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Combined treatment of A β immunization with statin in a mouse model of Alzheimer's disease

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Abstract

We evaluated the therapeutic efficacy of combined treatment of A β -immunization with simvastatin in an Alzheimer mouse model at age 22 months. DNA prime-adenovirus boost immunization induced modest anti-A β titers and simvastatin increased the seropositive rate. A β -KLH was additionally administered to boost the titers. Irrespective of simvastatin, the immunization did not decrease cerebral A β deposits but increased soluble A β and tended to exacerbate amyloid angiopathy in the hippocampus. The immunization increased cerebral invasion of leukocytes and simvastatin counteracted the increase. Thus, modest anti-A β titers can increase soluble A β and simvastatin may reduce inflammation associated with vaccination in aged Alzheimer mouse models.

Keywords

Alzheimer's disease; statin; adenovirus; vaccine; amyloid; inflammation

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the elderly. Patients with AD develop deposits of abnormally aggregated amyloid β -peptide (A β) as forms of amyloid plaques and cerebral amyloid angiopathy (CAA). A β is produced from a larger transmembrane glycoprotein, amyloid β -protein precursor (APP), by proteolytic processing. A β found in the AD brain is heterogeneous at its carboxyl-terminus, resulting in peptides of 39–43 amino acids. Amyloid plaques, however, mainly contain 2 forms of A β consisting of 40 (A β 40) and 42 (A β 42) amino acids (Iwatsubo et al., 1994; Savage et al., 1995). A β 42 is considered to be highly amyloidogenic, and oligomeric forms of A β are neurotoxic (Lambert et al., 2001; Walsh et al., 2002). The amyloid hypothesis states that accumulation of A β in the brain is the primary influence driving AD pathogenesis (Hardy and Selkoe, 2002). Thus,

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the amyloid hypothesis provides the prime target for AD therapy and preventing A β production and aggregation as well as removing A β from the brain are considered to be preventive and therapeutic (Citron, 2010).

Anti-A β immunotherapy is considered to be one of the most efficacious means of clearing A β deposits from the brain. Immunization of AD mouse models with synthetic A β prevented or reduced A β deposits and improved their memory and learning deficits (Janus et al., 2000; Morgan et al., 2000; Schenk et al., 1999). These impressive results prompted initiation of phase I/II clinical trials to evaluate the safety and tolerability of aggregated A β vaccination. Clinical trials of peptide A β vaccine (AN1792) brought to light the problems associated with the modality. Approximately, 6% of AD patients subjected to the A β vaccine developed aseptic T-lymphocyte meningoencephalitis and/or Fc-mediated brain inflammation, which caused discontinuation of the trial (Check, 2002; Orgogozo et al., 2003). Only 20% of AD patients developed anti-A β antibodies in the vaccine trial (Gilman et al., 2005), indicating that most AD patients might not have an adequate immune response to A β vaccination due to aging and low immunogenicity of A β (Monsonego et al., 2001; Monsonego, 2005). Cerebral hemorrhages were found in patients subjected to the A β vaccine trial (Ferrer et al., 2004; Uro-Coste et al., 2010). Such hemorrhages appeared to be associated with increases in vascular amyloid deposits after immunization, which may be detrimental to endothelial cells and blood-brain barrier (BBB) (Ferrer et al., 2004; Lee et al., 2005; Pfeifer et al., 2002; Racke et al., 2005; Wilcock et al., 2004). Therefore, the next generation of vaccines will need to overcome these problems associated with A β (AN-1792) vaccination.

To avoid the T-cell mediated side effects, we previously constructed an adenovirus vector encoding 11 tandem repeats of A β 1-6 fused to the receptor-binding domain (Ia) of Pseudomonas exotoxin A, AdPEDI-(A β 1-6)₁₁ (Kim et al., 2005). A β 1-6 lacks T cell epitopes of A β (Agadjanyan et al., 2005; Cribbs et al., 2003; Ghochikyan et al., 2006; Seabrook et al., 2007). Nasal vaccination of 2-month-old AD model mice with AdPEDI-(A β 1-6)₁₁ for 10 months induced Th2-biased responses and reduced A β deposits in the brain. Thus, nasal vaccination of mice with AdPEDI-(A β 1-6)₁₁ is preventive against cerebral β -amyloidosis without inducing T cell responses. Additionally, we demonstrated that a DNA prime-adenovirus boost regimen enhanced Th2-biased responses by increasing the seropositive rate and titers in 2–3 month-old C57BL/6 mice using AdPEDI-(A β 1-6)₁₁ and a plasmid DNA encoding PEDI-(A β 1-6)₁₁. Therefore, in this study, we have nasally vaccinated an aged AD mouse model with the DNA prime-adenovirus boost regimen followed by boosters of Keyhole limpet hemocyanin (KLH)-conjugated A β to evaluate its therapeutic efficacy in reducing cerebral A β deposits.

Statins inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and reduce biosynthesis of cholesterol. Statins are extensively used as lipid-lowering drugs in clinical practice and have been proven to be effective in reducing cardiovascular-related morbidity and mortality. Increasing evidence suggests a link between AD, vascular risk factors, and atherosclerosis (Altman and Rutledge, 2010; Bhat, 2010; Luzzi et al., 2010). Retrospective epidemiological studies indicated that subjects taking statins had a lower prevalence and incidence of AD (Jick et al., 2000; Rockwood et al., 2002; Wolozin et al., 2000). Data, however, from clinical trials of statins for AD are inconsistent. The conflicting results of the clinical trials can be explained by the differences in doses between the different trials (Miida et al., 2007; Wolozin et al., 2006). The beneficial effects of statins for AD may be exerted by the pleiotropic mechanisms of statin action at high doses (Menge et al., 2005; Miida et al., 2007; Wolozin et al., 2006). Such pleiotropic effects of statins include reducing A β production, suppressing inflammatory responses, stabilizing the BBB integrity, protecting neurons from excitotoxins, apoptosis and oxidative stresses, and promoting synaptogenesis (Fonseca et al., 2009; Willey and Elkind, 2010). Previously, we showed that simvastatin was

effective in reversing learning and memory deficits of an AD mouse model at 11–15 months of age without altering cerebral A β load (Li et al., 2006) and that simvastatin boosted the seropositive rate and titers in 2–5 month-old C57BL/6 mice vaccinated with AdPEDI-(A β 1-6)₁₁ (Kou et al., 2010). Here, we have evaluated the therapeutic efficacy of combined treatment of DNA prime-adenovirus boost regimen followed by A β -KLH boosters with simvastatin in reducing cerebral A β load in an AD mouse model at 11–22 months of age.

2. Materials and methods

2.1. Preparation of an adenovirus vector, AdPEDI-(A β 1-6)₁₁, and plasmid vector, pCA-PEDI-(A β 1-6)₁₁

AdPEDI-(A β 1-6)₁₁ and pCA-PEDI-(A β 1-6)₁₁ were prepared as previously described (Kim et al., 2005).

2.2. Animals and Treatments

B6.Cg-Tg(APPswe, PSEN1dE9) 85Dbo/J mice (TgAPPswe/PS1dE9 mice) mice (Jankowsky et al., 2004) were purchased from Jackson Laboratory (Bar Harbor, ME) and used as an AD mouse model. Eleven-month-old TgAPPswe/PS1dE9 mice were divided into 4 groups: PBS group, mice fed with regular food and no vaccination (treated with PBS) (n = 14, 7 males and 7 females); statin-only group, mice fed with food containing simvastatin with no vaccination (n = 14, 7 males and 7 females); vaccine-only group, mice fed with regular food with vaccination (n = 11, 5 males and 6 females); vaccine-plus-statin group, mice fed with food containing simvastatin with vaccination (n = 14, 7 males and 7 females). The simvastatin treatment groups were fed with a diet admixture containing 0.04% of simvastatin so that each mouse consumed a daily dose of approximately 50 mg simvastatin/kg body weight. One month after simvastatin treatment, mice in vaccination groups were nasally immunized with plasmid DNA, pCA-PEDI-(A β 1-6)₁₁ (100 μ g/mouse, twice, weekly), followed by an adenovirus vector, AdPEDI-(A β 1-6)₁₁ (1×10^8 pfu/mouse, twice weekly). Mice in the PBS group were subjected to only nasal treatment with PBS. Due to the decline of antibody titers 2 months after the initial vaccination, 50 μ g of synthetic A β 1-8 conjugated with KLH was given to the mice subcutaneously for further boosting at 3 to 5 week intervals for 5 months. The treatment schedule is shown in Fig 1. Two months after the final vaccination, the experimental mice were euthanized to determine the therapeutic effects of the treatment. All animal protocols used for this study were prospectively reviewed and approved by the Institutional Animal Care and Use Committee of the University of Illinois College of Medicine at Peoria.

2.3. Determination of total cholesterol levels in plasma

Blood samples were collected using heparinized capillary tubes at 1 and 2 months after simvastatin treatment. Plasma was prepared by centrifuging blood samples at $1500 \times g$ for 10 min at room temperature. The total cholesterol levels in plasma were determined colorimetrically with commercial reagents (InfinityTM cholesterol reagent, Thermo Electron Corporation, Melbourne, Australia).

