T cells promote the regeneration of neural precursor cells in the hippocampus of Alzheimer's disease mice

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Abstract

Alzheimer's disease is closely associated with disorders of neurogenesis in the brain, and growing evidence supports the involvement of immunological mechanisms in the development of the disease. However, at present, the role of T cells in neuronal regeneration in the brain is unknown. We injected amyloid-beta 1–42 peptide into the hippocampus of six BALB/c wild-type mice and six BALB/c-nude mice with T-cell immunodeficiency to establish an animal model of Alzheimer's disease. A further six mice of each genotype were injected with same volume of normal saline. Immunohistochemistry revealed that the number of regenerated neural progenitor cells in the hippocampus of BALB/c wild-type mice was significantly higher than that in BALB/c-nude mice. Quantitative fluorescence PCR assay showed that the expression levels of peripheral T cell-associated cytokines (interleukin-2, interferon-γ) and hippocampal microglia-related cytokines (interleukin-1β, tumor necrosis factor-α) correlated with the number of regenerated neural progenitor cells in the hippocampus. These results indicate that T cells promote hippocampal neurogenesis in Alzheimer's disease and T-cell immunodeficiency restricts neuronal regeneration in the hippocampus. The mechanism underlying the promotion of neuronal regeneration by T cells is mediated by an increased expression of peripheral T cells and central microglial cytokines in Alzheimer's disease mice. Our findings provide an experimental basis for understanding the role of T cells in Alzheimer's disease.

Key Words: nerve regeneration; neurodegeneration; Alzheimer's disease; beta-amyloid 1–42 peptide; neuronal precursors; mice; microglia; interleukin-2; interferon-gamma; interleukin-1β; tumor necrosis factor-α; microtubule associated protein; NSFC grant; neural regeneration

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Introduction

Alzheimer's disease (AD), a degenerative disease of the central nervous system, is characterized pathologically by extracellular senile plaques, intracellular neurofibrillary tangles, and a reduction in the number of neurons in the cerebral cortex and hippocampus. The clinical manifestations for AD include loss of memory, and cognitive and behavioral disorders (Finder, 2010). AD is the most common type of dementia, but its etiology and pathogenesis remain unclear. At present, AD is considered a complex pathological process involving several factors. The amyloid cascade hypothesis, or amyloid-beta (Aβ) toxicity hypothesis, has dominated research for decades, and postulates that the deposition of Aβ peptide in the brain is a central event in AD (Honjo et al., 2012). Although AD is not recognized as a classic immune response-mediated disease, growing evidence highlights the immunological mechanisms closely involved in the occurrence and development of AD (Schroeter et al., 2008; Tabira, 2010). According to the immune hypothesis of AD pathogenesis, when immune dysfunction occurs, Aβ metabolism is disrupted. Subsequently, inflammatory and neurotoxic cascade reactions occur, leading to synaptic damage, and neuronal degeneration and death in the brain, ultimately inducing AD (Chopra et al., 2011).

As the mechanism underlying neuronal death in AD has been investigated, researchers have begun to focus on the opposite aspect, newborn neurons, in AD pathogenesis (Donovan et al., 2006; Zhang et al., 2007; Yu et al., 2009; Biscaro et al., 2012). The precursor cells in the subventricular zone of the brain have the ability to regenerate (Alvare-Buylla et al., 2004). In adults, neurogenesis provides a specific mechanism for plasticity of the nervous system (Lazarov et al., 2010). AD pathology studies have revealed that damage to the regions where adult neural cells form (subventricular zone and subgranular zone) leads to a dysfunction in neuronal regeneration; if the dead neurons cannot be replaced by new neurons in time, memory and cognitive disorders will inevitably occur (Demars et al., 2010). AD pathogenesis is closely associated with disorders of neuronal regeneration in the brain, and the effect of immunological mechanisms on neuronal regeneration has become a focus of current AD research. Patients with AD have a significantly higher num-

