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·Original Article·

Autophagy is involved in oral rAAV/Aβ vaccine-induced Aβ clearance in APP/PS1 transgenic mice

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ABSTRACT

The imbalance between β-amyloid (Aβ) generation and clearance plays a fundamental role in the pathogenesis of Alzheimer's disease (AD). The sporadic form of AD is characterized by an overall impairment in Aβ clearance. Immunotherapy targeting Aβ clearance is believed to be a promising approach and is under active clinical investigation. Autophagy is a conserved pathway for degrading abnormal protein aggregates and is crucial for Aβ clearance. We previously reported that oral vaccination with a recombinant AAV/Aβ vaccine increased the clearance of Aβ from the brain and improved cognitive ability in AD animal models, while the underlying mechanisms were not well understood. In this study, we first demonstrated that oral vaccination with rAAV/Aβ decreased the p62 level and up-regulated the LC3B-II/LC3B-I ratio in APP/PS1 mouse brain, suggesting enhanced autophagy. Further, inhibition of the Akt/mTOR pathway may account for autophagy enhancement. We also found increased anti-Aβ antibodies in the sera of APP/PS1 mice with oral vaccination, accompanied by elevation of complement factors C1q and C3 levels in the brain. Our results indicate that autophagy is closely involved in oral vaccination-induced Aβ clearance, and modulating the autophagy pathway may be an important strategy for AD prevention and intervention.

Keywords: oral vaccination; autophagy; Akt/mTOR pathway; Aβ clearance; Alzheimer's disease

INTRODUCTION

Alzheimer's disease (AD) is characterized by increased amounts of β-amyloid (Aβ) species and has threatened millions of aged individuals. There is an urgent need for the development of novel therapeutic strategies for the prevention of AD, considering the increasingly serious aging trend[1]. The amyloid hypothesis proposes that dyshomeostasis in Aβ generation and clearance is essential for disease progression[2]. The central role of the Aβ cascade in the etiology of AD provides an important target for drug research and development. The sporadic form of AD, which accounts for >95% of all AD cases[3], has

been reported to show impaired clearance of Aβ from the brain[4], indicating that Aβ clearance is a potent target for the treatment of AD.

Aβ immunotherapy was initially developed in 1999 by Schenk *et al.*[5] and has attracted much interest for its therapeutic effects on brain Aβ pathology[6]. However, the failure of AN1792 due to the emergence of severe adverse reactions in clinical trials calls for the development of safer vaccines[7]. Phase III clinical trials of bapineuzumab and solanezumab also found no improvement in clinical outcomes in AD patients, while suggesting that anti-Aβ intervention should focus on the early stage of disease progression[8,9]. We previously developed an oral vaccine with a recombinant adeno-associated viral vector carrying Aβ1-43 cDNA (referred to as rAAV/Aβ), and animal studies demonstrated that oral vaccination significantly reduces the brain Aβ burdens without causing neuroinflammation and lymphocytic infiltration[10,11], suggesting it is a safe and effective vaccine for the treatment of AD. However, the underlying mechanism responsible for oral vaccination-induced Aβ clearance is still incompletely understood.

Autophagy plays a vital role in maintaining intracellular homeostasis and degrading abnormal protein aggregates[12]. Autophagy deficiency in the brain may contribute to Aβ accumulation and worsen cognition in AD mouse models[13]. We previously found that a presenilin-1 mutation results in pronounced intracellular Aβ42 deposition in neurons without causing amyloid plaque formation, which is correlated with accelerated neurodegeneration in aged mice[14]. The enhancement of autophagy by chemical or genetic approaches can protect against Aβ-induced neuronal damage and improve cognition[15,16]. Recent findings have uncovered a novel function of autophagy in the immune response, including the processing or elimination of antigens[17]. Besides, there is increasing evidence that autophagy can be activated in the context of viral infection or vaccination, which is in turn beneficial to the immune response[18,19].

We have previously found a dramatic reduction in brain Aβ content in Tg2576 mice with oral vaccination. However, whether autophagy is involved in the oral vaccination-induced Aβ clearance from the brain remains to be elucidated. The present study was designed to address the hypothesis that activation of autophagy participates in oral vaccination-related Aβ clearance.

MATERIALS AND METHODS

Construction of the Vaccine

The rAAV/Aβ vaccine and rAAV/GFP were designed and kindly provided by Takeshi Tabira (Juntendo University) and Hideo Hara (Saga University). The construction procedures are described in detail in previous studies[10,11].

Cell Culture and Treatment

Human embryonic kidney (HEK) 293 cells were cultured in DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) at 37°C in an atmosphere of 5% CO_2. Cells were passaged at a ratio of 1:6 when grown to 90% confluence. To verify that the vaccine was successfully constructed, cells at 70% confluence were divided into 3 groups, two were incubated with 10 μL rAAV/Aβ vaccine (containing 5×10^{11} genomes) or the same titer of rAAV/GFP for 48 h. Cells in the control group were treated with the same volume of PBS (pH 7.4). Sodium butyrate (Sigma-Aldrich, St. Louis, MO) at a final concentration of 2 mmol/L was added to all three groups of cells to promote gene expression[20]. Media and cells were harvested at the indicated time points. Complete proteinase inhibitor cocktail (Roche, Indianapolis, IN) was added into the media to prevent Aβ degradation and the cell debris was removed by centrifugation at 3000 × g for 10 min at 4°C[21].

Animals and Oral Vaccination

Male APPswe/PS1dE9 (referred to as APP/PS1) transgenic mice (5–6 months old) were raised in groups of 3 to 4 per cage under standardized housing conditions on a 12 h/12 h light/dark cycle, with food and water *ad libitum*. APP/PS1 mice develop pathology typical of Aβ deposition and cognitive impairment at the age of 5 to 6 months[22,23]. The experimental procedures were approved by Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch. Mice were divided into 3 groups (n = 10/group): PBS (control); rAAV/GFP (vehicle), and rAAV/Aβ (vaccine).

