Table 1. Aβ Peptide and Non-Aβ Peptide Vaccine.

1) Aβ1-15-BSAT + CFA *S.C.* (Monsonego et al. 2001)
2) 3 × (Aβ1-15)-BSAT + CFA *I.P.* (Bard et al. 2003)
3) 3 × (Aβ1-15)-PADRE + Alum *S.C.* (Agadjanyan et al. 2005)
4) Aβ1-15-Mannan *S.C.* (Ghochikyan et al. 2006)
5) Aβ1-42 *Nasal* (Weiner et al. 2000)
6) Aβ1-15-K-K-Aβ1-15, RGD-Aβ1-15-K-K-Aβ1-15, T1-Aβ1-15, T1-RGD-K-K-Aβ1-15 + *E. coli* heat-labile enterotoxin *Nasal* (Maier et al. 2006)
7) Aβ1-15-Parmitoyl liposome *I.P.* (Muhs et al. 2007)
8) Aβ1-42-Chorela Toxin *Transcutaneous* (Nikolic et al. 2007)
9) Aβ1-15-PDGFR in MLV particle *I.V.* (Bach et al. 2009)
10) Aβ affitope + Alum *S.C.* (Schneeberger A et al. 2009)
11) Copolymer1 (Glatiramer Acetate) *S.C.* with adjuvant (Butovsky et al. 2006) *Nasal* with Protollin *(Frenkel et al. 2005)

BSAT, bovine serum albumin T cell epitope; CFA, complete Freund's adjuvant; I.P., intraperitoneal injection; I.V., intravenous injection; MLV, *murine leukemia virus*; PADRE, pan-HLA-DR binding peptide; PDGFR, platelet-derived growth factor receptor; Protollin, non-covalent formulation of outer membrane proteins (proteosomes) of *Neisseria meningitides* and LPS from *Shigella flexneri*; RGD, cell attachment motif; S.C., subcutaneous injection; T1, T cell epitope of *HIV* IIIB gp120.

Table 2. DNA Vaccine.

1) Plasmid-Aβ1-42-MHC class II-targeting sequence *I.D.* (Qu et al. 2004)
2) Activator Plasmid-GAL4 and Responder Plasmid-3 × (Aβ1-42)-MHC class II-targeting sequence *I.D.* (Lambracht-Washington et al. 2009)
3) Plasmid-Aβ1-42 *I.M.* (Okura et al. 2006)
4) Plasmid-CCL22-3 × (Aβ1-11)-PADRE *I.D.* (Movsesyan et al. 2008)
5) Plasmid-Aβ1-42-mutant caspase *I.M.* (DaSilva et al. 2009)

I.D, intradermal injection; I.M., intramuscular injection.

Table 3. Recombinant Vegetable, Bacteria and Phage.

1) Recombinant potato expressing 5 × (Aβ1-42) Protein Extract + CT *Oral* (Youm et al. 2005)
2) Recombinant Tomato expressing 5 × (Aβ1-42) *Oral alone*: No immune response *Oral* + Aβ *S.C.*: High immune response (Youm et al. 2008)
3) Recombinant *Salmonella Oral* (Boutajangout et al. 2009)
4) Recombinant *phage* expressing Aβ3-6 (EFRH) in pIII or pVIII *I.V. or I.P.* (Frenkel et al. 2000; Lavie et al. 2004)

CT, *cholera* toxin; EFRH, aminoacid sequence of Aβ3-6.

Table 4. Recombinant Viral Vectors.

1) AAV-Aβ1-43 or 1-21 *Oral* (Hara et al. 2004; Mouri et al. 2007)
2) AAV- Aβ1-42 – CTB *Oral* (Zhang et al. 2003)
3) SeV-Aβ1-43 – IL-10 Nasal (Hara et al. in preparation)
4) pCA-PEDI-11 × (Aβ1-16) boosted with rAV- PEDI*-11 × (Aβ1-16) *Nasal* (Kim et al. 2007a, 2007b)
5) pHSV-Aβ1-42-IL-4 *S.C.* (Frazer et al. 2008)

AAV, *adeno-associated virus* vector; AV, *adenovirus* vector; CTB, *cholera* toxin B; pCA, a plasmid; pHSV, *herpes simplex virus* amplicon; SeV, *Sendai virus* vector; PEDI, receptor binding domain of *Pseudomonas* exotoxin A.

2006). Mannan is a molecular adjuvant that enhances uptake of antigens by dendritic cells and production of IgG1, IL-10 and IL-4. However, their later report said that it increased microhemorrhage in APP tg mice carrying the Swedish mutation (APPsw) (Petrushina et al. 2008).

