapy for AD has showed some efficacy in pilot human trials [9, 10], but the underlying mechanism is unclear. We investigated here the effects of Infliximab on the pathological changes in the APP/PS1 double transgenic mouse model of AD. We found that ICV administration of Infliximab reduced amyloid plaques and tau phosphorylation in the transgenic mouse brains. The increase of local CD11c-positive dendritic-like cells, which are located near amyloid plaques in the brain after Infliximab administration, suggested that they might play a pivotal role in the clearance of SP and hyperphosphorylation of tau.

There have been some controversies with regard to the role of TNF-\(\alpha\) in AD. It has been shown that TNF-\(\alpha\) was necessary for the synaptic plasticity in cortical neurons [Beattie et al., 2002;...]

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**Fig. 5** – Immunofluorescence staining with rabbit anti-\(A\beta_{1-42}\) (green) and rabbit anti-CD11c (red) of the temporal cortex of 12-month old APP/PS1 mice after ICV injections of normal human IgG (Ctrl) or Infliximab for 3 days. D3, D7, and D14 indicate mice examined 3, 7, and 14 days, respectively, after the last injection. Magnification, 400×.

**Fig. 6** – Determination of TNF-\(\alpha\). (a) Western blots of TNF-\(\alpha\) in the crude brain extracts of wild-type mice and APP/PS1 mice after ICV injections of normal human IgG (Ctrl) or Infliximab for 3 days. D3, D7, and D14 indicate mice examined 3, 7, and 14 days, respectively, after the last injection. GAPDH blot was included as a loading control. (b) Quantification of the blots as shown in (a).
A mouse model lacking TNF-α receptors was more susceptible than wild-type animals to brain injury and neuronal damage (Bruce et al., 1996; Sullivan et al., 1999; Thompson et al., 2004). However, more studies suggest neurotoxic roles of TNF-α. TNF-α increases apoptosis of neurons treated with Aβi (Blasko et al., 1997) and may increase Aβp production through upregulation of both β- and γ-secretase expression (Yamamoto et al., 2007) and γ-secretase activity (Liao et al., 2004; Kuo et al., 2008), as well as the expression of APP (Lahiri et al., 2003). Knock-out of TNF-α receptor 1 prevents Aβp generation and learning deficits (He et al., 2007). Similarly, pharmacological blockade of TNF-α reduced behavioral deficit in another mouse model (Medeiros et al., 2007). The clinical efficacy and the reduction of AD-like pathological changes observed here in the APP/PS1 double transgenic mouse model of AD support the neurotoxic roles of TNF-α in the brain. Our observations suggest that excessive TNF-α may play a role in the progress of AD.

We found that ICV administration of Infliximab decreased TNF-α in the mouse brain as early as three days after injection, but the reduction disappeared 14 days after injection (Fig. 6). We also found less reduction of Aβi and tau phosphorylation 14 days after injection as compared with 3 or 7 days (Figs. 2 and 3). These observations further support the role of TNF-α in AD pathogenesis in the transgenic mice. The half-life of Infliximab is approximately 8.5 days (Giansanti et al., 2008), which could explain why, at 14 days after injection, the TNF-α level returned to that seen before injection.

A recent study in which anti-TNF-α was administered to PDAPP mice by intraperitoneal injection did not observe significant effect of anti-TNF-α on amyloid burden (Giuliani et al., 2009). These observations suggest that anti-TNF-α drug could not pass through the blood–brain barrier. In this study, we injected Infliximab directly into the lateral ventricle of the brain. Infliximab is a human–mouse chimeric TNF-α monoclonal antibody, the variable region of which is derived from mouse. Here, we demonstrated the beneficial effect of Infliximab. We speculated that over-production of TNF-α may inhibit the function of some specific cells, i.e. microglia, which could help clear Aβi. There were two possibilities about the origin of CD11c-positive dendritic-like cells. Either they were derived from local microglia or they were from bone marrow-derived dendritic cells. Interestingly, microglia also originated from the same precursors as those of dendritic cells, and specific environmental cytokines decide the type of the cell, either microglia or DCs (Fischer et al., 2006; Santambrogio et al., 2001). Recent data showed that bone marrow-derived activated microglia were very efficient in restricting amyloid deposits in AD (Simard et al., 2006). Our previous study has suggested that expression of TNF-α is involved in the function of dendritic cells (Xu et al., 2007). Collectively, both activated CD11c-positive microglia and bone marrow-derived dendritic cells play a role in clearing amyloid plaques in AD. We found that the action of Infliximab may attribute partly to the rapid increase of CD11c-positive dendritic-like cells in the brain. But further work was needed to explore the detailed origin of Infliximab-induced CD11c-positive dendritic-like cells.