Oral immunization was carried out based on our previous research[10,11]. In brief, rAAV/GFP and rAAV/Aβ were diluted in PBS to give 5×10^{11} genomes/100 μL. APP/PS1 mice (5 to 6 months old) in the vaccine group were given 5×10^{11} genomes of rAAV/Aβ once through an orogastric tube. Mice in the vehicle group were treated with

5×10^{11} genomes of rAAV/GFP in the same manner. Age-matched control mice received 100 μL PBS once *via* an orogastric tube.

Sample Preparation

For ELISA and immunoblotting analysis, mice were sacrificed 4 weeks after oral vaccination. Brain tissues were dissected immediately and stored at −80°C until use. Tissues for ELISA assessment were further prepared as described[21,24]. In brief, brain tissues were homogenized in 5 volumes (*w*/*v*) of 1% Triton X-100 in Tris-buffered saline (25 mmol/L Tris and 137 mmol/L NaCl, pH 7.6) containing protease inhibitor cocktail (Roche). Homogenates were centrifuged at 100 000 g for 60 min at 4°C and the supernatant was collected as the Triton X-100-soluble fraction. The pellets were sonicated in 5 mol/L guanidine HCl in 50 mmol/L Tris (pH 8.0) in the presence of protease inhibitor cocktail (Roche). After incubation for 2 h at 25°C, the mixture was centrifuged at 13 000 g for 20 min at 4°C. The supernatant was 10-fold diluted and used as the guanidine HCl-soluble fraction.

ELISA

Aβ40 and Aβ42 levels in media and cell lysates were measured by sandwich ELISA according to the manufacturer's instructions (Wako, Japan). The Aβ concentration was normalized based on the number of cells in each culture (as determined by protein content in cell lysates)[21,25].

Aβ40 and Aβ42 levels in the detergent-soluble and guanidine HCl-soluble fractions were measured using commercially-available ELISA kits as noted in previous reports[21,25], with minor modifications, and calibrated with synthetic Aβ peptides. The described method is able to measure the Aβ40 or Aβ42 contents in brain samples with high specificity[21,25,26]. The final values of Aβ content were expressed as pmol per wet weight of brain tissue.

Complement C1q and C3 levels in brain tissues were assessed according to the manufacturer's instructions (Cloud-Clone Crop, China). Data were normalized to control. All ELISA assays were performed in duplicate.

Detection of Serum Anti-Aβ Antibody and Characterization of Antibody Isotypes

The concentration of anti-Aβ antibody in serum was determined as described[11], with minor modifications. Plates were coated with 4 μg/mL synthetic human Aβ42 dissolved in 55 mmol/L $NaHCO_3$ (pH 8.3) overnight at 4°C. Plates were washed three times with washing buffer and blocked with 1% BSA and 2% normal goat serum in PBS for 1 h at 37°C. After three washes, plates were incubated with mouse serum samples diluted in blocking buffer for 1 h with shaking. Then plates were washed and incubated with HRP-conjugated goat anti-mouse IgG for 1 h at 37°C. The chromogen substrate 3,3',5,5'-tetra-methylbenzidine was added to the plates after three washes, and incubated at 37°C for 30 min in the dark. H_2SO_4 (2 mol/L, 50 μL) was added to stop the reaction. The absorbance of each well at 450 nm was read with a microplate reader. The isotypes of IgG antibodies were further determined using mouse immunoglobulin isotyping ELISA kits (BD Bioscience PharMingen, San Jose, CA) according to the manufacturer's instructions. All measurements were performed in duplicate and data were normalized to control.

Immunofluorescence Imaging

Samples for immunofluorescence imaging were prepared as reported[27]. At 4 weeks after oral vaccination, mice were anesthetized with 5% chloral hydrate and perfused intracardially with 0.9% saline followed by ice-cold phosphate-buffered 4% paraformaldehyde (PFA, Sigma-Aldrich) at pH 7.4. Tissue samples were post-fixed overnight in 4% PFA and equilibrated in phosphate-buffered 30% sucrose for 48 h at 4°C. The brains were then sectioned on a frozen microtome (Leica, Bannockburn, IL) at 30 μm in the coronal plane. Sections were collected in 12-well plate and stored at −20°C in an antifreeze solution of phosphate buffer containing 30% glycerol and 30% ethylene glycol.

Immunofluorescence staining was conducted as described previously, with minor modifications[25,27]. Free-floating sections were incubated in PBS at room temperature for 30 min, then rinsed with PBS-Triton X-100 before incubation in 0.3% H_2O_2 for 30 min. Sections were then incubated overnight at 4°C with 6E10 (1:120; Covance, Princeton, NJ), a monoclonal antibody against Aβ, followed by incubation with Alexa-Fluor 488 conjugated secondary antibodies (Molecular Probes, Eugene, OR) for 1 h at room temperature. Nuclei were stained with Hoechst 33258 (Invitrogen, Carlsbad, CA). Sections were

washed and then imaged using a confocal laser scanning microscope (Olympus, Japan). No signal was detected when the primary antibody was omitted.

Western Blotting Analysis

Western blotting analyses were performed according to previous reports[10,11,28]. The dissected brain tissues were lysed on ice in lysis buffer containing 50 mmol/L Tris-HCl, pH 6.8, 8 mol/L urea, 5% β-mercaptoethanol, 2% SDS, and protease inhibitors. Lysates were collected and centrifuged at 12 000 g for 10 min at 4°C. The protein content was determined using a BCA protein assay kit (Pierce, Rockford, IL). Proteins (40 to 60 μg) were separated by SDS-PAGE electrophoresis and then transferred to 0.45 μm polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked for 1 h in 5% (*m*/*v*) nonfat milk in Tris-buffered saline (pH 7.5) supplemented with 0.1% Tween-20, and incubated with primary antibodies against LC3B (1:4 000, Novus, Littleton, CO), p62 (1:5 000, MBL, Nagoya, Japan), mTOR (1:1 000, CST, Danvers, MA), pmTOR (Ser2448, 1:1 000, CST), Akt (1:1 000, CST) or pAkt (Ser473, 1:1 000, CST) overnight at 4°C. β-actin (1:5 000, Sigma-Aldrich) was used as an internal control. The membranes were then incubated with peroxidase-conjugated secondary antibodies, and protein bands were developed with the ECL system (Millipore).