2.4. Determination of anti-A β antibodies in sera

Blood samples were collected at 2 or 3 weeks after each vaccination booster by cutting the tail tips or puncturing the saphenous veins. The blood samples were incubated at room temperature for 1 h then transferred to 4°C. After overnight incubation, sera were separated by centrifugation at $10,000 \times g$ for 10 min. Sera were stored at -80°C and thawed at the time of assay. Enzyme-linked immunosorbent assay (ELISA) was carried out to determine the titers of anti-A β antibodies and the immunoglobulin isotypes in the sera as previously

described (Kim et al., 2005). To be brief, a 96-well plate was coated with 500 ng synthetic A β 1-42 peptide per well at 4°C overnight, followed by incubation with blocking buffer (1× PBS containing 0.5% BSA, 0.05% Tween-20 and 5% goat serum) at room temperature for 1 h. Then, diluted serum samples were added to microtiter wells and incubated at 4°C overnight. The next day, microtiter wells were washed 5 times using washing buffer (1× PBS containing 0.05% Tween-20), and then incubated with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 h. The microtiter wells were washed again with the washing buffer 5 times and then incubated with 100 μ l of 3,3',5,5'-tetramethylbenzidine (TMB) for 15 min. The reaction was stopped by adding 100 μ l of 1 N H₂SO₄. The optic densities were determined at 450 nm using Microplate Reader. Serial dilutions of 6E10 (monoclonal anti-A β antibody) were used as the standard to determine the titer of anti-A β antibodies in the sera. Therefore, the concentrations (μ g/ml) of the serum titers presented here reflect the concentrations of 6E10 antibody, which produce the same ELISA readings, and may not accurately represent the absolute amounts. Comparison of treatment groups was performed by one-way analysis of variance (ANOVA) and two-tailed Student's t-test. $P < 0.05$ was considered statistically significant.

2.5. Quantification of brain A β by ELISA

Female mice ($n = 5$ for the PBS, statin-only, and vaccine-only group; and $n = 7$ for the vaccine-plus-statin group) were terminated at 22 months of age by intraperitoneal injection of pentobarbital (100 mg/kg). The neocortex and hippocampus were dissected and lysed using the Bio-Plex cell lysis kit (Bio-Rad Laboratories, Hercules, CA). The brain tissues were dounce-homogenized in carbonate buffer (100 mM Na₂CO₃, 50mM NaCl, pH 11.5) containing protease inhibitors [10 μ g/ml aprotinin and 1 mM 4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride (AEBSF)] and centrifuged at 16,000 g for 30 min at 4°C. The supernatants containing buffer soluble A β were collected and the protein concentrations in the supernatants were determined by Bio-Rad Protein Assay (Bio-Rad). The pellets containing insoluble A β were further dounce-homogenized in guanidine hydrochloride (final concentration, 5 M) and then rock-shaken for 3–4 h at room temperature. Levels of buffer-soluble and insoluble A β in the neocortex and hippocampus were quantified by the A β 42 and A β 40 ELISA kits (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

2.6. Immunohistochemistry and histochemistry

Five male mice for each group were deeply anesthetized with pentobarbital and cardinally perfused with cold PBS followed by 4% paraformaldehyde. The brains were quickly removed and fixed in 4% paraformaldehyde for 16 h. The brains were then stored overnight in 30% sucrose in 0.1M PBS and frozen in Tissue-Tek optimal cutting temperature compound. Frozen sections (35 μ m thickness) were prepared and subjected to immunohistochemical staining to determine the brain A β load and the activation of microglial cells and astrocytes using the avidin-biotin-peroxidase method (Vectastatin ABC kit, Vector, Burlingame, CA). Endogenous peroxidase was eliminated by treatment with 3% H₂O₂/10% methanol Tris-buffered saline (TBS) for 60 min at room temperature. After washing with water and 0.1 M TBS (pH 7.4), slides were blocked with blocking buffer for 60 min at room temperature to prevent non-specific protein binding. The slides were then incubated with primary antibody 6E10 (1: 2000; Signet Laboratories, Dedham, MA), CD45 (1:100; Serotec, MCA1388, Raleigh, NC), GFAP (1:500; Dako, Carpinteria, CA) or CD11b (1:200; Serotec, MCA711, Raleigh, NC) overnight at 4°C. The sections were rinsed in 0.1 M TBST and incubated with appropriate biotinylated secondary antibody for 1 h at room temperature. Finally, the avidin biotin peroxidase method using 3,3'-diaminobenzidine as a substrate (Vector, Burlingame, CA) was performed according to manufacturer's protocol.

Histomorphometry for quantification of amyloid deposition, astrocyte and microglial activation was performed using an Olympus BX61 automated microscope, Olympus Fluoview system and the Image Pro Plus v4 image analysis software (Media Cybernetics, Silver Spring, MD) capable of color segmentation and automation via programmable macros. For each mouse, 4–5 coronal brain sections starting at 1.2 mm posterior to the bregma to caudal, each separated by approximately 300 μ m intervals, were analyzed. Areas stained with specific antibodies were expressed as a percentage of total hippocampus or neocortex examined.

For detection of CAA, 3 coronal brain sections for each male mouse ($n = 5$ for each group) starting at 2.7 mm posterior to the bregma to caudal, each separated by 300 μ m intervals, were stained with 1% thioflavin S followed by destaining in 70% ethanol. Blood vessels positive for thioflavin fluorescence in the hippocampus were enumerated as described previously (Van Dorpe et al., 2000).

2.7. Prussian blue reaction

For detection of possible cerebral hemorrhages that might be associated with vaccination treatment, Prussian blue reaction was carried out on brain sections using an Iron Stain kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. For each male mouse ($n = 5$ for each group), 8 coronal brain sections starting at 1.5 mm posterior to the bregma to caudal, each separated by approximately 150 μ m intervals, were analyzed.

2.8. Immunoblot analysis

Hippocampal homogenates (30 μ g, $n = 5$ females for each group) were mixed with 5 $\times$ sample buffer (final concentration: 60mM Tris-HCl, 2% SDS, 10% glycerol, 0.001% bromophenol blue, pH: 6.8) and heated at 100°C for 5 min. Samples were subjected to 10–20% Tris-HCl gradient SDS-PAGE and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membrane was blocked by PBS containing 5% non-fat dried milk (w/v), 0.02% sodium azide, and 0.02% Tween 20 for 1 h at room temperature, incubated at 4°C overnight with anti-CD45 antibody (Serotec, MCA1388, Raleigh, NC). The membrane was then incubated with appropriate biotinylated secondary antibody for 1 h at room temperature and visualized by the western lighting chemiluminescence reagent plus (Perkin Elmer, Boston, MA) according to the manufacturer's protocol. The membranes were reprobed with monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon, Temecula, CA). The optical densities of protein bands from the membranes were determined by densitometric scanning using a HP Scanjet G3010 Photo Scanner and the Image J V1.40 (National Institutes of Health, USA). The optical density of each protein band was divided by that of the GAPDH band on the same lane from the same membrane for normalization.

2.9. Cytokine/chemokine microarray

The neocortex and hippocampus tissue lysates were prepared and their protein concentrations were determined as described above. Levels of cytokines and chemokines in the lysates were determined by the bead-based suspension microarray technology (AssayGate, Ijamsville, MD) (Opalka et al., 2003). The threshold of detection was determined by adding two standard deviations to the mean fluorescence intensity of twenty zero standard (background) replicates. The minimum detectable concentrations were 3.6 pg/ml for IL-1 α , 3.8 pg/ml for IL-1 β , 2.3 pg/ml for IL-2, 4.3 pg/ml for IL-4, 1.3 pg/ml for IL-6, 8.2 pg/ml for IL-10, 2.3 pg/ml for IFN- γ , 16 pg/ml for TGF- β 1, 2.5 pg/ml for monocyte chemoattractant protein (MCP)-1 (CCL2), and 1.3 pg/ml for macrophage inflammatory protein (MIP)-1 α (CCL3). The results were expressed in picograms per milligram of brain

protein and analyzed by ANOVA and two-tailed Student's t-test using SigmaStat for Windows Version 3.00. $P < 0.05$ was considered statistically significant.

2.10. Assessments of potential liver and muscle injury

To evaluate potential liver damage caused by long-term administration of simvastatin, aspartate transaminase (AST) and alanine transaminase (ALT) levels in the plasma were determined using AST and ALT enzymatic assay kit (Bio Scientific Corporation, Austin, TX) according to the manufacturer's protocol.

Skeletal muscle injury possibly associated with simvastatin treatment was assessed by examining thigh muscle sections stained with hematoxylin and eosin and by determining creatine kinase (CK) levels (Charles River research animal diagnostic service).

2.11. Statistical analysis

Data were expressed as mean $\pm$ SEM. ANOVA and two-tailed Student's t-test were used to determine the intergroup differences except for seropositive rates where chi-square analysis was used. Correlations between the antibody titers and the areas (%) stained with specific antibodies (6E10, CD11b, and CD45) were estimated using Prism 3.02 program (GraphPad Software, Inc., San Diego, CA). $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Simvastatin treatment

Two groups of mice were treated daily with 50 mg/kg of simvastatin for 11 months. This daily dose was chosen for this study because this dose lowered the total plasma cholesterol levels in mice and produced learning and memory improvement without detectable toxicity after 3–4 months of simvastatin treatment in our previous studies (Kou et al., 2010; Li et al., 2006). The total cholesterol levels in plasma were measured 1 and 2 months after the initiation of simvastatin treatment. Simvastatin significantly reduced total plasma cholesterol levels by 10% only after 2 months of treatment compared to mice consuming regular diet without simvastatin (Table 1). We previously showed that plasma total cholesterol levels in young C57Bl/6 mice were reduced after 1-month treatment with simvastatin (Kou et al., 2010). However, 2-month treatment was required for aged mice to achieve the same cholesterol-lowering effect of simvastatin. There was no difference in the total cholesterol levels between the statin-only and vaccine-plus-statin groups. No difference in the total cholesterol levels was found between the PBS and vaccine-only groups, also. During the treatment period, there were no differences in physical appearance, body weight, food consumption, or mortality among the groups (data not shown). Long-term treatment with 50 mg/kg of simvastatin did not cause any generalized toxicity, muscle and liver damage assessed by histology and plasma levels of AST, ALT and CK (Table 1).