ber of T cells in the brain than healthy people. Immune cells cross the blood-brain barrier and enter the brain, participating in its physiological and pathological functions (Togo et al., 2002; Cao et al., 2009; Monsonego et al., 2013). Additionally, immune cells are shown to maintain nerve cell regeneration function (Ziv et al., 2008). Central-specific T cells play an important role in the maintenance of adult learning and memory capacity, and a deficiency of T cells leads to severe impairments in spatial learning and memory in adult rats (Ziv et al., 2006). We hypothesize that, in AD pathology, T cells are involved in the maintenance of nervous system plasticity, which is also related to neuron regeneration. To our knowledge, no studies have examined the correlation between T cells and neuronal regeneration in the brain. Therefore, the aim of the present study was to investigate the role of T cells in hippocampal neurogenesis in AD pathogenesis, and the underlying molecular mechanisms, in an effort to reveal the contribution of T cells in neuronal regeneration, using immunohistochemistry and quantitative PCR techniques.

Materials and Methods

Animals
Twelve BALB/c wild-type (WT) mice and 12 BALB/c-nude mice, all specific pathogen free, with T lymphocyte deficiency were provided by Guangdong Medical Laboratory Animal Center, China (license No. SCXK (Yue) 2008-0002). The mice were all male, aged 8 weeks, weighing 20–28 g, and housed for 1 week prior to experimentation. Experimental procedures were in accordance with the Guidelines of the Use of Experimental Animals, issued by the Ministry of Science in China.

Animal grouping
The mice were randomly divided into an experimental group and a control group ($n = 6$ per group). In experimental group I (WT + Aβ) and experimental group II (nude + Aβ), oligomeric state $Aβ_{1–42}$ was injected bilaterally into the hippocampal CA1 region, to establish a model of AD. In control group I (WT + NS) and control group II (nude + NS), mice received equivalent volumes of normal saline instead of $Aβ_{1–42}$.

On day 7 after modeling, peripheral blood samples collected from the mice were harvested for quantitative PCR detection of interleukin-2 (IL-2) and interferon-γ (IFN-γ) expression. The mouse brain was divided symmetrically along the midline. The left hemisphere was used for immunohistochemistry of hippocampal neuronal regeneration, and the right for quantitative PCR assay of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) expression in hippocampal tissue.

Establishment of AD models using hippocampal injection of $Aβ_{1–42}$
To prepare oligomeric state $Aβ_{1–42}$, freeze-dried $Aβ_{1–42}$ powder (500 μg; AnaSpec, San Jose, CA, USA) was dissolved in 100 μL of 1% NH₄OH solution for a stock solution at a concentration of 500 μg/100 μL, which was then aliquoted

(50 μg/10 μL) and stored at −20°C. At the time of experimentation, an aliquot was thawed and 15 μL normal saline was added to prepare the working solution (2 μg/μL, 50 μg/25 μL), which was incubated at 37°C for 24 hours. This allowed aggregation of $Aβ_{1–42}$ to toxic oligomeric Aβ (Dahlgren et al., 2002).

Mice were anesthetized by intraperitoneal injection of 0.4% sodium pentobarbital at a dose of 0.2 mL/10 g body weight. The heads were fixed onto a stereotaxic frame, and then the skull was drilled to create a hole at 2.3 mm posterior to bregma and 1.8 mm lateral to the midline, to 1.0 mm depth. A 25 μL microsyringe was inserted 2.0 mm into the brain, and 2 μL $Aβ_{1–42}$ working solution (experimental groups I and II) or saline (control groups I and II) was slowly (0.4 μL/minute) injected bilaterally into the hippocampal CA1 *via* micropipette (KDS Model 310 Plus, KD Scientific Holliston, MA, USA). The needles were maintained in place for 5 minutes and then slowly withdrawn to prevent leakage. The skin was sutured and disinfected with alcohol, followed by intramuscular injections of sodium penicillin (40,000 units) for 3 consecutive days. For the remainder of the experiment, mice were housed in specific-pathogen-free cages.