Statistical Analysis

Data are expressed as mean ± SEM and were analyzed by GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). All data were tested for normal distribution and for homogeneity of variance before analysis of variance (ANOVA). Data that fit a normal distribution and with homogeneous variance were analyzed using one-way ANOVA followed by Tukey's *post hoc* test. Otherwise, data were analyzed by the Kruskal-Wallis non-parametric test with Dunn's Multiple Comparison Tests. P <0.05 was considered statistically significant.

RESULTS

rAAV/Aβ Vaccine Transfection Increased Aβ Secretion in HEK293 Cells

To verify the successful construction of the vaccine, we transfected it into HEK 293 cells and evaluated Aβ expression. We found that co-incubation of 5×10^{11} genomes of vaccine particles (rAAV/Aβ) for 48 h resulted in a significant increase in Aβ40 and Aβ42 content in both cell lysates and the media (Fig. 1, P <0.001). No significant difference was found in Aβ40 and Aβ42 levels between the rAAV/GFP and PBS groups (Fig. 1). Therefore, the constructed vaccine was able to express large amounts of Aβ peptides in mammalian cells, indicating that it may be an efficient antigen.

Oral Vaccination with rAAV/Aβ Significantly Attenuated Brain Aβ Burden in APP/PS1 Transgenic Mice

ELISA analysis (Fig. 2A, B) showed a remarkable reduction in soluble Aβ40 and Aβ42 contents in the brain of mice with oral rAAV/Aβ vaccination, compared with rAAV/GFP- and PBS-treated mice (Fig. 2A, P <0.01). Similar results were found for insoluble Aβ40 and Aβ42 (Fig. 2B, P <0.001). Mice treated with rAAV/GFP had a brain Aβ burden similar to PBS-treated mice (P >0.05). The decrease in the brain Aβ burden was further supported by immunofluorescent imaging. The brain Aβ load in the cerebral cortex and hippocampus of APP/PS1 mice following oral rAAV/Aβ vaccination was markedly lower than that in rAAV/GFP- and PBS-treated mice (Fig. 2C). These results together demonstrated that the developed vaccine was capable of improving Aβ pathology in APP/PS1 mice.

Oral Vaccination with rAAV/Aβ Increased Concentrations of Serum Anti-Aβ Antibody and Elevated Brain Complement Levels in APP/PS1 Mice

Antibodies against Aβ are known to be capable of preventing Aβ deposition and disrupting Aβ assembly[29]. Here, serum anti-Aβ antibodies were significantly elevated in APP/PS1 mice with rAAV/Aβ vaccination (Fig. 3A). The titers of the antibodies were much higher than those in the vehicle and control groups (Fig. 3A; P < 0.001), indicating the efficacy of the developed vaccine in triggering the immune response in APP/PS1 mice. We next determined the isotypes of the antibodies in sera from all three groups. We found that IgG1, IgG2a, and IgG2b, main immunoglobulins of the IgG family, were increased after oral vaccination (Fig. 3A). IgG2b was the predominant Ig isotype in rAAV/Aβ-vaccinated mice, followed by IgG1, while the concentration of IgG2a was slightly increased. The pattern of antibody isotypes