3.2. Anti-A β antibody titers and IgG isotyping

Sera were collected from all groups at 2 to 7 weeks after each vaccine booster (Fig. 1). The titers of anti-A β antibodies and the immunoglobulin isotypes in the sera were determined by ELISA. Anti-A β antibody titers in the mouse sera were calculated using serial dilutions of 6E10 (anti-A β antibody) as the standard. The data on immune responses, anti-A β antibody titers, isotypes and IgG1/IgG2a ratios are summarized in Table 2. The DNA prime-adenovirus boost regimen elicited modest anti-A β titers in both vaccine groups. One month after the initial DNA vaccination, only 2 mice in each vaccine group developed anti-A β antibody titers (seropositive) with the average of 1.53 ± 0.05 and 1.28 ± 0.05 $\mu\text{g/ml}$ for the vaccine-only and vaccine-plus-statin groups, respectively. The numbers of seropositive mice increased to 7 and 8 out of 14 mice at 6 and 8 weeks after the initial vaccination,

respectively, in the vaccine-plus-statin group but remained the same in the vaccine-only group. Thus, simvastatin increased the seropositive rates in an aged AD mouse model ($P = 0.09$ and 0.048 , respectively, chi-squared test). IgG isotype profiles of anti-A β antibodies showed that the DNA prime-adenovirus boost regimen predominantly elicited IgG1 and IgG2b isotypes in both groups regardless of simvastatin treatment. Anti-A β IgG2a titers in both groups were below detectable levels by ELISA. Anti-A β titers continuously decreased 4 to 8 weeks after the initial vaccination in the vaccine-only group. The vaccine-plus-statin group had peak anti-A β titers at 6 weeks and, then, a decline at 8 weeks after the initial vaccination. Although the vaccine-plus-statin group had approximately 2- to 3-fold higher antibody titers than the vaccine-only group at 6 and 8 weeks after the initial vaccination, the differences were not significant. Due to the modest immune responses and the declines in the anti-A β antibody titers after cessation of the DNA prime-adenovirus boost regimen, A β ₁₋₈ conjugated with KLH (A β ₁₋₈-KLH) was subcutaneously given to both vaccination groups at intervals of 3 to 5 weeks for 5 months in order to increase the antibody titers. The peptide boosters increased the antibody titers by 8- to 17-fold in both groups and no differences were found in the anti-A β titers between the 2 vaccination groups. Almost all mice in both groups became seropositive after A β ₁₋₈-KLH administration (Table 2). IgG isotyping revealed that A β ₁₋₈-KLH increased anti-A β IgG1 titers but induced anti-A β IgG2a titers in both groups. The IgG1/IgG2a ratios in both groups were consistently more than 3, indicating Th2-polarized immune responses. Simvastatin shifted the immune responses further toward Th2-type only 1.5 weeks after the 4th A β ₁₋₈-KLH booster; the IgG1/IgG2a ratios were 3.2 ± 0.69 and 7.5 ± 1.81 for the vaccine-only and vaccine-plus-statin groups, respectively ($P = 0.05$). Similar to the way the anti-A β titers declined after cessation of adenovirus boosters, termination of A β ₁₋₈-KLH boosters resulted in reductions in anti-A β antibody titers 7 weeks after the 2nd A β ₁₋₈-KLH boosters, as well as 6 weeks after the 5th A β ₁₋₈-KLH boosters in both groups (Table 2).

3.3. Quantification of A β load by ELISA in the brain

Ten months after the initial immunization, mice were euthanized at 22 months of age. Levels of buffer-soluble and insoluble A β in the neocortex and hippocampus were quantified by A β 42- and A β 40-specific ELISA and were expressed as pg of A β per mg of total protein. The amounts of hippocampal buffer-soluble A β 42 were 86.5 ± 13.0 and 82.9 ± 12.9 pg/mg in the vaccine-only and vaccine-plus-statin groups, respectively, and 2 times greater than those in the PBS group (41.0 ± 5.9 pg/mg, $P < 0.05$ for both, Fig. 2A). No difference was found in the amounts of hippocampal buffer-soluble A β 42 between the PBS and statin-only (65.4 ± 13.8 pg/mg) groups ($P > 0.05$). Similarly, hippocampal buffer-soluble A β 40 in the vaccine-only and vaccine-plus-statin groups (88.0 ± 14.8 and 95.3 ± 16.8 pg/mg, respectively) increased 2-fold compared to the PBS group (47.2 ± 9.2 pg/mg, $P < 0.05$ for both) (Fig. 2B). There was no difference between the statin-only (79.8 ± 21.0 pg/mg) and PBS groups in the levels of hippocampal soluble A β 40. Levels of neocortex buffer-soluble A β 42 were 42.1 ± 6.5 , 33.0 ± 3.6 , 43.5 ± 6.7 and 29.2 ± 4.2 pg/mg for the PBS, statin-only, vaccine-only and vaccine-plus-statin groups, respectively. Levels of neocortex buffer-soluble A β 40 were 34.3 ± 3.0 , 44.2 ± 7.8 , 30.3 ± 5.0 and 53.1 ± 11.6 pg/mg for the PBS, statin-only, vaccination-only and vaccine-plus-statin groups, respectively. There were no significant differences in the levels of buffer-soluble A β 42 and A β 40 in the neocortex between groups. Levels of buffer-insoluble A β 42 and A β 40 were determined by ELISA, also. No significant differences were identified in either buffer-insoluble A β 42 or A β 40 in both hippocampus and neocortex between groups (Table 3).

3.4. Quantification of A β load by immunohistochemistry and morphometry in the brain

The A β load in the hippocampus and neocortex was determined by immunohistochemistry using anti-A β 6E10 antibody followed by morphometric analysis (Fig. 3). Average

percentages of areas showing A β immunoreactivity measured by morphometry in the hippocampus and neocortex are shown in Figure 3B. The A β loads in the hippocampus were 0.69 ± 0.08 , 0.75 ± 0.15 , 0.82 ± 0.14 and $0.74 \pm 0.16\%$ for the PBS, statin-only, vaccine-only and vaccine-plus-statin groups, respectively. The A β loads in the neocortex were 1.00 ± 0.11 , 1.31 ± 0.21 , 1.06 ± 0.22 and $1.02 \pm 0.20\%$ for the PBS, statin-only, vaccine-only and vaccine-plus-statin groups, respectively. No significant differences were found in the A β loads in the hippocampus and neocortex between groups ($P > 0.05$).

3.5. Detection of neuroinflammation by immunohistochemistry and immunoblotting

To assess potential alterations in inflammatory responses in the brain after vaccination and/or statin treatment, brain sections were subjected to immunohistochemistry using anti-CD45 antibody for blood-derived leukocytes and CD11b antibody for activated microglia/monocytes, and anti-GFAP antibody for reactive astrocytes followed by morphometric analysis. The CD45-immunoreactive areas in both hippocampus ($1.00 \pm 0.06\%$) and neocortex ($1.11 \pm 0.15\%$) in the vaccine-only group were greater than those in the PBS group (0.64 ± 0.05 and $0.83 \pm 0.03\%$ in the hippocampus and neocortex, respectively, $P < 0.05$ for both) (Fig. 4). No differences were found in CD45-immunoreactive areas between the vaccine-plus-statin group (0.65 ± 0.10 and $0.86 \pm 0.14\%$ in the hippocampus and neocortex, respectively) and the PBS group (Fig. 4). In the hippocampus, the CD45-immunoreactive areas in the vaccine-plus-statin group were smaller than those in the vaccine-only group ($P < 0.05$) but this difference was not observed in the neocortex ($P = 0.13$). The statin-only group (0.82 ± 0.11 and $1.05 \pm 0.22\%$ in the hippocampus and neocortex, respectively) was not different from the PBS group in CD45 immunoreactivity ($P > 0.05$).

To confirm an increase in CD45-immunoreactivity in the vaccine-only group and counteraction of simvastatin against CD45-immunoreactive cells, immunoblot analysis was performed using hippocampal homogenates and anti-CD45 antibody (Fig. 4C and D). Expression levels of CD45 in the vaccine-only group increased by 34% compared with those in the PBS group ($P = 0.01$). CD45 expression levels in the vaccine-only group were greater than those in the vaccine-plus-statin group in the hippocampus ($P = 0.05$). No difference was found in CD45 expression levels between the PBS and vaccine-plus-statin groups ($P > 0.05$). Thus, simvastatin counteracted an increase in CD45 expression, which was caused by vaccination.

In the hippocampus, CD11b-immunoreactive areas were 0.44 ± 0.05 , 0.34 ± 0.04 , 0.46 ± 0.07 and $0.44 \pm 0.04\%$ in the PBS, statin-only, vaccine-only and vaccine-plus-statin groups, respectively. CD11b-immunoreactive areas in the neocortex were 0.34 ± 0.07 , 0.34 ± 0.01 , 0.41 ± 0.05 and $0.27 \pm 0.09\%$ in the PBS, statin-only, vaccine-only and vaccine-plus-statin groups, respectively. There were no differences in CD11b immunoreactivity between groups. (Fig. 5A).

GFAP immunoreactivity was not significantly different across groups ($P > 0.05$). CD11b-immunoreactive area percentages of the PBS, statin-only, vaccine-only, vaccine-plus-statin groups were 11.7 ± 4.6 , 10.1 ± 2.9 , 11.0 ± 3.3 and $10.5 \pm 2.1\%$ in the hippocampus and 12.4 ± 4.8 , 12.2 ± 2.8 , 11.5 ± 3.6 and $11.2 \pm 2.1\%$ in the neocortex (Fig. 5B).

3.6. Detection of neuroinflammation by cytokines and chemokines

To further assess potential alterations in inflammatory responses in the brain after vaccination and/or statin treatment, levels of cytokines and chemokines in the hippocampus and neocortex were determined by the bead-based suspension microarray technology. Cytokines included IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, IFN- α , IFN- γ , and TGF- β 1. Chemokines determined were MCP-1 (CCL2) and MIP-1 α (CCL3). IL-4, IL-6, and IFN- γ

were the minimum detectable levels and omitted from analysis. The levels of some cytokines are shown in Fig. 6. Levels of IL-2 decreased in the vaccine-only group compared to the PBS group ($P < 0.01$ for the hippocampus and $P < 0.05$ for the neocortex). In the hippocampus and neocortex, levels of IL-10 consistently increased in the statin-only group compared to the PBS group ($P < 0.05$). There were no significant differences in any of the other cytokines and chemokines between any groups.