Harvesting the specimens
The brain tissue was harvested 7 days after injection. In brief, mice were anesthetized with 0.4% sodium pentobarbital *via* intraperitoneal injection, and 1 mL cardiac blood was collected and placed into a tube containing the anticoagulant EDTA. The sample was stored at −20°C for gene expression analysis. After the blood sample was collected, the mice were quickly decapitated, and the brain was removed and cut in two along the middle. The left hemisphere was fixed in 4% paraformaldehyde and embedded in paraffin for the detection of hippocampal neuronal regeneration. The right hippocampus was removed and preserved in pre-cooling preservation tubes, then frozen in liquid nitrogen and stored at −80°C for the detection of microglial cytokine expression.

Immunohistochemistry of doublecortin (DCX) expression in hippocampal neurons
The brain sections were dewaxed and hydrated through an alcohol series and rinsed three times with double-distilled water (ddH₂O). Antigen retrieval was performed in 0.01 mol/L citrate buffer (pH 6.0) for 20 minutes using a microwave before the sections were cooled to room temperature, rinsed three times with ddH₂O, incubated with 3% H₂O₂ at room temperature for 15 minutes, and rinsed with 0.01 mol/L PBS three times for 5 minutes each time. The sections were then blocked with 10% bovine serum albumin in a humid chamber at 37°C for 30 minutes, incubated with rabbit anti-DCX polyclonal antibody (1:100, Santa Cruz Biotechnology) at 4°C overnight, rinsed with PBS three times for 5 minutes each time, incubated with biotin-labeled donkey anti-rabbit IgG (Boster Biological Engineering Co., Ltd,, Wuhan, Hubei Province, China) at 37°C for 30

minutes, and rinsed as before with PBS. The sections were then incubated with HRP-conjugated streptavidin working solution at 37°C for 30 minutes, rinsed as before with PBS, developed with DAB for 2–3 minutes and counterstained with hematoxylin for 1–2 minutes, before being dehydrated, cleared with xylene, and mounted with neutral gum. Specimens were observed and photographed under a BX51 microscope (Olympus, Tokyo, Japan). The mean integrated absorbance of positive products for DCX immunostaining was measured using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA) and used for statistical analysis.

Fluorescence quantitative PCR detection of mRNA expression in peripheral blood and hippocampus of mice
Specific primers in the coding region were designed using Primer Express v2.0 software (Applied Biosystems, Foster, CA, USA) according to the target gene mRNA sequences in GenBank. The primers were synthesized using the ABI 3900 High-Throughput DNA synthesizer (Applied Biosystems).

Primer sequences and product sizes are as follows:

Primer	Sequence	Product size (bp)
IL-2	5′-GCA CCT GGA GCA GCT GTT G-3′ 5′-AGG TTC CTG TAA TTC TCC ATC CTG-3′	66
IFN-γ	5′-CAA GTT TGA GGT CAA CAA CCC A-3′ 5′-GCT GGA TTC CGG CAA CAG-3′	105
IL-1β	5′-TGC CAC CTT TTG ACA GTG AT-3′ 5′-GCT CTT GTT GAT GTG CTG CT-3′	146
TNF-α	5′-CCC CAA AGG GAT GAG AAG TTC-3′ 5′-GGC TTG TCA CTC GAA TTT TGA GA-3′	101
β-Actin	5′-ATG GTG GGA ATG GGT CAG AA-3′ 5′-TCT CCA TGT CGT CCC AGT TG-3′	121

The total RNA in whole blood and brain tissue was extracted with TRIzol, and then reverse-transcribed from an RNA template (4 μL) at 37°C for 1 hour and at 95°C for 3 minutes using the ABI 9700 PCR system (Applied Biosciences). For the quantitative PCR reactions, a positive standard and its gradient were prepared using a standard curve, and the samples and standards were subjected to the reaction system. Reaction conditions were 40 cycles of 93°C for 3 minutes, 93°C for 30 seconds, and 55°C for 45 seconds. Automated analysis was carried out on the results. Total RNA concentration was calculated according to the following formula: $A = B_1/B_2$, where A is the statistical value, B_1 is the number of copies of the target gene per μL cDNA, and B_2 is the number of copies of the reference gene per μL cDNA.

Statistical analysis
Data were analyzed using SPSS 16.0 statistical software (SPSS, Chicago, IL, USA) and expressed as mean ± SD. Groups were compared using one-way analysis of variance followed by the least significant difference tests. Statistical significance was set at $P < 0.05$.