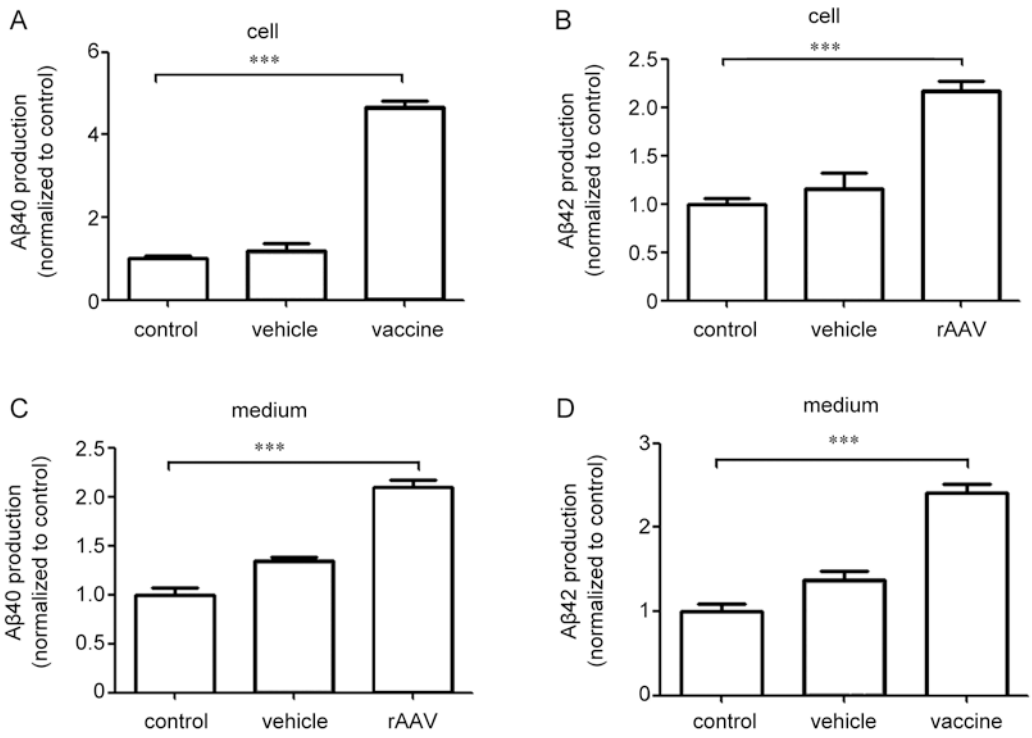

Fig. 1. ***In vitro*** **characterization of recombinant AAV/Aβ vaccine. HEK293 cells were co-incubated with rAAV virus for 48 h and cell lysates and media were harvested to quantify Aβ levels using specific ELISA kits. Aβ40 (A) and Aβ42 levels (B) in cell lysates; Aβ40 (C) and Aβ42 (D) in the media were assessed and compared in all three groups (mean ± SEM; ******P* **<0.001** ***vs*** **control,** ***n*** **= 3; one-way ANOVA).**

implied that oral vaccination induced Th2-based immune responses.

Activation of the complement system is a common physiological reaction in the immune response[30]. It has been reported that brain Aβ deposits are surrounded by small amounts of IgG and complement factors C1q and C3[31], indicating a possible role of these components in limiting Aβ pathology[32]. To determine whether oral vaccination with rAAV/Aβ activates the complement system, we assessed the content of C1q and C3 in the brains of mice from all three groups. The C1q and C3 content was notably higher in rAAV/Aβ-treated mice than in rAAV/GFP- and PBS-treated mice (Fig. 3B, P <0.05; Fig. 3C, P < 0.01). There was no difference in the C1q and C3 content between the rAAV/GFP and PBS groups. The changes of brain C1q and C3 levels after oral vaccination also indicate a possible participation of the complement system in vaccine-induced Aβ clearance.

Activation of Autophagy Function in Brains of APP/PS1 Mice with Oral rAAV/Aβ Vaccination

Autophagy has been found to closely participate in Aβ metabolism[33]. However, little is known about the role of autophagy in AD immunotherapy. In order to clarify whether oral vaccination with rAAV/Aβ vaccine enhanced autophagy in the brain, we examined several markers of autophagy (LC3B and p62) in all the three groups. We found that the p62 levels in the brains of rAAV/Aβ-vaccinated mice were strikingly lower than those of the rAAV/GFP and PBS mice (Fig. 4B, P <0.05). The autophagosome marker LC3B-II and the LC3B-II/LC3B-I ratio were markedly increased in brains of mice undergoing rAAV/Aβ vaccination (Fig. 4C, P <0.05). Note that the rAAV/GFP (vehicle) itself did not alter autophagy as the LC3B and p62 levels were unchanged after its administration (compared with PBS-treated mice). These results imply that the activation of autophagy may play a part in oral vaccination-induced Aβ clearance.