The time course study of the level of TNF-α in the Infliximab-injected mouse brains indicated a clean up of TNF-α over-production 3 and 7 days after injection. This clean up of TNF-α coincided with the reduction of Aβi level, amyloid plaques, and hyperphosphorylated tau, which supports the role of TNF-α in these pathological changes in the mouse brains. Probably due to the turnover of Infliximab in the brain, the TNF-α level returned to the initial high level 14 days after injection. Consistent with the recovery of TNF-α level, the levels of Aβi level, amyloid plaques, and hyperphosphorylated tau were also higher at 14 days as compared to 3 and 7 days after injection. The smaller extents of the relapse suggest that a longer time is required for Aβi buildup and tau hyperphosphorylation than that of TNF-α over-production. These observations also suggest that Aβi buildup and tau hyperphosphorylation are reversible, at least at early stages of pathogenesis, and that continuous treatment may be required to achieve therapeutic efficacy. Thus, our data suggested that CD11c-positive dendritic-like cells might contribute to the Infliximab-induced reduction of AD-like pathology.

In summary, we provide data supporting the use of anti-TNF-α for the treatment of AD. Anti-TNF-α reduced amyloid plaques and tau phosphorylation in the mouse brains and the increase of local CD11c-positive dendritic-like cells in the brain after Infliximab administration might play a pivotal role in the clearance of SP and hyperphosphorylation of tau. Anti-Aβi active and passive immunotherapy markedly reduced Aβi burden and reversed behavioral impairment in mouse models of AD, but significant adverse effects occur in human trials. A 6-month pilot study has demonstrated the safety of anti-TNF-α therapy (Tobinick and Gross, 2008). Given its efficacy and safety, anti-TNF-α immunotherapy would offer a superior alternative therapeutic approach for AD and potentially for other neurodegenerative disorders, of which TNF-α is involved in the pathogenesis, such as Parkinson’s disease and amyotrophic lateral sclerosis.

4. Experimental procedures

4.1. Reagents

The following reagents were used in this study: Infliximab (Remicade) (Cilag AG, Switzerland); Normal Human IgG control (R&D Systems, United States); Thiouanilin (T) (Sigma-Aldrich, United States); rabbit anti-p-Tau (pThr181) (Sigma-Aldrich, United States); rabbit anti-Tau (Tau-5) (Santa Cruz, United States); rabbit anti-Aβi1-42, rabbit anti-CD11c, rabbit anti-TNF-α (Abcam, United States); rabbit anti-APP (Aldrich, United States); rabbit anti-AP1 (A,42, rabbit anti-CD11c, rabbit anti-TNF-α (Abcam, United States); goat anti-rabbit GAPDH antibody (Wuhan Boster Biological Technology Co., Ltd, China), rhodamine (TRITC)-conjugated AffiniPure goat anti-mouse IgG (H+L), fluorescein (FITC)-conjugated AffiniPure goat anti-rabbit IgG (H+L) (Jackson Immunoresearch Laboratories, Inc., USA); and Supervision™ Universal (Anti-Mouse/Rabbit) Detection Reagent (HRP) (Shanghai Changdao Biotech Co., Ltd, China).

4.2. Mice and study design

Twenty APP/PS1 transgenic mice (12 months old) were obtained from the Institute of Zoology, Chinese Academy of Sciences, and were housed in an air-conditioned room under a photoperiod of a 12:12 h-cycle (lights on, 8:00 am through 8:00 pm). Food and water were provided ad libitum. The mice were
randomly divided into 4 groups (Table 1). The protocol was approved by the Nanjing Medical University Experimental Animal Care and Use Committee.

4.3. ICV injection

Mice were anesthetized with intraperitoneal injection of 10% chloral hydrate (0.4 g/kg) and then placed on a stereotaxic apparatus. A total of 150 μg Infliximab in 7.5 μl of phosphate buffered saline (PBS) or, as a control, normal human IgG alone was injected into the lateral ventricle of each mouse, as described elsewhere (Skovronsky et al., 2000). Each mouse received daily injections for three consecutive days. On days 3, 7, and 14 after the last injection, the mice were sacrificed (Table 1) as described below.