Results

Neuronal regeneration in the hippocampus of AD mice
The mice were injected with $A\beta_{1–42}$ peptide or normal saline for 7 days, and neuronal regeneration in the hippocampus was examined using DCX immunohistochemistry (**Figure 1**). Under a high magnification microscope, DCX-immunopositive cells were stained brown or yellow, with unilateral radial filament-like projections. The results revealed significant differences in DCX expression between the groups ($F = 460.707$, $P < 0.01$). In the WT groups (**Figure 1A, C**), the number of positive cells was significantly higher than that in the nude groups (**Figure 1B, D**). DCX expression was the highest in the WT + NS group, then in the WT + Aβ group, nude + NS group, and the lowest in the nude + Aβ group. Statistical analysis showed that the WT groups had a significantly higher number of DCX-positive cells than the nude groups ($P < 0.01$), and that nude + Aβ mice had a higher expression of DCS than nude + NS mice ($P < 0.01$; **Figure 2**). The findings confirm that injection of $A\beta_{1–42}$ peptide inhibits the regeneration of hippocampal neurons. Furthermore, they indicate that T cells promote hippocampal neuron regeneration, and that such regeneration does not notably occur without T cells.

IL-2 and IFN-γ expression in peripheral blood of AD mice
To explore the correlation between hippocampal neuronal regeneration and T cell-associated cytokine expression in AD mice, we measured the expression of cytokines in peripheral blood using quantitative PCR 7 days after modeling. A significant difference in IL-2 gene expression was found ($F = 120.109$, $P < 0.01$). IL-2 expression in the WT mice was significantly higher than that in nude mice ($P < 0.01$; **Figure 3A**). The results of IL-2 expression were consistent with those of DCX expression in each group, suggesting that IL-2 expression might be related to neuronal regeneration. Significant differences in IFN-γ expression were also found among the groups ($F = 55.663$, $P < 0.01$). IFN-γ expression was greatest in the WT + NS group, and significantly lower in the WT + Aβ and nude + NS groups ($P < 0.01$; **Figure 3B**). These results indicate that intrahippocampal injection of Aβ1-42 inhibited the expression of IFN-γ in BALB/c-WT mice, and that the BALB/c-nude genotype was associated with lower expression of IFN-γ.