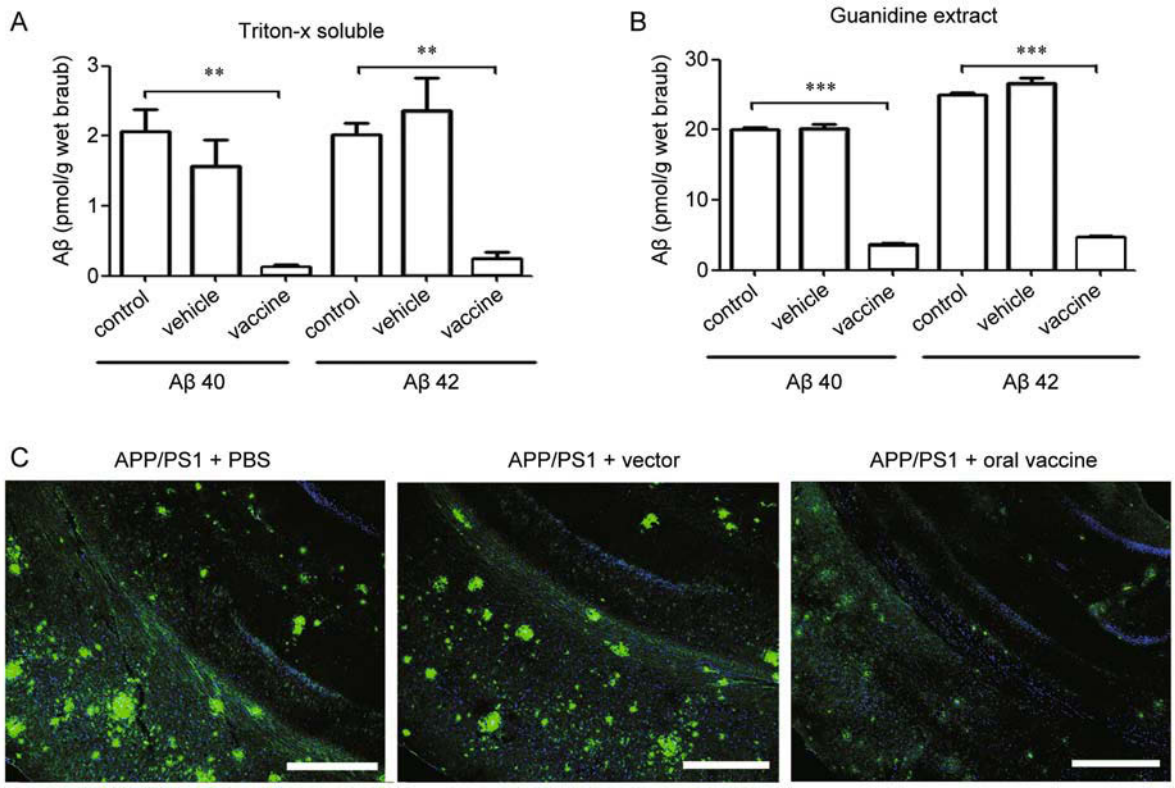

Fig. 2. Oral vaccination with rAAV/Aβ vaccines effectively reduced the brain Aβ burden in APP/PS1 mice. (A,B) The changes in brain Aβ content at 4 weeks after oral vaccination were examined by ELISA and immunofluorescence staining. ELISA quantification of Triton X-soluble Aβ (A) and guanidine HCl-soluble Aβ (B) content in brain tissues from control, vehicle and vaccine groups. (C) Representative immunofluorescence staining of Aβ load in brain regions (cerebral cortex and hippocampus) using 6E10 antibody in coronal sections from APP/PS1+PBS (control), APP/PS1+rAAV/GFP (vehicle), and APP/PS1+rAAV/Aβ (vaccine) groups (mean ± SEM, normalized to control; **P <0.01, ***P <0.001 *vs* control, n = 5; one-way ANOVA; scale bar, 200 μm).

Akt/mTOR Pathway Suppressed in Brains of APP/PS1 Mice with Oral rAAV/Aβ Vaccination

The Akt/mTOR pathway is well-recognized in regulating autophagy[34]; recent studies have also proposed a role of this pathway in the virus-related immune response[35]. To further investigate the potential mechanisms associated with oral vaccination-induced autophagy, we assessed the Akt/mTOR pathway in the brains of all three groups of mice. We discovered that the phosphorylated Akt (Ser473) level was significantly down-regulated following oral vaccination (Fig. 5A, C, P <0.01). Consistent results were obtained for phosphorylated mTOR (Ser2448) levels (Fig. 5B, D, P <0.01). The total protein levels of Akt and mTOR were not altered in the three groups. The rAAV/GFP had no influence on the Akt/mTOR pathway (Fig. 5, P >0.05). These results were in line with the finding that oral vaccination induced autophagy activation in the brain, suggesting that such activation may depend on Akt/mTOR inhibition.

DISCUSSION

Oral Vaccination with rAAV/Aβ Initiated the Immune Response and Increased Serum Anti-Aβ Antibody in APP/PS1 Mice

Amyloidopathy is one of the major pathological changes in the progression of AD. It is generally accepted that Aβ imbalance occurs in the early stage of disease progression[36], and appears to act as a trigger for neuronal damage[37]. An overall dysfunction in Aβ clearance in the brain has been emphasized in the pathogenesis of late-onset AD, the

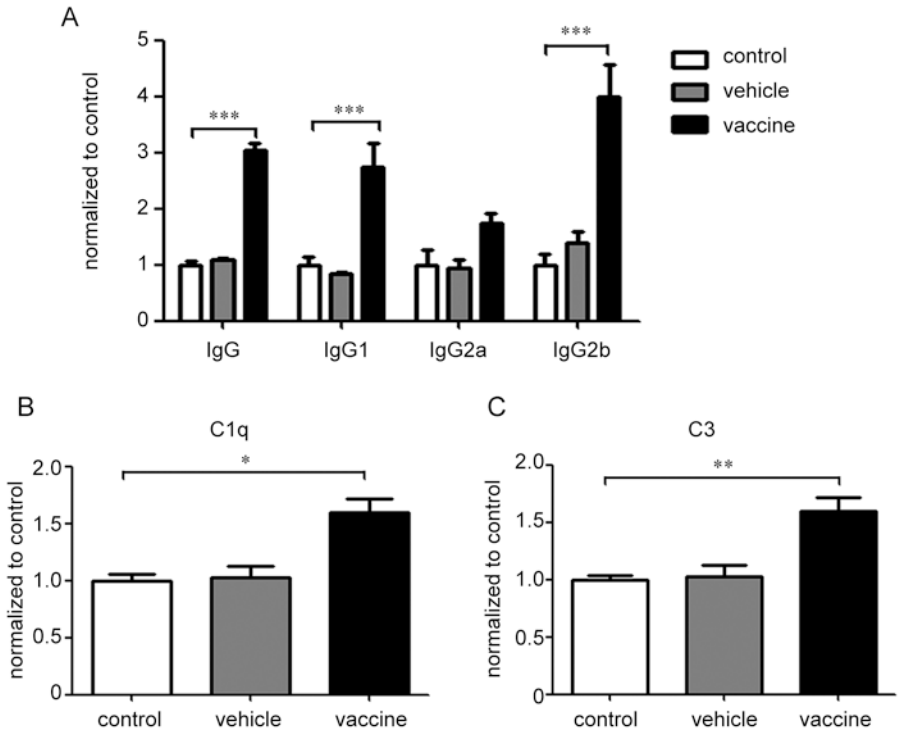

Fig. 3. Oral vaccination with rAAV/Aβ generated a strong immune response by increasing serum anti-Aβ antibodies and elevating brain complement levels. The titers of anti-Aβ antibody and the subclasses of these antibodies in serum samples from all three groups of mice were determined using specific ELISA. (A) Concentrations of antibody against Aβ and the contents of IgG1, IgG2a, and IgG2b in sera from control, vehicle, and vaccine groups were measured. The levels of complement factors C1q (B) and C3 (C) in brains from control, vehicle, and vaccine groups were measured by ELISA (mean ± SEM, normalized to control; *P <0.05, **P <0.01, ***P <0.001 *vs* control, n = 5; one-way ANOVA).