3.7. No correlation between the antibody titers and A β load or activated microglia

The relationships between the anti-A β titers and A β load (6E10-stained area) in the hippocampus and neocortex were analyzed in the vaccine-only and vaccine-plus-statin groups using the titers 1.5 weeks (Fig. 7) and 2 weeks (data not shown) after the 4th A β ₁₋₈-KLH booster. The anti-A β titers were not correlated with A β load in the hippocampus and neocortex in these mouse groups. Additionally, there was no correlation between the anti-A β titers and immunoreactive areas (%) with antibodies against activated microglial markers (CD11b and CD45) (data not shown).

3.8. Detection of cerebral amyloid angiopathy and cerebral hemorrhages

Because anti-A β immunotherapy was reported to cause cerebral microhemorrhages in aged AD mouse models and patients with AD, possible cerebral hemorrhages were investigated by iron staining. Eight brain sections were examined from each mouse. Hemorrhages associated with blood vessels were rare in any group (0–1/section) and no differences were found in the numbers of hemorrhages between groups.

Hemorrhages associated with A β immunotherapy are often accompanied by an increase in CAA. Three brain sections were analyzed from each mouse. The numbers of hippocampal blood vessels positive for thioflavin fluorescence per brain section are 3.2 ± 0.98 , 3.8 ± 1.28 , 5.5 ± 1.00 , 3.3 ± 0.80 for the PBS, statin-only, vaccine-only, and vaccine-plus-statin groups, respectively (Fig. 8). Thus, the vaccine-only group had more thioflavin-positive blood vessels in the hippocampus than the 3 other groups but the differences were not significant ($P = 0.14$, 0.33 , and 0.12 for the PBS, statin-only, and vaccine-plus-statin groups, respectively).

4. Discussion

Here, we have investigated the therapeutic efficacy of anti-A β immunotherapy with and without statin in a DNA prime-adenovirus boost regimen followed by A β peptide boosters using TgAPPswe/PS1dE9 mice at 22 months of age. The DNA prime-adenovirus boost regimen induced very modest anti-A β titers and simvastatin increased the seropositive rate. Because anti-A β titers declined after cessation of the adenovirus inoculations, we administered A β ₁₋₈-KLH as additional boosters. A β ₁₋₈-KLH boosters increased anti-A β titers. Regardless of simvastatin treatment, the DNA prime-adenovirus boost regimen followed by A β ₁₋₈-KLH boosters increased soluble A β and revealed a trend toward increased CAA in the hippocampus without decreasing A β deposits in the brain. The immunization increased CD45-positive leukocytes in the brain and simvastatin treatment counteracted the increase. Our results suggest that modest anti-A β titers in advanced AD patients can increase soluble A β and simvastatin may reduce inflammation associated with vaccination by inhibiting monocyte invasion.

Previously, we demonstrated that the DNA prime-adenovirus boost regimen increased the seropositive rate (100%) and anti-A β titers ($19.5 \mu\text{g/ml}$) compared with its homologous prime-boost regimens (0 and 57% seropositive rate, and 0 and $2.8 \mu\text{g/ml}$ anti-A β titers for DNA-only and adenovirus-only, respectively) in C57BL/6 mice at age 2–3 months (Kim et

al., 2007a). In contrast to young C57BL/6 mice, only 18% of TgAPPswe/PS1dE9 mice older than 11 months developed anti-A β titers by the DNA prime-adenovirus boost regimen in this study. These poor responses in aged TgAPPswe/PS1dE9 mice could be explained by immunological tolerance and immunosenescence. Tg2576 mice, another AD mouse model overexpressing APP, showed a decreased antibody response when immunized with synthetic A β compared with non-transgenic controls, which is attributed to hyporesponsiveness of A β -reactive T or B cells caused by the high levels of A β in the blood of Tg2576 mice (Monsonogo et al., 2001). We, however, showed that a homologous prime-boost regimen with AdPEDI-(A β 1-6)₁₁ in young TgAPPswe/PS1dE9 mice induced immune responses comparable to young C57BL/6 mice (Kim et al., 2007b). Therefore, our results suggest that the poor responses to the DNA prime-adenovirus boost regimen in the aged TgAPPswe/PS1dE9 mice are mainly due to immunosenescence and that the DNA prime-adenovirus boost regimen may not be effective in overcoming poor responsiveness to A β immunization in the elderly. Poor immune responses to A β in aged mice (20-month-old) compared to young mice (3-month-old) were reported (Pifer et al., 2002). However, the DNA prime-adenovirus boost regimen with AdPEDI-(A β 1-6)₁₁ followed by A β ₁₋₈-KLH boosters increased the seropositive rate and titers in aged mice. Thus, it is possible to induce low to moderate levels of anti-A β antibodies by active immunization modalities in aged mice (older than 18 months), which include intraperitoneal injection of A β 42 in Freund's adjuvant (Das et al., 2001b), subcutaneous injection of mannan conjugated to A β 1-28 (Petrushina et al., 2008), and subcutaneous injection of A β 1-6 conjugated to the virus-like particle (Wiessner et al., 2011).

In the phase II clinical trial of aggregated A β (AN-1792) vaccination, only 19.7% of AD patients developed positive A β titers (Gilman et al., 2005). We previously found that simvastatin enhanced both seropositive rates and anti-A β titers in young C57BL/6 mice subjected to a homologous prime-boost regimen with AdPEDI-(A β 1-6)₁₁ (Kou et al., 2010). Here, we showed that simvastatin increased the seropositive rates of the DNA prime-adenovirus boost regimen in aged AD model mice without boosting the titers. Thus, simvastatin may be effective in improving immune responses to A β immunization in the elderly.

Because meningoencephalitis associated with the AN1792 trial is thought to be T-cell-mediated auto-immune responses, immunization modalities that elicit predominantly T helper (Th) type 2 (humoral) immune responses are considered to be safer for AD prevention and treatment (Gelinis et al., 2004; Goni and Sigurdsson, 2005; Monsonogo and Weiner, 2003; Schenk et al., 2004; Wisniewski and Frangione, 2005). In mice, the production of IgG1 is primarily induced by Th2-type anti-inflammatory cytokines (IL-4 and IL-10), while IgG2a is produced through Th1-type pro-inflammatory cytokines (IL-2 and IFN- γ). IgG1 was predominant in anti-A β antibodies elicited by the DNA prime-adenovirus boost regimen in aged TgAPPswe/PS1dE9 mice and IgG2a was indiscernible by ELISA, indicating induction of Th2 immune responses. These results are consistent with those in young C57BL/6 mice in which Th2-biased immune responses were induced by the DNA prime-adenovirus boost regimen (Kim et al., 2007a) and with a decrease in cerebral IL-2 levels in the vaccine-only group (Fig. 6). Additional administration of A β ₁₋₈-KLH increased anti-A β titers in aged TgAPPswe/PS1dE9 mice but induced IgG2a anti-A β antibodies, suggesting that a minor Th1 component might be instrumental for boosting the titers. Simvastatin temporarily shifted the immune responses further toward Th2-type without reducing the titers 1.5 weeks after administration of the 4th A β ₁₋₈-KLH booster (Table 2). Statins promote Th2 differentiation by inducing expression of GATA-binding protein 3 (GATA3) and inhibit Th1 differentiation by downregulating activation of nuclear factor- γ B (NF- γ B) and T-bet (a T-box transcription factor) in CD4⁺ T cells (Arora et al., 2006). We and others showed that statins suppress expression of Th1 type cytokines including

interferon (IFN)- γ (Arora et al., 2006; Hakamada-Taguchi et al., 2003; Kou et al., 2010). Naïve CD4 T-cells activated in the presence of IL-2 and IFN- γ tend to develop into Th1-cells and IFN- γ inhibits the proliferation of Th2-cells. Statins promote Th2 biased immune responses through the induction of STAT6 phosphorylation and Th2 cytokines including IL-10 and inhibition of STAT4 phosphorylation, also (Shimada et al., 2006). Furthermore, statins prevent or reverse paralysis in experimental autoimmune encephalomyelitis (EAE) (an animal model of multiple sclerosis) which is mediated by myelin-specific CD4⁺ T cells (Youssef et al., 2002). In line with this view, cerebral levels of IL-10 increased or tended to increase in the statin-only or vaccine-plus-statin group, respectively (Fig. 6). Thus, statins suppress Th1 and augment Th2 responses resulting in increased production of antigen-specific IgG1 in mice (Arora et al., 2006) and young healthy adults (Lee et al., 2006). Because induction of meningoencephalitis (infiltration of A β -specific T cells) by A β vaccination requires expression of IFN- γ in AD mouse models (Monsonogo et al., 2006), statins may mitigate meningoencephalitis associated with A β vaccination by inhibiting IFN- γ signaling.

The DNA prime-adenovirus boost regimen followed by A β -KLH injections increased CD45-positive cells (migratory leukocytes and microglia) in the brain and simvastatin treatment counteracted the increase. A β immunotherapy activates microglia and recruits them to amyloid plaques (Morgan, 2006). Microglial phagocytosis of A β is one of the postulated mechanisms by which A β deposits are cleared from the brain (Bard et al., 2000; Nicoll et al., 2006). However, the role of microglia in clearing A β deposits is debatable (Rezai-Zadeh et al., 2011). In our study, the vaccination increased CD45-positive cells but did not reduce A β load in the brain, suggesting that CD45-positive cells have a limited ability to clear A β deposits from the brain. Such a limited role of microglia in antibody-mediated amyloid plaque clearance was previously demonstrated by enhancing and eliminating activation of microglia by IFN- γ and minocycline, respectively (Garcia-Alloza et al., 2007).