**IL1-β and TNF-α gene expression in the hippocampus of AD mice**
To further explore the possible mechanism of T cells on neuronal regeneration in AD mouse models, we used quantitative PCR to measure the expression of microglial cytokines in the hippocampus 7 days after modeling (**Figure 4**). Significant differences in IL-1β gene expression were found among the groups ($F = 1217.713$, $P < 0.01$). Expression levels of IL-1β and TNF-α in wild-type mice were higher than those in nude mice ($P < 0.01$). The highest level of IL-1β and TNF-α expression was found in the WT + Aβ group, suggesting that hippocampal injection of Aβ peptides may produce
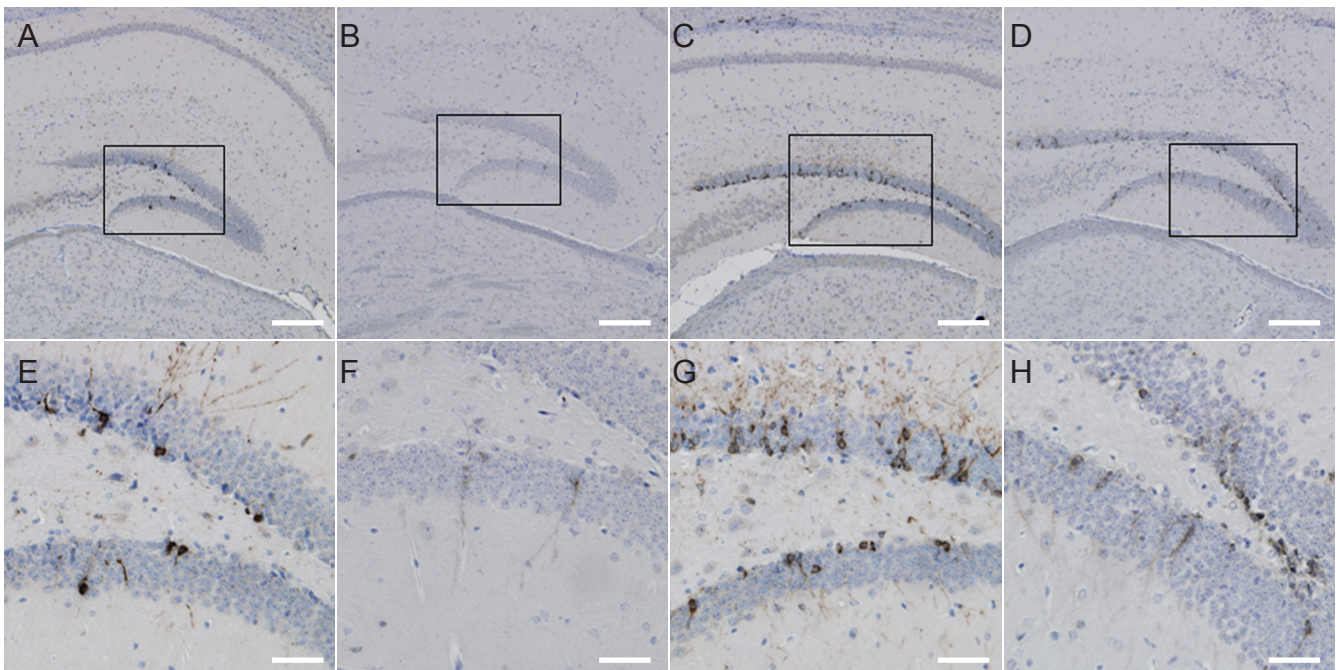
Figure 1 Immunolabeling for doublecortin-positive (newborn) neurons in the hippocampal dentate gyrus 7 days after hippocampal injection of Aβ$_{1-42}$ or saline.
The number of doublecortin-positive cells in the dentate gyrus of wide-type mice (A, C, E, G) was significantly higher than that in nude mice (B, D, F, H). Expression was greatest in control group I (wide-type + saline; C, G), followed by experimental group I (wide-type + Aβ; A, E), control group II (nude + saline; D, H), and minimal in experimental group II (nude + Aβ; B, F). (E, F, G, H) Enlarged view (× 40; scale bars: 200 μm) of the boxed areas in A, B, C, D (× 10; scale bars: 50 μm), respectively.
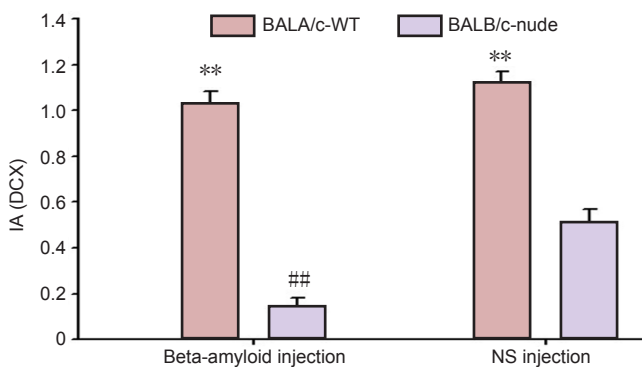
Figure 2 Quantitative analysis of doublecortin (DCX) expression in hippocampal dentate gyrus neurons 7 days after modeling.
Y-axis represents the integrated absorbance (IA) value of doublecortin-positive cells. Data are expressed as mean ± SD ($n = 6$) and compared using one-way analysis of variance and the least significant difference test. **$P < 0.01$, *vs.* nude + Aβ group; ##$P < 0.01$, *vs.* nude + NS group. NS: Normal saline; WT: wide-type.

some toxicity, and that WT mice with normal immune function react more severely to Aβ peptide (and produced more cytokines) than nude mice with T-cell immunodeficiency, which respond weakly to Aβ peptide, producing fewer reactive cytokines.