major form of dementia[4]. Targeting Aβ cascades has been the focus of AD drug development. Among the ongoing research projects directed at Aβ clearance, immunotherapy has emerged as a promising strategy[38]. Our previous studies have shown that oral Aβ vaccine using an rAAV vector is effective in decreasing the Aβ burden and improving cognitive ability in Tg2576 mice[10,11]. In parallel, the present study on APP/PS1 mice obtained similar results showing that oral vaccination reduced both the soluble and insoluble Aβ species in the brain. Oral vaccination with rAAV/Aβ may stimulate the immune response *via* gut-associated lymphoid tissues[39] since rAAV/Aβ-induced Aβ expression is detectable in the lamina propria of the stomach and duodenum of mice, and transduction of rAAV occurs in intestinal cells[10,11]. Antibodies against Aβ are regarded to be necessary for Aβ clearance[40], and we indeed found an increase in serum IgG antibodies against Aβ species in a previous study[10]. Similarly, Zhang *et al*. reported that single oral immunization with an Aβ vaccine induces the strong production of anti-Aβ antibodies in a mouse model of AD[39]. In accordance with these findings, we found a significant increase in serum anti-Aβ antibody titers in APP/PS1 mice treated with rAAV-Aβ, confirming that oral vaccination successfully initiated an immune response. Of note, we characterized the antibody isotypes after oral vaccination, and revealed that IgG1 and IgG2b were the predominant isotypes rather than IgG2a. The robust elevation of IgG1 and IgG2b, which are typically used as markers to determine the types of humoral immune response[41], indicates a Th2-based immune response induced by this vaccine. Besides, the gut immune system, a possible mediator for initiating the oral vaccination-induced

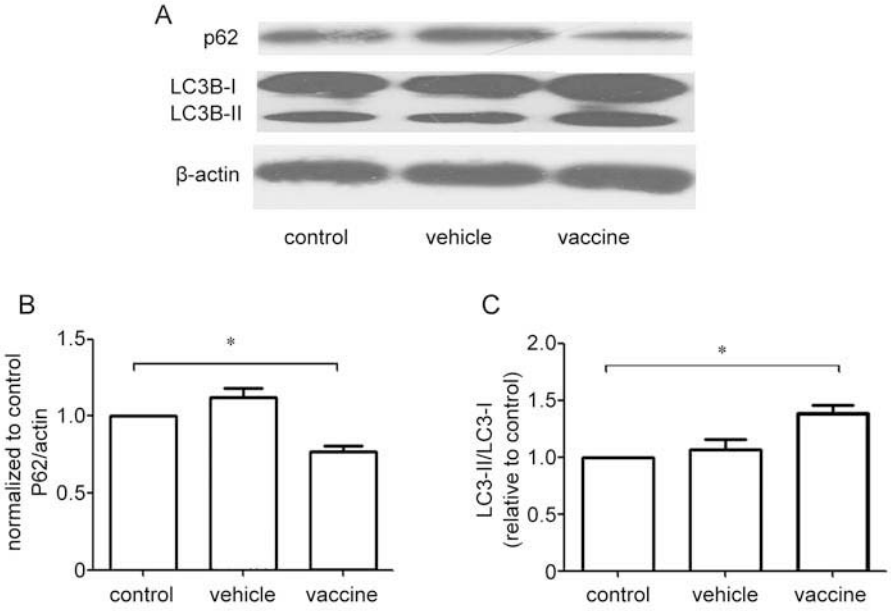

Fig. 4. Activation of autophagic functions in the brains of mice following oral vaccination. The autophagic markers LC3B and p62 in brain tissues were determined by western blotting analysis. The ratio of LC3B-II/LC3B-I and p62 protein levels were evaluated and compared in all three groups. Representative LC3B and p62 western blotting images are shown in (A); quantitative results of p62 protein levels in (B); and the ratio of LC3B-II/LC3B-I in (C) (mean ± SEM, normalized to control; *P <0.05 *vs* control, n = 5; one-way ANOVA).

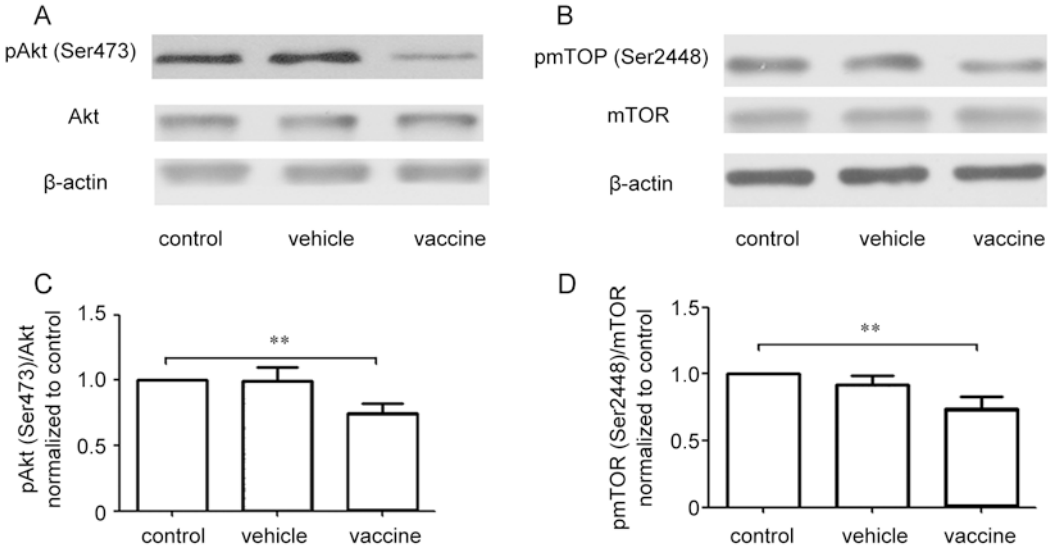

Fig. 5. Oral vaccination with rAAV/Aβ inhibited the brain Akt/mTOR pathway in APP/PS1 mice. The changes in pAkt (Ser473)/Akt and pmTOR (Ser2448)/mTOR were analyzed by western blotting. Representative immunoblotting images of pAkt (Ser473)/Akt (A) and pmTOR (Ser2448)/mTOR (B). Quantitative results of the alteration in pAkt (Ser473)/Akt (C) and pmTOR (Ser2448)/mTOR (D) (mean ± SEM, normalized to control; **P <0.01 *vs* control, n = 5; one-way ANOVA).