CD45 positive cells in the brain are thought to be migratory leukocytes from the circulation. Statins downregulate expression of chemokines and their receptors such as CCL2, CX3CL1, CCR2, and CX3CR1 via inhibition of the geranylgeranylpyrophosphate pathway in endothelial cells and leukocytes (Damas et al., 2005; Han et al., 2005; Veillard et al., 2006), leading to reduced BBB permeability and restrict leukocyte migration (Ghittoni et al., 2007; Ifergan et al., 2006; Zeng et al., 2005). In line with these observations, simvastatin inhibited invasion of CD45-positive cells, which was induced by vaccination. Thus, statins may be effective in ameliorating meningoencephalitis associated with A β vaccination by inhibiting leukocyte migration, also.

Parenchymal amyloid plaques were focally depleted in the brains of AD patients after A β 42 vaccine (AN1792) treatment (Nicoll et al., 2003) but the severity of CAA increased (Boche et al., 2008). Levels of soluble A β in the cerebral cortex remarkably increased in AD patients subjected to AN1792 vaccination (Patton et al., 2006). These observations after AN1792 vaccination are explained by relocation of plaque A β to blood vessels based on one of the postulated mechanisms for amyloid plaque clearance by A β immunotherapy, which states that anti-A β antibodies directly interact with amyloid plaques, resulting in disaggregation and solubilization of A β fibrils (Bacskai et al., 2002; Boche et al., 2010). In this study, levels of buffer-soluble A β 40 and A β 42 increased in the hippocampus in mice subjected to the vaccination regardless of simvastatin treatment but no reduction was found in cerebral A β deposits and insoluble A β levels. There was no correlation between the anti-A β titers and cerebral A β load in the vaccine-only and vaccine-plus-statin groups (Fig. 7). The mice in the vaccine-only group showed an increasing trend in the severity of CAA and simvastatin treatment appeared to counteract the trend. The observed discrepancies in A β

immunization outcomes between this study and the AN1792 clinical trial may be explained by relatively low levels of anti-A β titers in this study. A positive correlation between levels of anti-A β titers and the reduction of amyloid plaques has been reported in an AD mouse model (Petrushina et al., 2007) as well as AD patients subjected to AN1792 vaccination (Gilman et al., 2005; Nicoll et al., 2003). Prophylactic treatment of TgAPPswe/PS1dE9 mice with AdPEDI-(A β 1-6)₁₁ in our previous study (Kim et al., 2007b) elicited several-fold higher anti-A β titers than those in the current study and decreased cerebral amyloid deposits. Movsesyan et al. (2010) reported that prophylactic vaccination of an AD mouse model with DNA epitope vaccine induced low anti-A β titers and failed to reduce cerebral amyloid deposits. Uro-Coste et al. (2010) reported a clinical case in which an AD patient subjected to AN1792 vaccination had increases in the severity of CAA and microhemorrhages without apparent reduction in amyloid plaques. Therefore, anti-A β antibodies produced in aged TgAPPswe/PS1dE9 mice in this study were able to augment hippocampal levels of soluble A β , resulting in an increasing trend of CAA but might not be high enough to reduce cerebral A β deposits. Another possible explanation is that outcomes of A β immunotherapy may depend on the disease stage and ages. Several studies reported that a correlation between anti-A β antibody titers and the effect on A β load could not be established in aged AD mouse models partly due to the high intrinsic variations of A β load as well as titers over time (Das et al., 2001a; Minami et al., 2010; Okura et al., 2006; Wiessner et al., 2011).

Minami et al. (2010) reported that passive immunization with anti-A β antibody in a 22-month-old AD mouse model did not reduce cerebral A β load but increased levels of CCL2 and pro-inflammatory cytokine, tumor necrosis factor (TNF)- α , suggesting induction of neuroinflammation in advanced stages of cerebral amyloidosis by A β immunotherapy without beneficial effects. We, however, did not observe increased levels of CCL2 and pro-inflammatory cytokines by active immunization (Fig. 6). In this regard, it is tempting to speculate that statins may be effective in preventing both neuroinflammation and exacerbation of CAA, which are induced by A β immunotherapy, because statins inhibit the induction of TNF- α and CCL2 in microglia and macrophages (Damas et al., 2005; Han et al., 2005; Pahan et al., 1997; Veillard et al., 2006) and increase expression of low density lipoprotein-related protein 1 (LRP1) in endothelial cells (Shinohara et al., 2010), which is thought to mediate A β clearance at the BBB (Zlokovic, 2008).

In conclusion, this study revealed that it was difficult to achieve high anti-A β titers and high seropositivity by a DNA prime-adenovirus boost vaccination regimen in a very old AD mouse model and that modest levels of anti-A β antibody did not decrease amyloid plaques but increased soluble A β in the hippocampus. AD mouse models have been criticized because they failed to predict side effects. Our study emphasizes the importance of testing A β immunotherapy in very old AD mouse models with advanced amyloid pathology. Our study also demonstrated that simvastatin counteracted an increase in CD45-positive leukocytes and tended to mitigate an increase in CAA, both of which were induced by A β vaccination. These results warrant further investigation of statins in possibly preventing the side effects associated with A β immunotherapy.

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REFERENCES

- Agadjanyan MG, Ghochikyan A, Petrushina I, Vasilevko V, Movsesyan N, Mkrtichyan M, Saing T, Cribbs DH. Prototype Alzheimer's disease vaccine using the immunodominant B cell epitope from

- beta-amyloid and promiscuous T cell epitope pan HLA DR-binding peptide. *J Immunol.* 2005; 174:1580–1586. [PubMed: 15661919]
- Altman R, Rutledge JC. The vascular contribution to Alzheimer's disease. *Clin Sci (Lond).* 2010; 119:407–421. [PubMed: 20684749]
- Arora M, Chen L, Paglia M, Gallagher I, Allen JE, Vyas YM, Ray A, Ray P. Simvastatin promotes Th2-type responses through the induction of the chitinase family member Ym1 in dendritic cells. *Proc Natl Acad Sci U S A.* 2006; 103:7777–7782. [PubMed: 16682645]
- Bacskai BJ, Kajdasz ST, McLellan ME, Games D, Seubert P, Schenk D, Hyman BT. Non-Fc-mediated mechanisms are involved in clearance of amyloid-beta in vivo by immunotherapy. *J Neurosci.* 2002; 22:7873–7878. [PubMed: 12223540]
- Bard F, Cannon C, Barbour R, Burke RL, Games D, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenko D, Lee M, Lieberburg I, Motter R, Nguyen M, Soriano F, Vasquez N, Weiss K, Welch B, Seubert P, Schenk D, Yednock T. Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. *Nat Med.* 2000; 6:916–919. [PubMed: 10932230]
- Bhat NR. Linking cardiometabolic disorders to sporadic Alzheimer's disease: a perspective on potential mechanisms and mediators. *J Neurochem.* 2010; 115:551–562. [PubMed: 20807313]
- Boche D, Denham N, Holmes C, Nicoll JA. Neuropathology after active Abeta42 immunotherapy: implications for Alzheimer's disease pathogenesis. *Acta Neuropathol.* 2010; 120:369–384. [PubMed: 20632020]
- Boche D, Zotova E, Weller RO, Love S, Neal JW, Pickering RM, Wilkinson D, Holmes C, Nicoll JA. Consequence of Abeta immunization on the vasculature of human Alzheimer's disease brain. *Brain.* 2008; 131:3299–3310. [PubMed: 18953056]
- Check E. Nerve inflammation halts trial for Alzheimer's drug. *Nature.* 2002; 415:462. [PubMed: 11823817]
- Citron M. Alzheimer's disease: strategies for disease modification. *Nat Rev Drug Discov.* 2010; 9:387–398. [PubMed: 20431570]
- Cribbs DH, Ghochikyan A, Vasilevko V, Tran M, Petrushina I, Sadzikava N, Babikyan D, Kesslak P, Kieber-Emmons T, Cotman CW, Agadjanyan MG. Adjuvant-dependent modulation of Th1 and Th2 responses to immunization with beta-amyloid. *Int Immunol.* 2003; 15:505–514. [PubMed: 12663680]
- Damas JK, Boullier A, Waehre T, Smith C, Sandberg WJ, Green S, Aukrust P, Quehenberger O. Expression of fractalkine (CX3CL1) and its receptor, CX3CR1, is elevated in coronary artery disease and is reduced during statin therapy. *Arterioscler Thromb Vasc Biol.* 2005; 25:2567–2572. [PubMed: 16224053]
- Das KP, Chao SL, White LD, Haines WT, Harry GJ, Tilson HA, Barone S Jr. Differential patterns of nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3 mRNA and protein levels in developing regions of rat brain. *Neuroscience.* 2001a; 103:739–761. [PubMed: 11274792]
- Das P, Murphy MP, Younkin LH, Younkin SG, Golde TE. Reduced effectiveness of Abeta1-42 immunization in APP transgenic mice with significant amyloid deposition. *Neurobiol Aging.* 2001b; 22:721–727. [PubMed: 11705631]
- Ferrer I, Boada RM, Sanchez Guerra ML, Rey MJ, Costa-Jussa F. Neuropathology and pathogenesis of encephalitis following amyloid-beta immunization in Alzheimer's disease. *Brain Pathol.* 2004; 14:11–20. [PubMed: 14997933]
- Fonseca AC, Proenca T, Resende R, Oliveira CR, Pereira CM. Neuroprotective effects of statins in an in vitro model of Alzheimer's disease. *J Alzheimers Dis.* 2009; 17:503–517. [PubMed: 19363255]
- Garcia-Alloza M, Ferrara BJ, Dodwell SA, Hickey GA, Hyman BT, Bacskai BJ. A limited role for microglia in antibody mediated plaque clearance in APP mice. *Neurobiol Dis.* 2007; 28:286–292. [PubMed: 17822910]
- Gelinas DS, DaSilva K, Fenili D, George-Hyslop P, McLaurin J. Immunotherapy for Alzheimer's disease. *Proc Natl Acad Sci U S A.* 2004; 101:14657–14662. [PubMed: 15297619]