Discussion

AD is caused by synaptic loss and neuronal death, which result from Aβ deposition-mediated chronic inflammation (Ferretti et al., 2012; Rubio-Perez et al., 2012). In the brain of patients with AD, Aβ deposition not only causes inflammation, but also allows specific T cells to cross the microvascular endothelial cells of the blood-brain barrier and enter the brain parenchyma, thus exacerbating inflammatory responses (Man et al., 2007; Li et al., 2009; Fisher et al., 2011). Neuronal regeneration, in particular its induction and maintenance, is attracting increasing attention in studies of AD pathogenesis.

DCX is a microtubule-associated protein, which is specifically expressed in neuronal precursors and is involved in the migration of immature neurons and neurite growth (Rao et al., 2004). DCX is transiently expressed for 2–3 weeks in the cytoplasm and projections of newly formed neuronal precursors, after which it begins to decline; it is not expressed in mature neurons (Brown et al., 2003). This characteristic allows the use of DCX as a specific marker of neuronal precursors. The present results indicate that T cells promote nerve cell regeneration in the subgranular zone of the hippocampus in WT mice, and a lack of T cells may impair regeneration. In addition, injection of Aβ$_{1-42}$ inhibits the production of hippocampal nerve cells, depending on the neurotoxicity of Aβ$_{1-42}$. Previous studies compared neuronal regeneration in WT BALB/c/Ola mice and BALB/c-nude mice with a deficiency of T cells but normal B cells. The evidence suggests that T-cells can not only promote proliferation of neural precursors in the dentate gyrus, but also affect the differentiation of precursor cells (Ziv et al., 2006). Immune deficiency in cells is considered to be the main cause of conflicting results. In the present study, we compared neuronal regenera-

A

B
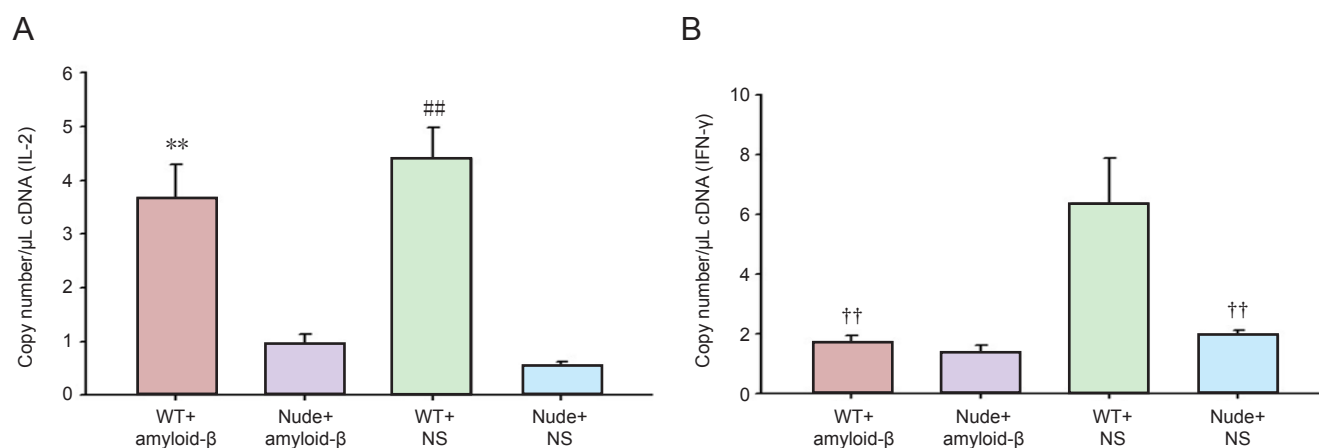
Figure 3 Quantitative fluorescence PCR for interleukin-2 (IL-2) and interferon-γ (IFN-γ) gene expression in peripheral blood of mice 7 days after modeling.

∗∗*P* < 0.01, *vs*. nude + amyloid-β group; ##*P* < 0.01, *vs*. nude + NS group; ††*P* < 0.01, *vs*. WT + NS group (one-way analysis of variance and the least significant difference test). Data are expressed as mean ± SD (*n* = 6). WT: Wide-type; NS: normal saline.