Nasal immunization also induces Th2 dominant immune responses. Weiner et al. immunized APP tg mice with Aβ1-42 nasally and showed efficient reduction of amyloid burden with an increase of microhemorrhage. (Weiner et al. 2000). Lemere et al. made N-terminal peptide vaccine using a lysine core (2 arms) conjugated with RGD or T1, and immunized mice intra-nasally with *E. coli* heat-labile enterotoxin [LT(R192G)] as an adjuvant (Maier et al. 2006). These constructs were Aβ1-15-K-K-Aβ1-15, RGD-Aβ 1-15-K-K-Aβ1-15, T1-Aβ1-15, and T1-RGD-K-K-Aβ1-15. Here RGD is a cell attachment motif and enhances antigen uptake and presentation by antigen presenting cells in the mucous membrane, and T1 is a T cell epitope of HIV IIIB gp120. Although these vaccines elicited Th2 immune responses predominantly, a certain amount of IgG2a antibody was also elevated, suggesting that Th1 T cells were also activated. We must be cautious that intra-nasally administered Aβ and adjuvant enter into the brain through the olfactory nerve, which may have some effects on the CNS.

Aβ peptide/liposome vaccine: A liposome vaccine was made by C16 parmitoylation at both end of Aβ1-15 peptide, and repeated intra-peritoneal inoculations of the vaccine without adjuvant elicited IgG1 and IgG2b antibodies recognizing β sheet structures of Aβ (Muhs et al. 2007). However, low titers of IgG2a antibodies were also elevated. They also made a liposome vaccine by adding polyethylene glycol (PEG) in both ends of Aβ1-15. In this case, antibodies recognizing α helix of Aβ were produced.

Transcutaneous vaccine using Aβ1-42 peptide and cholera toxin: The precursor cells of $CD14^+$ Langerhans cells in the epidermis are antigen presenting cells that induce Th2 immune responses predominantly. In order to activate these cells, Nikolic et al. immunized mice by applying Aβ1-42 peptide solution with cholera toxin as an adjuvant repeatedly on the carefully shaved skin for 2 hours every 1-2 weeks (Nikolic et al. 2007). A transcutaneous patch vaccine using a sticky tape containing small needles and antigens is being developed (Nakagawa S. et al., personal communication).

Murine leukemia virus (MLV) particles expressing Aβ 1-15 peptide and PDGF receptor protein: Plasmid pHIT60 containing murine leukemia virus (MLV) gag and pol genes and plasmid pDisplay containing Aβ1-15, PDGF receptor and an Igκ signal sequence were co-transfected in HEK-293 T cells, which produced virus like particles expressing Aβ 1-15-PDGFR fusion protein. When 1×10^{10} particles were injected intravenously in APP23 mice, they produced high titers of anti-Aβ antibodies, and the subtypes were IgG1 and IgG2b. The immunized mice showed significant reduction of SPs, soluble and insoluble Aβ (Bach et al. 2009). Each virus-like particle expresses several thousands epitopes of Aβ1-15 and the injected particles were calculated as containing 150 ng of Aβ1-15. However, it was not reported whether it reduced Aβ oligomers and improved cognitive functions.

Human trials of N-terminal peptide vaccine: ACC-001, an N-terminal Aβ peptide vaccine conjugated with diphtheria toxin and adjuvant QS21 is now under the phase II clinical trial in the world including Japan by Wyeth. The precise construct of the vaccine is unknown. Since the encephalitogenic epitope mainly exists in the C-terminal portion of Aβ, the N-terminal peptide may be safe. However, the encephalitogenic epitope was examined in Caucasians, and nothing is known in Orientals. Humans are hybrid and the encephalitogenic epitope could exist in the N-terminal portion. Also concerned is that QS21 is the Th1 adjuvant, which may induce Th1 responses to the peptide and induce continuous inflammatory responses in the CNS.

Recently, a new technology to express non-self structures of self antigens, named "affitope" vaccine was invented (Schneeberger et al. 2009). This is easy to break immune barrier and elevates antibodies to N-terminal Aβ peptide. A human trial of this vaccine is planned by GlaxoSmithKline.

Non-Aβ peptide and adjuvant vaccine

Copolymer1 (Cop1), Glatiramer Acetate® is now used for treatment of multiple sclerosis (MS). Autoimmune encephalitogenic T cells that recognize myelin antigens are involved in MS. Cop1 is a mixture of randomly polymerized peptides composed of Glutamic acid, Lysine, Alanine and Tyrosine, and activates regulatory T cells reactive to myelin antigen-reactive and encephalitogenic T cells. It is interesting to note that APP tg mice immunized with Cop1 and adjuvant showed significant reduction of SPs in association with an increase of Th2 T cells (Butovsky et al. 2006). It is speculated that Cop1-activated-Th2 T cells in the brain activated IGF1-producing $CD11c^+$ microglia cells, which in turn cleared amyloid deposits. Cop1 is proven safe in humans, so it seems to be easier to go to the human trial. However, tolerability may be the problem, because it must be injected subcutaneously every day for MS patients.