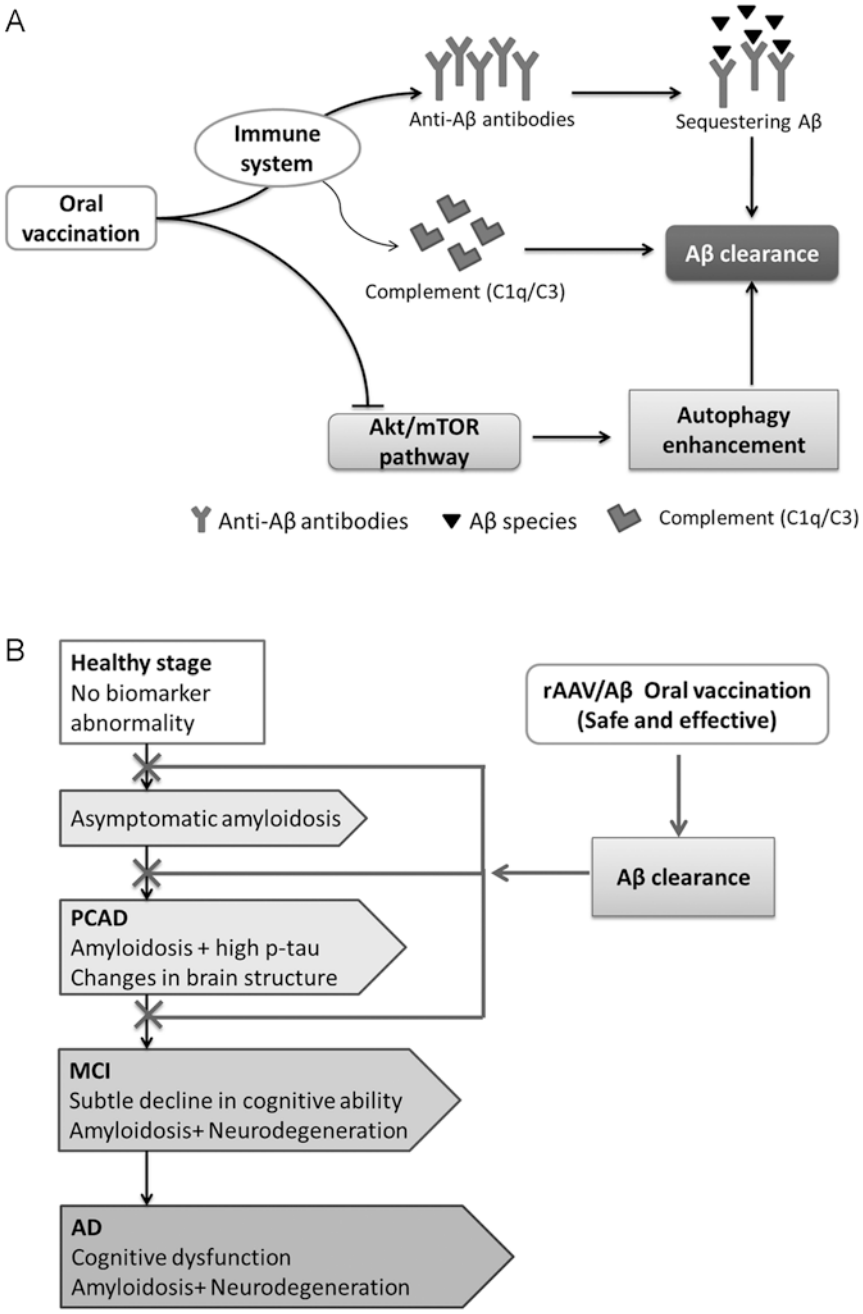

Fig. 6. (A) Schema of the established effects of oral rAAV/Aβ vaccination on Aβ clearance. Here, we demonstrated that activation of brain autophagy was closely involved in oral vaccination-induced Aβ clearance, and this effect might be mediated by inhibition of the Akt/mTOR pathway. We also found that the decrease of Aβ was accompanied by elevation of C1q and C3. In addition, elevation of antibodies against Aβ also played an important part in sequestering and eliminating Aβ species. All three pathways may play a role in oral vaccination-induced Aβ clearance. (B) Oral vaccination is likely to be a safe and effective strategy for early intervention in AD. The hypothetical staging of AD based on the progression of the pathological changes is shown. Amyloidopathy has been regarded to be a key early event in the pathophysiological process of AD[71,74], and recent evidence suggests that aberrant clearance of Aβ species may be critical to the etiology of AD[4]. Based on our study on rAAV/Aβ, we propose that oral immunization with this vaccine might be effective in improving Aβ pathology and retarding disease progression.

immune response, can also suppress the Th1 response and enhance the Th2 response[42]. The Th2 immune response is regarded to be non-inflammatory and is closely linked to the safety of the vaccine[40,43]. Our results that oral vaccination preferentially induced a Th2-based immune response suggested that rAAV/Aβ has the potential to avoid adverse reactions such as neuroinflammation and microhemorrhage.

The complement system is an essential element in the immune response for its function in regulating immune reactions and antigen elimination[44]. It has also been demonstrated that complement factors are widely expressed in the central nervous system[45]. The function of complement in AD is controversial regarding its influence on Aβ pathology, while accumulating studies support a protective effect of complement on Aβ clearance[46]. C1q and C3 are key factors in the complement cascade. Activation of C1q in the early stage protects AD mouse models from Aβ-related neurotoxicity[47]. In addition, C3 deficiency in AD mice models results in age-associated increase in brain Aβ deposition[48], and elevated brain C3 levels can reduce Aβ accumulation[49]. Complement factors are known to activate microglial cells and elevate phagocytic capacity of macrophages or microglia by binding with their receptors, which may contribute to Aβ clearance[30]. We showed here that oral vaccination increased brain C1q and C3 levels, indicating that complement activation might be an auxiliary pathway in oral vaccination-induced Aβ clearance (Fig. 6A).