A

B
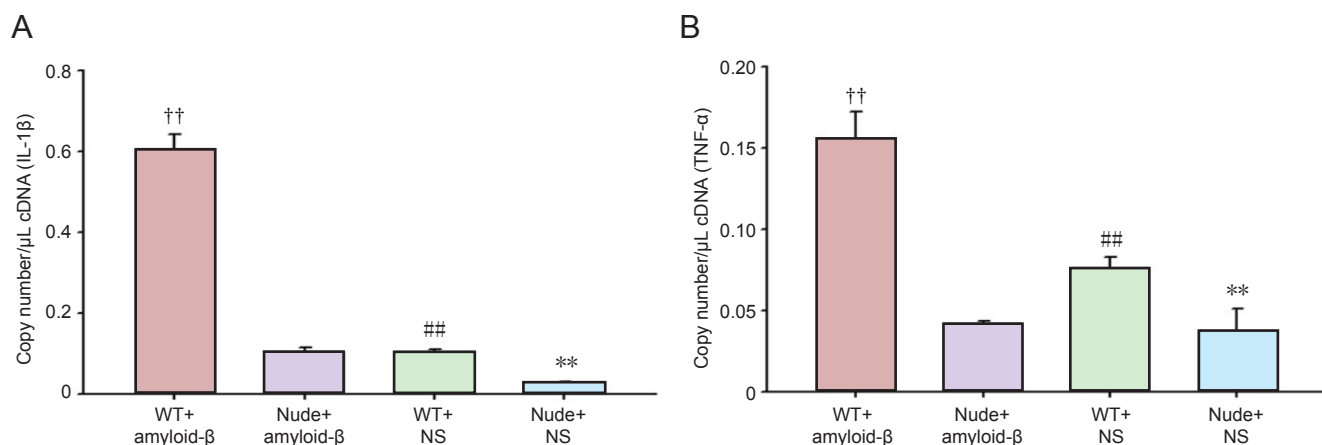
Figure 4 Interleukin-1β (IL-1β; A) and tumor necrosis factor-α (TNF-α; B) gene expression in the hippocampus of mice 7 days after modeling.

∗∗*P* < 0.01, *vs*. nude + amyloid-β group; ##*P* < 0.01, *vs*. nude + NS group; ††*P* < 0.01, *vs*. all other groups (one-way analysis of variance and the least significant difference test). Data are expressed as mean ± SD (*n* = 6). WT: Wide-type; NS: normal saline.

tion in normal BALB/c-WT mice and T-cell-deficient BALB/c-nude mice; our results highlight the contribution of T cells to neuronal regeneration in the hippocampus of AD mice, consistent with previous findings. Thus, hippocampal neuronal regeneration is presumably mediated by the activation of microglial cells. The mechanisms underlying T cell promotion of hippocampal neuronal regeneration also depend on microglial cell-secreted cytokines (Monsonego et al., 2003; Pellicanò et al., 2010; Swardfager et al., 2010).

IL-2 is a T-cell growth factor, mainly produced by activated T-cells. It plays a crucial role in maintaining the growth of T-cell subsets and promoting the proliferation of activated B cells. In the present study, we used PCR to show that the secretion of IL-2 from T cells (IL-2 expression) was positively correlated with the regeneration of hippocampal neurons. IL-2 is an important pro-inflammatory cytokine, and its expression correlates closely with the degree of inflammation and neuronal loss in AD and other degenerative diseases (Meola et al., 2013). The quantity of CD4^{+} T-cells is signifi-

cantly increased in the peripheral blood of AD patients, consistent with upregulated expression of IL-2, IL-6 and other inflammatory cytokines in peripheral blood, all of which are secreted by T cells (Becher et al., 2006; Wolf et al., 2009). Interestingly, IL-2 expression correlates with neuronal regeneration, indicating that the inflammatory and neuroprotective effects exist simultaneously in AD, and peripheral IL-2 gene expression can reflect the activation state of T-cells.