4.4. Immunohistochemistry

Mice were anesthetized and sacrificed. Then, the brains were removed and were cut into halves. One half was used for Western blotting, the other for immunohistochemistry. The half then was fixed in 4% paraformaldehyde for 20 h. After being dehydrated in alcohol, the brains were embedded in paraffin and cut into 3–4 μm sections. Sections were deparaffinized, hydrated in distilled water, treated with 3% H2O2 for 10 min to remove residual peroxidase activity, and then rinsed with PBS. Sections were permeabilized with 1% NP-40/0.1% TritonX-100 for 10 min, rinsed with PBS, and blocked with 10% normal goat serum. Two consecutive sections were then incubated overnight with rabbit anti-Aβ1-42 and rabbit anti-CD11c at a dilution of 1:600 and 1:150, respectively. Then, the consecutive sections were developed using rhodamine (TRITC)-conjugated AffiniPure goat anti-rabbit IgG (H+L) and fluorescein (FITC)-conjugated AffiniPure goat anti-rabbit IgG (H+L), respectively. The fluorescence immunostaining was detected by a fluorescence microscope, and the two consecutive images were merged by Adobe Photoshop CS3.

4.7. ELISA assay for Aβ42

Soluble and insoluble Aβ42 in brain samples was quantified using ELISA, as previously described (Kawarabayashi et al., 2001). Briefly, brain tissue was homogenized in extraction buffer consisting of 50 mM Tris (pH 7.4), 2 mM EDTA, 400 mM NaCl, and complete protease inhibitor cocktail (Roche). The homogenates were centrifuged at 20,000 × g for 5 min at 4 °C. The resulting supernatants were analyzed for soluble Aβ. The pellets were homogenized in 70% formic acid and centrifuged at 44,000 × g for 5 min at 4 °C. The resulting supernatants were neutralized with 1 M Tris and then diluted in ELISA buffer for the measurement of insoluble Aβ. Samples were prepared from 5 animals for each group. All samples were analyzed in triplicates. Standard curves were made using human Aβ42 standards provided in the ELISA kit (Wako, United States). All antibodies that used in ELISA were provided by Beijing Biosynthesis Biotechnology Co., Ltd, China.

4.8. Western blotting

Western blotting was performed as previously described (Lu et al., 2008). Samples were prepared from 5 animals for each group. Brain tissues were homogenized in homogenization buffer containing 50 mM Tris/HCl, pH 7.5, 0.1% dithiothreitol, 0.2 mM EDTA, 0.2 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1.25 μg/ml pepstatin A, 0.2 μg/ml aprotinin, 0.2 μg/ml leupeptin, 5 nM tetrahydrobiopterin, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 2 mM sodium pyrophosphate, and 1% Nonidet P-40 (NP-40). Homogenates were then centrifuged at 14,000 rpm at 4 °C for 10 min. Protein concentrations of the supernatants were determined by Bradford method using BSA as standard. Extract proteins (50 μg/lane) were separated in 8% SDS-PAGE, and proteins were transferred to polyvinylidene difluoride (PVDF) membranes and incubated with primary polyclonal antibodies against TNF-α (1:1000), p-Tau (1:2500), Tau (1:2000), CD11c (1:2000), or GAPDH (1:500) at 4 °C.

| Table 1 – Experimental design of the APP/PS1 transgenic mice. |
|----------------|----------------|----------------|----------------|
| Group | N | Injection/day | Frequency of injection | Time the brains were collected after the last injection (days) |
| Ctrl | 5 | 150 μg normal human IgG | daily × 3 days | 7 |
| D3 | 5 | 150 μg Infliximab | daily × 3 days | 3 |
| D7 | 5 | 150 μg Infliximab | daily × 3 days | 7 |
| D14 | 5 | 150 μg Infliximab | daily × 3 days | 14 |
overnight. Membranes were then incubated with secondary antibodies (1:1000) at room temperature for 1 h. Finally, membranes were developed with the enhanced chemiluminescence (ECL) system. The immunoreactivity was quantitated using Image J software.

4.9. Immunohistochemistry semi-quantification and statistical analysis

All procedures were performed by an individual blinded to the treatments. Semi-quantitative analysis of immunohistochemistry was performed by counting the number of positive plaques or cells in the same plane of the temporal cortices in 5 randomly selected fields (×100 fields for anti-Aβ1-42 or ×400 fields for anti-p-Tau and anti-CD11c) in every section using Image Pro-plus software. A total of 5 sections per brain were counted. A total of 5 animals per group were counted. All results were expressed as mean±SD and were followed by the LSD procedure. If variances were unequal, were evaluated by one-way analysis of variance (ANOVA) was performed using computer software SPSS 13.0. If variances were equal, Games–Howell procedure was followed by ANOVA. A value of P<0.05 is considered to be statistically significant.

5. Author contributions

JX, YDZ, and WS participated in the concept and design of the study. JQS, JC, BRW, LLZ, and YWZ participated in the acquisition of raw data, analysis and interpretation of data, and drafting the manuscript. HZ and QZ participated in the acquisition of raw data, analysis and interpretation of data, and the critical revision of the manuscript.

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REFERENCES