- Ghittoni R, Lazzerini PE, Pasini FL, Baldari CT. T lymphocytes as targets of statins: molecular mechanisms and therapeutic perspectives. *Inflamm Allergy Drug Targets*. 2007; 6:3–16. [PubMed: 17352684]
- Ghochikyan A, Petrushina I, Lees A, Vasilevko V, Movsesyan N, Karapetyan A, Agadjanyan MG, Cribbs DH. Abeta-immunotherapy for Alzheimer's disease using mannan-amyloid-Beta peptide immunoconjugates. *DNA Cell Biol*. 2006; 25:571–580. [PubMed: 17132088]
- Gilman S, Koller M, Black RS, Jenkins L, Griffith SG, Fox NC, Eisner L, Kirby L, Rovira MB, Forette F, Orgogozo JM. Clinical effects of Abeta immunization (AN1792) in patients with AD in an interrupted trial. *Neurology*. 2005; 64:1553–1562. [PubMed: 15883316]
- Goni F, Sigurdsson EM. New directions towards safer and effective vaccines for Alzheimer's disease. *Curr Opin Mol Ther*. 2005; 7:17–23. [PubMed: 15732525]
- Hakamada-Taguchi R, Uehara Y, Kuribayashi K, Numabe A, Saito K, Negoro H, Fujita T, Toyo-oka T, Kato T. Inhibition of hydroxymethylglutaryl-coenzyme a reductase reduces Th1 development and promotes Th2 development. *Circ Res*. 2003; 93:948–956. [PubMed: 14563711]
- Han KH, Ryu J, Hong KH, Ko J, Pak YK, Kim JB, Park SW, Kim JJ. HMG-CoA reductase inhibition reduces monocyte CC chemokine receptor 2 expression and monocyte chemoattractant protein-1-mediated monocyte recruitment in vivo. *Circulation*. 2005; 111:1439–1447. [PubMed: 15781755]
- Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science*. 2002; 297:353–356. [PubMed: 12130773]
- Ifergan I, Wosik K, Cayrol R, Kebir H, Auger C, Bernard M, Bouthillier A, Moundjian R, Duquette P, Prat A. Statins reduce human blood-brain barrier permeability and restrict leukocyte migration: relevance to multiple sclerosis. *Ann Neurol*. 2006; 60:45–55. [PubMed: 16729291]
- Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, Ihara Y. Visualization of A beta 42(43) and A beta 40 in senile plaques with end-specific A beta monoclonals: evidence that an initially deposited species is A beta 42(43). *Neuron*. 1994; 13:45–53. [PubMed: 8043280]
- Jankowsky JL, Fadale DJ, Anderson J, Xu GM, Gonzales V, Jenkins NA, Copeland NG, Lee MK, Younkin LH, Wagner SL, Younkin SG, Borchelt DR. Mutant presenilins specifically elevate the levels of the 42 residue beta-amyloid peptide in vivo: evidence for augmentation of a 42-specific gamma secretase. *Hum Mol Genet*. 2004; 13:159–170. [PubMed: 14645205]
- Janus C, Pearson J, McLaurin J, Mathews PM, Jiang Y, Schmidt SD, Chishti MA, Horne P, Heslin D, French J, Mount HT, Nixon RA, Mercken M, Bergeron C, Fraser PE, George-Hyslop P, Westaway D. A beta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease. *Nature*. 2000; 408:979–982. [PubMed: 11140685]
- Jick H, Zornberg GL, Jick SS, Seshadri S, Drachman DA. Statins and the risk of dementia. *Lancet*. 2000; 356:1627–1631. [PubMed: 11089820]
- Kim HD, Jin JJ, Maxwell JA, Fukuchi K. Enhancing Th2 immune responses against amyloid protein by a DNA prime-adenovirus boost regimen for Alzheimer's disease. *Immunol Lett*. 2007a; 112:30–38. [PubMed: 17686533]
- Kim HD, Maxwell JA, Kong FK, Tang DC, Fukuchi K. Induction of anti-inflammatory immune response by an adenovirus vector encoding 11 tandem repeats of Abeta1-6: toward safer and effective vaccines against Alzheimer's disease. *Biochem Biophys Res Commun*. 2005; 336:84–92. [PubMed: 16126169]
- Kim HD, Tahara K, Maxwell JA, Lalonde R, Fukuiwa T, Fujihashi K, Van Kampen KR, Kong FK, Tang DC, Fukuchi KI. Nasal inoculation of an adenovirus vector encoding 11 tandem repeats of Abeta1-6 upregulates IL-10 expression and reduces amyloid load in a Mo/Hu APPswe PS1dE9 mouse model of Alzheimer's disease. *J Gene Med*. 2007b; 9:88–98. [PubMed: 17219449]
- Kou J, Kim HD, Jin J, Cao D, Li L, Lalonde R, Fukuchi K. Simvastatin enhances immune responses to Abeta vaccination and attenuates vaccination-induced behavioral alterations. *Brain Res*. 2010; 1356:102–111. [PubMed: 20691674]
- Lambert MP, Viola KL, Chromy BA, Chang L, Morgan TE, Yu J, Venton DL, Krafft GA, Finch CE, Klein WL. Vaccination with soluble Abeta oligomers generates toxicity-neutralizing antibodies. *J Neurochem*. 2001; 79:595–605. [PubMed: 11701763]

- Lee EB, Leng LZ, Lee VM, Trojanowski JQ. Meningoencephalitis associated with passive immunization of a transgenic murine model of Alzheimer's amyloidosis. *FEBS Lett.* 2005; 579:2564–2568. [PubMed: 15862291]
- Lee PY, Scumpia PO, Byars JA, Kelly KM, Zhuang H, Shuster JS, Theriaque DW, Segal MS, Reeves WH, Brantly ML. Short-term atorvastatin treatment enhances specific antibody production following tetanus toxoid vaccination in healthy volunteers. *Vaccine.* 2006; 24:4035–4040. [PubMed: 16464519]
- Li L, Cao D, Kim H, Lester R, Fukuchi K. Simvastatin enhances learning and memory independent of amyloid load in mice. *Ann Neurol.* 2006; 60:729–739. [PubMed: 17192930]
- Luzzi S, Vella L, Bartolini M, Provinciali L, Silvestrini M. Atherosclerosis in the evolution of Alzheimer's disease: can treatment reduce cognitive decline? *J Alzheimers Dis.* 2010; 20:893–901. [PubMed: 20182031]
- Menge T, Hartung HP, Stuve O. Statins--a cure-all for the brain? *Nat Rev Neurosci.* 2005; 6:325–331. [PubMed: 15803163]
- Miida T, Takahashi A, Ikeuchi T. Prevention of stroke and dementia by statin therapy: experimental and clinical evidence of their pleiotropic effects. *Pharmacol Ther.* 2007; 113:378–393. [PubMed: 17113151]
- Minami SS, Sidahmed E, Aid S, Shimoji M, Niikura T, Mocchetti I, Rebeck GW, Prendergast JS, Dealwis C, Wetzel R, Bosetti F, Matsuoka Y, Hoe HS, Turner RS. Therapeutic versus neuroinflammatory effects of passive immunization is dependent on Abeta/amyloid burden in a transgenic mouse model of Alzheimer's disease. *J Neuroinflammation.* 2010; 7:57. [PubMed: 20920207]
- Monsonogo A. Immunogenic aspects of amyloid B-peptide: implications for pathogenesis and treatment of Alzheimer's disease; *Proceedings VIII Conference on Alzheimer's Disease and Related Disorders*; 2005. p. 423
- Monsonogo A, Imitola J, Petrovic S, Zota V, Nemirovsky A, Baron R, Fisher Y, Owens T, Weiner HL. Abeta-induced meningoencephalitis is IFN-gamma-dependent and is associated with T cell-dependent clearance of Abeta in a mouse model of Alzheimer's disease. *Proc Natl Acad Sci U S A.* 2006; 103:5048–5053. [PubMed: 16549802]
- Monsonogo A, Maron R, Zota V, Selkoe DJ, Weiner HL. Immune hyporesponsiveness to amyloid beta-peptide in amyloid precursor protein transgenic mice: implications for the pathogenesis and treatment of Alzheimer's disease. *Proc Natl Acad Sci U S A.* 2001; 98:10273–10278. [PubMed: 11517335]
- Monsonogo A, Weiner HL. Immunotherapeutic approaches to Alzheimer's disease. *Science.* 2003; 302:834–838. [PubMed: 14593170]
- Morgan D. Modulation of microglial activation state following passive immunization in amyloid depositing transgenic mice. *Neurochem Int.* 2006; 49:190–194. [PubMed: 16740342]
- Morgan D, Diamond DM, Gottschall PE, Ugen KE, Dickey C, Hardy J, Duff K, Jantzen P, DiCarlo G, Wilcock D, Connor K, Hatcher J, Hope C, Gordon M, Arendash GW. A beta peptide vaccination prevents memory loss in an animal model of Alzheimer's disease. *Nature.* 2000; 408:982–985. [PubMed: 11140686]
- Movsesyan N, Davtyan H, Mkrtichyan M, Petrushina I, Tiraturyan T, Ross T, Agadjanyan MG, Ghochikyan A, Cribbs DH. Low concentrations of anti-Abeta antibodies generated in Tg2576 mice by DNA epitope vaccine fused with 3C3d molecular adjuvant do not affect AD pathology. *Hum Gene Ther.* 2010; 21:1569–1576. [PubMed: 20528468]
- Nicoll JA, Barton E, Boche D, Neal JW, Ferrer I, Thompson P, Vlachouli C, Wilkinson D, Bayer A, Games D, Seubert P, Schenk D, Holmes C. Abeta species removal after abeta42 immunization. *J Neuropathol Exp Neurol.* 2006; 65:1040–1048. [PubMed: 17086100]
- Nicoll JA, Wilkinson D, Holmes C, Steart P, Markham H, Weller RO. Neuropathology of human Alzheimer disease after immunization with amyloid-beta peptide: a case report. *Nat Med.* 2003; 9:448–452. [PubMed: 12640446]
- Okura Y, Miyakoshi A, Kohyama K, Park IK, Staufenbiel M, Matsumoto Y. Nonviral Abeta DNA vaccine therapy against Alzheimer's disease: long-term effects and safety. *Proc Natl Acad Sci U S A.* 2006; 103:9619–9624. [PubMed: 16769900]