IFN-γ is generated by a variety of immune cells including T-cells, the crucial immune regulators *in vivo*. IFN-γ can increase the expression of MHC-II molecules on the macrophage surface and promote phagocytosis. In AD model mice, IFN-γ produced by Aβ-specific T cells activates microglia to stimulate inflammation and aggravate abnormal Aβ protein deposition (Browne et al., 2013). Another study showed that Aβ-specific T-cells induce immune cells to clear Aβ protein deposition in the brain through the secretion of IFN-γ (Fisher et al., 2010). Hippocampal injection of $Aβ_{1-42}$ downregulates the expression of IFN-γ and inhibits neuro-

nal regeneration (Zheng et al., 2013). The major adaptive immune cytokine, IFN-γ, not only promotes regeneration of hippocampal neurons and improves spatial learning and memory capacity in adult WT mice, but also plays a crucial role in the regulation of brain inflammation, repair of damaged neurons, and maintenance of normal nervous system function (Baron et al., 2008; Mastrangelo et al., 2009). The present study shows that hippocampal injection of $A\beta_{1-42}$ peptide inhibits the expression of peripheral blood IFN-γ, and the expression level in BALB/c-nude mice is lower than that in WT mice. Changes in IFN-γ expression reflect the complexity of immunoregulation.

There may be cross-talk between brain-infiltrated T-cells and microglia, activating brain microglia. Microglia secretes various cytokines, which affect the function of nerve cells and other glial cells. In the process of AD pathology, activated microglia may release a series of proinflammatory cytokines, primarily IL-1β, TNF-α, IFN-γ and IL-6. Among these, IL-1β exerts important and diverse biological roles (Farfara et al., 2008; Graber et al., 2009), including its action as an acute inflammatory cytokine in the brain, its expression sharply increasing within a few hours of injury; its autocrine effects on microglia, promoting microglial proliferation and increasing its own secretion as well as that of TNF-α and IL-6; its enhancement of APP-α lyase activity and promotion of its metabolism, reducing Aβ deposition; and at moderate levels it can promote the growth of cultured neurons, but at high levels IL-1β is neurotoxic (Tachida et al., 2008; Moore et al., 2009; Matousek et al., 2012; Song et al., 2013). The present study demonstrated that BALB/c-WT mice have a strong response to Aβ peptide, showing high IL-1β expression, whereas BALB/c-nude mice react weakly to Aβ peptide, with little IL-1β expression. IL-1β expression levels correlated with neuron regeneration throughout the four groups.

TNF-α is an important inflammatory factor that can kill tumor cells or cytokines that induce tumor tissue necrosis. It is mainly produced by activated microglia (Montgomery et al., 2012). The biological activity of TNF-α is diverse, and includes direct killing of cells, as well as immune regulation and the promotion of cell proliferation and differentiation (McCoy et al., 2008; Alvarez et al., 2011). Studies have shown that the loss of neurons in AD is mainly due to the activation of microglial cells by oligomerized Aβ protein, altering the cell cycle via the TNF-α and c-Jun kinase signaling pathways, affecting normal neuronal differentiation and ultimately leading to apoptosis (Bhaskar et al., 2014). The present results suggest that TNF-α expression correlates positively with neuronal regeneration in each group.

As AD progresses, the Aβ-activated microglia produce inflammatory cytokines (such as IL-1α, IL-1β and TNF-α), inducing neuronal death and memory impairment (Fang et al., 2010). Neurotrophic factors (including NGF, BDNF and GDNF) are also produced, to maintain neuronal regeneration and learning and memory functions (Scharfman et al., 2005; Ji et al., 2011; Lilja et al., 2013). Changes in the balance of inflammatory cytokines and neurotrophic factors will accelerate or delay the AD process. Here, we have examined the regeneration of hippocampal neurons and the expression of pro-inflammatory cytokines (IL-2, IFN-γ, IL-1β, TNF-α) in AD mice at 7 days, and found a positive correlation, that is, significant neuronal regeneration and high expression levels of cytokines are found in the same group. The present results suggest that both nerve inflammation and neuroprotective effects might be concomitant in the AD pathological state, and they may maintain a dynamic balance under normal immune function; when an immune imbalance or defect occurs, neuroprotective effects are decreased, affecting nerve regeneration. Our experimental results showed that neuronal regeneration in BALB/c-WT mice was notably better than in T-cell-deficient BALB/c-nude mice. The contribution of T cells to neuronal regeneration in AD mice is closely related to immune status, microglial activation, and the secretion of cytokines.

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