Potential Mechanisms Responsible for Oral Vaccination-Induced Antibody- Mediated Aβ Clearance

Several hypotheses have been proposed to explain the mechanism by which active immunization removes Aβ from the central nervous system. One possible route is the "peripheral sink hypothesis": anti-Aβ antibodies generated following vaccination are able to sequester and eliminate peripheral Aβ species, and this process may further facilitate the efflux of Aβ peptides, favoring the decline of Aβ burden[50]. It is known that the activation of microglia is related to Aβ pathology in the progression of AD. The microglial attraction induced by Aβ species may exert a positive effect on the clearance of Aβ by phagocytosis[51–53]. The presence of anti-Aβ antibodies in the brain could bind with Aβ and form antibody-Aβ complexes, which would further interact with Fc receptors on phagocytic microglia, thereby promoting the uptake and internalization of Aβ species[54-56]. In addition, the complement factors C1q and C3 were able to facilitate the phagocytic response of microglia as noted above (Fig. 6A). Therefore, microglia-dependent phagocytosis may also be an additional mechanism for the oral vaccination-induced elimination of Aβ from the brain, regarding the elevation of both antibodies and complement factors induced by the rAAV/Aβ vaccine.

Enhancement of Autophagy in the Brain May Play a Novel Role in Oral Vaccination-Induced Aβ Clearance

Increasing evidence supports the pivotal role of autophagy in Aβ metabolism[12,33]. During normal aging or the progression of AD, autophagy gradually declines in the central nervous system[57,58], likely causing the accumulation of aberrant proteins. The impairment of autophagy in AD has been substantiated by the accumulation of autophagosomes in dystrophic neurites[59,60]. Autophagy enhancement is beneficial in ameliorating Aβ-related pathological changes by promoting Aβ clearance[61]. Here, we discovered that rAAV/Aβ vaccination enhanced autophagy as demonstrated by an increase in the LC3B-II/LC3B-I ratio, suggesting the up-regulation of autophagosome formation. Meanwhile, the p62 protein levels were significantly decreased in mice subjected to rAAV/Aβ vaccination; this could furthermore support the enhancement of autophagy. Lysosomal function in the brain plays an essential role in regulating autophagy[62], but whether oral immunization with rAAV/Aβ influences lysosomal function in mouse brains remains to be determined. Enhanced autophagy is closely associated with Aβ clearance[15]. Based on our results, we tentatively conclude that activation of autophagy is an important pathway for mediating oral vaccination-induced Aβ clearance (Fig. 6A).

The Akt/mTOR pathway is fundamental in modulating various cellular responses, including autophagy[63]. Suppression of the Akt/mTOR pathway can improve autophagy function[63,64]. A recent clinical study found that the Akt/mTOR pathway is hyperactive in patients with mild cognitive impairment or Alzheimer's disease[34], and such hyperactivation is closely linked to autophagy failure in these individuals[34]. Genetic or chemical approaches that inhibit the Akt/mTOR pathway successfully rescue

Aβ pathology and improve cognitive function in AD mouse models[61,65]. Consistent with these findings, the Akt/mTOR pathway was also inhibited in the brains of mice following oral rAAV/Aβ vaccination. Given the mechanistic link between autophagy and the Akt/mTOR pathway, it is likely that oral vaccination may induce autophagy *via* Akt/mTOR inhibition.

The detailed functions of autophagy in the vaccination-related immune response are largely unknown, although it is well-established that autophagy is actively involved in antigen-processing or elimination in immune cells, and in maintaining immunological memory[66–68]. It has been reported that viral infections may activate autophagy with unknown mechanisms[69]. Recent research on a recombinant BCGΔureC::hly (rBCG) vaccine pointed out that rBCG vaccination induces autophagy in macrophages *via* an AIM2-dependent pathway[18]. Since our rAAV was detected only in the gut after oral vaccination[10], and rAAV/GFP did not activate autophagy, it is unlikely that oral administration of rAAV directly activated autophagy in the brain. Nevertheless, the molecular mechanism behind the inhibition of the Akt/mTOR pathway by oral vaccination requires further study.

Oral Vaccination Appears to Be a Safe Strategy for the Early Prevention of AD

Amyloidosis, which occurs in the preclinical stage of AD[70,71], has been regarded to be an upstream trigger for many neuropathological changes[72]. Although the relationship between Aβ dysmetabolism and neurodegeneration has not been totally clarified, the prolonged existence of Aβ pathology that begins in the preclinical stage suggests that targeting Aβ clearance could be a potential approach for early intervention[73,74]. The safety of the developed vaccine is also of particular importance, considering the adverse reactions of AN1792[75]. In previous animal studies we did not observe lymphocytic infiltration and microhemorrhage in mouse brains[11]. In the present study, we monitored the changes in body weight following oral vaccination and found no significant reduction compared with mice treated with PBS (data not shown). The survival rate in the rAAV/Aβ group was 100% during the experiment. These results support the safety of the developed vaccine and the route of vaccination. Moreover, oral vaccination may have a better tolerability than other immunization routes in aged patients with mild cognitive impairment or AD, especially when considering the long-term course of the disease[76]. We propose that oral rAAV/Aβ vaccination may be a safe and potent approach for early intervention in AD (Fig. 6B). Further investigations on the safety of the vaccine are underway to better address this concern.

Taken together, the present study demonstrated for the first time that autophagy activation is closely involved in oral rAAV/Aβ vaccination-induced Aβ clearance, and this activation is likely due to the inhibition of the Akt/mTOR pathway. Our research may provide new insights into vaccine-induced autophagy-dependent Aβ clearance and the development of early interventional strategies for AD.

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