- Opalka D, Lachman CE, MacMullen SA, Jansen KU, Smith JF, Chirmule N, Esser MT. Simultaneous quantitation of antibodies to neutralizing epitopes on virus-like particles for human papillomavirus types 6, 11, 16, and 18 by a multiplexed luminex assay. *Clin Diagn Lab Immunol*. 2003; 10:108–115. [PubMed: 12522048]
- Orgogozo JM, Gilman S, Dartigues JF, Laurent B, Puel M, Kirby LC, Jouanny P, Dubois B, Eisner L, Flitman S, Michel BF, Boada M, Frank A, Hock C. Subacute meningoencephalitis in a subset of patients with AD after Abeta42 immunization. *Neurology*. 2003; 61:46–54. [PubMed: 12847155]
- Pahan K, Sheikh FG, Namboodiri AM, Singh I. Lovastatin and phenylacetate inhibit the induction of nitric oxide synthase and cytokines in rat primary astrocytes, microglia, and macrophages. *J Clin Invest*. 1997; 100:2671–2679. [PubMed: 9389730]
- Patton RL, Kalback WM, Esh CL, Kokjohn TA, Van Vickle GD, Luehrs DC, Kuo YM, Lopez J, Brune D, Ferrer I, Masliah E, Newel AJ, Beach TG, Castano EM, Roher AE. Amyloid-beta peptide remnants in AN-1792-immunized Alzheimer's disease patients: a biochemical analysis. *Am J Pathol*. 2006; 169:1048–1063. [PubMed: 16936277]
- Petrushina I, Ghochikyan A, Mkrtichyan M, Mamikonyan G, Movsesyan N, Ajdari R, Vasilevko V, Karapetyan A, Lees A, Agadjanyan MG, Cribbs DH. Mannan-Abeta28 conjugate prevents Abeta-plaque deposition, but increases microhemorrhages in the brains of vaccinated Tg2576 (APPsw) mice. *J Neuroinflammation*. 2008; 5:42. [PubMed: 18823564]
- Petrushina I, Ghochikyan A, Mkrtichyan M, Mamikonyan G, Movsesyan N, Davtyan H, Patel A, Head E, Cribbs DH, Agadjanyan MG. Alzheimer's disease peptide epitope vaccine reduces insoluble but not soluble/oligomeric Abeta species in amyloid precursor protein transgenic mice. *J Neurosci*. 2007; 27:12721–12731. [PubMed: 18003852]
- Pfeifer M, Boncristiano S, Bondolfi L, Stalder A, Deller T, Staufenbiel M, Mathews PM, Jucker M. Cerebral hemorrhage after passive anti-Abeta immunotherapy. *Science*. 2002; 298:1379. [PubMed: 12434053]
- Pifer J, Hennes JL, Lee JM, Witte PL. Age-related differences in the immune response to immunization with human Abeta42 peptide. *J Gerontol A Biol Sci Med Sci*. 2002; 57:B355–B358. [PubMed: 12242310]
- Racke MM, Boone LI, Hepburn DL, Parsadainian M, Bryan MT, Ness DK, Pirooz KS, Jordan WH, Brown DD, Hoffman WP, Holtzman DM, Bales KR, Gitter BD, May PC, Paul SM, DeMattos RB. Exacerbation of cerebral amyloid angiopathy-associated microhemorrhage in amyloid precursor protein transgenic mice by immunotherapy is dependent on antibody recognition of deposited forms of amyloid beta. *J Neurosci*. 2005; 25:629–636. [PubMed: 15659599]
- Rezai-Zadeh K, Gate D, Gowing G, Town T. How to get from here to there: macrophage recruitment in Alzheimer's disease. *Curr Alzheimer Res*. 2011; 8:156–163. [PubMed: 21345166]
- Rockwood K, Kirkland S, Hogan DB, MacKnight C, Merry H, Verreault R, Wolfson C, McDowell I. Use of lipid-lowering agents, indication bias, and the risk of dementia in community-dwelling elderly people. *Arch Neurol*. 2002; 59:223–227. [PubMed: 11843693]
- Savage MJ, Kawooya JK, Pinsker LR, Emmons TL, Mistretta SSR, Greenberg BD. Elevated A-beta levels in Alzheimer's disease brain are associated with selective accumulation of A-beta 42 in parenchymal amyloid plaques and both A-beta40 and A-beta42 in cerebrovascular deposits. *Int J Exp Clin Invest*. 1995; 2:234–240.
- Schenk D, Barbour R, Dunn W, Gordon G, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenko D, Lee M, Liao Z, Lieberburg I, Motter R, Mutter L, Soriano F, Shopp G, Vasquez N, Vandeventer C, Walker S, Wogulis M, Yednock T, Games D, Seubert P. Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature*. 1999; 400:173–177. [PubMed: 10408445]
- Schenk D, Hagen M, Seubert P. Current progress in beta-amyloid immunotherapy. *Curr Opin Immunol*. 2004; 16:599–606. [PubMed: 15342006]
- Seabrook TJ, Thomas K, Jiang L, Bloom J, Spooner E, Maier M, Bitan G, Lemere CA. Dendritic Abeta1-15 is an effective immunogen in wildtype and APP-tg mice. *Neurobiol Aging*. 2007; 28:813–823. [PubMed: 16725229]
- Shimada K, Park JK, Daida H. T helper 1/T helper 2 balance and HMG-CoA reductase inhibitors in acute coronary syndrome: statins as immunomodulatory agents? *Eur Heart J*. 2006; 27:2916–2918. [PubMed: 17098759]

- Shinohara M, Sato N, Kurinami H, Takeuchi D, Takeda S, Shimamura M, Yamashita T, Uchiyama Y, Rakugi H, Morishita R. Reduction of brain A β by fluvastatin, an HMG-CoA reductase inhibitor, through increase in degradation of APP-CTFs and A β clearance. *J Biol Chem*. 2010; 285:22091–22102. [PubMed: 20472556]
- Uro-Coste E, Russano de Paiva G, Guilbeau-Frugier C, Sastre N, Ousset PJ, da Silva NA, Lavalie-Guilloteau V, Vellas B, Delisle MB. Cerebral amyloid angiopathy and microhemorrhages after amyloid beta vaccination: case report and brief review. *Clin Neuropathol*. 2010; 29:209–216. [PubMed: 20569670]
- Van Dorpe J, Smeijers L, Dewachter I, Nuyens D, Spittaels K, Van Den Haute C, Mercken M, Moechars D, Laenen I, Kuiperi C, Bruynseels K, Tesseur I, Loos R, Vanderstichele H, Checler F, Sciot R, Van LF. Prominent cerebral amyloid angiopathy in transgenic mice overexpressing the london mutant of human APP in neurons. *Am J Pathol*. 2000; 157:1283–1298. [PubMed: 11021833]
- Veillard NR, Braunersreuther V, Arnaud C, Burger F, Pelli G, Steffens S, Mach F. Simvastatin modulates chemokine and chemokine receptor expression by geranylgeranyl isoprenoid pathway in human endothelial cells and macrophages. *Atherosclerosis*. 2006; 188:51–58. [PubMed: 16321392]
- Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, Selkoe DJ. Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature*. 2002; 416:535–539. [PubMed: 11932745]
- Wiessner C, Wiederhold KH, Tissot AC, Frey P, Danner S, Jacobson LH, Jennings GT, Luond R, Ortman R, Reichwald J, Zurini M, Mir A, Bachmann MF, Staufenbiel M. The second-generation active Abeta immunotherapy CAD106 reduces amyloid accumulation in APP transgenic mice while minimizing potential side effects. *J Neurosci*. 2011; 31:9323–9331. [PubMed: 21697382]
- Wilcock DM, Rojiani A, Rosenthal A, Subbarao S, Freeman MJ, Gordon MN, Morgan D. Passive immunotherapy against Abeta in aged APP-transgenic mice reverses cognitive deficits and depletes parenchymal amyloid deposits in spite of increased vascular amyloid and microhemorrhage. *J Neuroinflammation*. 2004; 1:24. [PubMed: 15588287]
- Wiley JZ, Elkind MS. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors in the treatment of central nervous system diseases. *Arch Neurol*. 2010; 67:1062–1067. [PubMed: 20837848]
- Wisniewski T, Frangione B. Immunological and anti-chaperone therapeutic approaches for Alzheimer disease. *Brain Pathol*. 2005; 15:72–77. [PubMed: 15779239]
- Wolozin B, Kellman W, Ruosseau P, Celestia GG, Siegel G. Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. *Arch Neurol*. 2000; 57:1439–1443. [PubMed: 11030795]
- Wolozin B, Manger J, Bryant R, Cordy J, Green RC, McKee A. Re-assessing the relationship between cholesterol, statins and Alzheimer's disease. *Acta Neurol Scand Suppl*. 2006; 185:63–70. [PubMed: 16866913]
- Youssef S, Stuve O, Patarroyo JC, Ruiz PJ, Radosovich JL, Hur EM, Bravo M, Mitchell DJ, Sobel RA, Steinman L, Zamvil SS. The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease. *Nature*. 2002; 420:78–84. [PubMed: 12422218]
- Zeng L, Xu H, Chew TL, Eng E, Sadeghi MM, Adler S, Kanwar YS, Danesh FR. HMG CoA reductase inhibition modulates VEGF-induced endothelial cell hyperpermeability by preventing RhoA activation and myosin regulatory light chain phosphorylation. *FASEB J*. 2005; 19:1845–1847. [PubMed: 16160062]
- Zlokovic BV. The blood-brain barrier in health and chronic neurodegenerative disorders. *Neuron*. 2008; 57:178–201. [PubMed: 18215617]

Fig. 1.
Simvastatin treatment and immunization schedule.

Fig. 2.

Levels of buffer-soluble A β in the hippocampus increased in 22-month-old AD model mice subjected to A β vaccination. The amounts of buffer-soluble A β 42 (A) and A β 40 (B) in the hippocampus and neocortex were determined by A β 42 and A β 40 specific ELISA, respectively. The amounts of buffer soluble A β 42 and A β 40 in the vaccine-only and vaccine-plus-statin groups increased in the hippocampus as compared with the PBS group (* $P < 0.05$). The values shown are the mean $\pm$ SEM. * $P < 0.05$

Fig. 3.

A β load in the brain by immunohistochemistry and ELISA. Ten months after the initial immunization, mice were euthanized at 22 months of age and A β deposits in the brain were visualized by anti-A β 6E10 antibody (A). Scale bars 500 μ m. Average percentages of areas showing A β immunoreactivity measured by morphometry in the hippocampus and neocortex are shown (B). There is no difference between any groups in the A β load. The values shown are the mean $\pm$ SEM.

Fig. 4.

CD45-positive cells in the brain by immunohistochemistry and immunoblot analysis. For detection of migratory leukocytes/microglia, brain sections were subjected to immunohistochemistry using anti-CD45 antibody (A). Scale bars 1 mm. Average percentages of areas showing A β immunoreactivity measured by morphometry in the hippocampus and neocortex are shown (B). The CD45 immunoreactive areas in both hippocampus ($P < 0.01$) and neocortex ($P < 0.05$) in the mice received vaccine only are greater than those in the PBS group, while simvastatin treatment counteracts the increase of CD45-immunoreactivity induced by vaccination in the hippocampus ($P < 0.05$) but not in the neocortex ($P = 0.13$). Levels of CD45 and GAPDH in the cerebral homogenates were determined by immunoblotting using anti-CD45 and anti-GAPDH antibodies, respectively (D). The bar graph represents densitometric quantification of CD45 after normalization with GAPDH (means $\pm$ SEM) (C). The mean of hippocampal CD45 levels in the vaccine-only group is greater than those in the PBS ($P = 0.01$) and vaccine-plus-statin groups ($P = 0.05$).

Fig. 5. Quantification of microglia and astrocytes in the hippocampus and neocortex. Brain sections were stained with anti-CD11b and anti-GFAP antibodies for detection of activated microglia and reactive astrocytes, respectively. Average percentages of areas showing immunoreactivity measured by morphometry in the hippocampus and neocortex are shown for CD11b (A) and GFAP (B). No differences in immunoreactivity were found between groups. The values shown are the mean $\pm$ SEM.

Fig. 6.

Levels of cytokines in the hippocampus and neocortex. Levels of cytokines in the tissue lysates were determined by multiplex cytokine/chemokine array analysis. The bar graphs represent levels of cytokines in the hippocampus (A) and neocortex (B). Mean concentrations $\pm$ SE are expressed in picograms per milligram of brain protein. Levels of IL-2 in the vaccine-only group are lower than those in the PBS group ($P < 0.01$ for the hippocampus and $P < 0.05$ for the neocortex). Levels of IL-10 in the statin-only group are greater than those in the PBS group ($P < 0.05$).

Fig. 7.

No correlation between the antibody titers and A β load. Correlation analyses were carried out between anti-A β antibody titers and A β load (6E10-immunoreactive areas) in the hippocampus (A) and neocortex (B) in the vaccine-only and vaccine-plus-statin groups. The anti-A β titers were not correlated with A β load in the hippocampus ($r^2 = 0.017$, $P = 0.834$ for the vaccine-only group; $r^2 = 0.295$, $P = 0.344$ for the vaccine-plus-statin group) and neocortex ($r^2 = 0.416$, $P = 0.240$ for the vaccine-only group; $r^2 = 0.278$, $P = 0.351$ for the vaccine-plus-statin group).

Fig. 8. Quantification of blood vessels bearing amyloid deposits in the hippocampus. Brain sections were stained with thioflavin S for detection of amyloid. Hippocampal blood vessels positive for thioflavin fluorescence were indicated by arrows (A). Scale bars 100 μ m. The numbers of blood vessels positive for thioflavin fluorescence per brain section are shown (B). On average, the vaccine-only group had more thioflavin-positive blood vessels in the hippocampus than the 3 other groups but the differences are not significant. The values shown are the mean $\pm$ SEM.

Table 1

Levels of plasma cholesterol and activities of transaminases and creatine kinase

Plasma Parameters	Treatment Groups	
	Non-simvastatin	Simvastatin
TC, mg/dl (1 month)	80.9 ± 16.9	82.8 ± 3.2
TC, mg/dl (2 month)	79.6 ± 2.7	72.0 ± 2.7*
AST, IU/L	47.7 ± 3.6	56.2 ± 4.5
ALT, IU/L	11.4 ± 1.4	13.6 ± 1.3
CK, U/L	390.6 ± 45	424.6 ± 34.8

TC = total cholesterol; AST = aspartate transaminase; ALT = alanine transaminase; CK = creatine kinase. Cholesterol levels were measured after 1 and 2 month treatment with simvastatin. The other parameters were determined at the time of animal termination.

* $P < 0.05$

Table 2Characterization of anti-sera induced by anti-A β vaccines with or without simvastatin

Treatment	No. of anti-A β seropositive mice	IgG (mean $\pm$ S.E.M.)	Anti-A β Ig isotypes and IgG1/IgG2a
4 weeks post initial vaccination			
Vaccine only	2 out of 11	1.53 $\pm$ 0.05	IgG1, IgG2b
Vaccine plus statin	2 out of 14	1.28 $\pm$ 0.05	IgG1, IgG2b
6 weeks post initial vaccination			
Vaccine only	2 out of 11	0.66 $\pm$ 0.4	IgG1, IgG2b
Vaccine plus statin	7 out of 14	1.77 $\pm$ 0.68	IgG1, IgG2b
8 weeks post initial vaccination			
Vaccine only	2 out of 11 ^a	0.5 $\pm$ 0.16	IgG1, IgG2b
Vaccine plus statin	8 out of 14 ^a	1.01 $\pm$ 0.39	IgG1, IgG2b
2 weeks post 1st A β -KLH			
Vaccine only	10 out of 11	6.59 $\pm$ 1.71	IgG1/IgG2a = 3.4 $\pm$ 0.89
Vaccine plus statin	14 out of 14	8.77 $\pm$ 1.78	IgG1/IgG2a = 3.5 $\pm$ 0.68
2 weeks post 2nd A β -KLH			
Vaccine only	11 out of 11	8.64 $\pm$ 1.36	ND ^b
Vaccine plus statin	14 out of 14	7.76 $\pm$ 1.44	ND ^b
7 weeks post 2nd A β -KLH			
Vaccine only	11 out of 11	1.93 $\pm$ 0.58	IgG1/IgG2a = 5.4 $\pm$ 1.46
Vaccine plus statin	14 out of 14	1.34 $\pm$ 0.37	IgG1/IgG2a = 3.7 $\pm$ 0.82
3 weeks post 3rd A β -KLH			
Vaccine only	11 out of 11	1.07 $\pm$ 0.31	IgG1/IgG2a = 4.01 $\pm$ 1.15
Vaccine plus statin	14 out of 14	1.67 $\pm$ 0.3	IgG1/IgG2a = 6.96 $\pm$ 1.63
1.5 weeks post 4th A β -KLH			
Vaccine only	11 out of 11	2.31 $\pm$ 1.01	IgG1/IgG2a = 3.2 $\pm$ 0.69 ^c
Vaccine plus statin	12 out of 12 ^d	4.73 $\pm$ 1.46	IgG1/IgG2a = 7.5 $\pm$ 1.81 ^c
2 weeks post 5th A β -KLH			
Vaccine only	9 out of 11	4.08 $\pm$ 2.24	IgG1/IgG2a = 6.03 $\pm$ 2.0
Vaccine plus statin	11 out of 12	2.21 $\pm$ 0.74	IgG1/IgG2a = 5.89 $\pm$ 1.32
6 weeks post 5th A β -KLH			
Vaccine only	6 out of 10 ^d	0.83 $\pm$ 0.22	IgG1/IgG2a = 3.66 $\pm$ 0.71
Vaccine plus statin	8 out of 12	1.86 $\pm$ 0.63	IgG1/IgG2a = 4.16 $\pm$ 0.82

^a $P < 0.05$ (chi square test).^bNot determined.^c $P = 0.05$ (t-test).^dMice died of unspecified causes.

Table 3Levels of buffer-insoluble A β 42 and A β 40

Treatment	Buffer-insoluble A β 42 (ng/mg)		Buffer-insoluble A β 40 (ng/mg)	
	hippocampus	neocortex	hippocampus	neocortex
PBS	2.08 $\pm$ 0.11	1.88 $\pm$ 0.17	2.67 $\pm$ 0.47	3.25 $\pm$ 0.33
Simvastatin only	2.46 $\pm$ 0.19	2.04 $\pm$ 0.19	3.64 $\pm$ 0.70	3.66 $\pm$ 0.69
Vaccine only	2.19 $\pm$ 0.11	1.53 $\pm$ 0.39	3.54 $\pm$ 0.34	4.18 $\pm$ 0.33
Vaccine + simvastatin	2.11 $\pm$ 0.12	1.90 $\pm$ 0.20	2.90 $\pm$ 0.49	3.15 $\pm$ 0.56