Intra-nasal administration of Cop1 with an adjuvant Protollin (IVX-908) effectively reduced amyloid burden in APP tg mice without inducing inflammatory change (Frenkel et al. 2005). Protollin is a non-covalent formulation of outer membrane proteins (proteosomes) of *Neisseria meningitides* and LPS from *Shigella flexneri*. Nasal vaccine is not painful, but regulatory T cells against encephalitogenic T cells may be mainly activated in the white matter where amyloid pathology is very few. Since this vaccine do not use Aβ, it is uncertain how long such nonspecific vaccines continue to work.

DNA vaccine

Qu et al. made a gene construct using an expression plasmid containing cDNA of monomer or dimer of Aβ1-42

under the control of SP72, a synthetic mammalian cell-specific promoter, an α-antitrypsin leader sequence and an MHC class II-targeting sequence. Gold particles coated with the DNA were given intra-dermally in the ear of mice by a helium-driven gene gun 3 times. Consequently, they could observe a good immune response to Aβ without activation of cytotoxic T cells (Qu et al. 2004). Later they changed the leader sequence to that of Adenovirus E3, and immunized APPsw × PS1$\triangle$E9 mice 15 times similarly using a gene gun. They showed a significant elevation of Th2 type antibodies and reduction of Aβ burden (Qu et al. 2007). Recently, they made a vaccine mixed with an activator plasmid carrying a GAL4 activator sequence under the control of CMV promoter and a responder plasmid carrying a GAL4 upstream activating system, 3-tandem repeat cDNAs of Aβ1-42, the E3 leader sequence and the MHC class II endosomal targeting sequence. They compared immune responses in wild type mice immunized with the DNA vaccine and those with Aβ1-42 peptide vaccine mixed with adjuvant Quil A. The antibody responses in the DNA-vaccinated mice showed a significantly higher ratio of IgG1/IgG2a than those in peptide-vaccinated mice (Lambracht-Washington et al. 2009). Thus, DNA vaccine seems to induce Th2 dominant immune responses. However, the IgG1/IgG2a ratio was about 10 in the DNA-vaccinated mice, the IgG2a response was not nil.

A similar DNA vaccine was made using an expression plasmid pTarget carrying an Igκ signal sequence and Aβ1-42 or Aβ1-42 with a fusion gene of Ig Fc (Okura et al. 2006). APP tg mice received this vaccine repeatedly into the muscle, and they showed a significant reduction of Aβ burden without T cell activation. However, antibody subtypes and cognitive functions were not examined.

Movsesyan et al. made a pCMV DNA vaccine carrying an IP10 signal sequence, a gene encoding CCL22 chemokine, 3 repeat of Aβ1-11 and PADRE gene (Movsesyan et al. 2008). This vaccine was given 3 times into the shaved abdominal skin of mutant APP tg × mutant PS1 tg × mutant tau tg (3X-Tg-AD) mice using a gene gun. The use of CCL22 further shifted the immune response to Th2 and produced IgG1 and IgG2b antibodies. It is good to know that SPs were significantly reduced and cognitive functions were improved. In addition, insoluble Aβ40, Aβ42 and soluble Aβ oligomers (3-mers and 6-mers) were significantly reduced, but tau pathology was unchanged.

Since post-mitotic muscle cells are poor for antigen presentation, DaSilva et al. made a DNA vaccine carrying wild type Aβ1-42 and a mutant caspase gene. The expression of the mutant caspase was not enough to induce apoptosis of muscle cells but enough to induce apoptosomes, which enhanced antigen presentation. TgCRND8 mice which carry APP_{SW} and $APP_{INDIANA}$ mutations were repeatedly injected with the vaccine intramuscularly. They showed Th2 type antibody responses and reduction of amyloid plaques and insoluble Aβ42. Cerebral amyloid angiopathy and soluble Aβ oligomers tended to show reduction, but not significant (DaSilva et al. 2009).

Recombinant vegetable, recombinant bacteria, recombinant phage

Recombinant potato expressing 5 tandem repeats of Aβ1-42 was made, and Tg2576 mice were fed with 25 mg of the protein extract, which contained 0.9 μg/mg of Aβ, and adjuvant cholera toxin B once a week for 3 weeks. The result showed elevated anti-Aβ antibodies and reduction of senile plaques (Youm et al. 2005). This suggested that in a certain condition orally given food extracts with a certain adjuvant can break immune tolerance. However, it is unknown whether taking recombinant food instead of food extract and an adjuvant is enough to induce the immune response. In addition, how much recombinant potato is required to be eaten, and how cholera toxin B is mixed with the potato before eating?

Since we eat cooked potato, antigenicity of Aβ may be inactivated by heat. Therefore, they produced recombinant tomato for eating without cooking. Mice just fed with the soluble extract of recombinant tomato did not develop any anti-Aβ antibodies. However, mice injected with a small amount of Aβ after feeding with transgenic tomato developed high titers of anti-Aβ antibodies, suggesting that mice acquired immune conditioning by eating Aβ-containing tomato (Youm et al. 2008). Similar effect may be obtained by eating potato infected with virus which has high homology with Aβ (Friedland et al. 2008).

APP tg mice fed with non-pathogenic recombinant Salmonella expressing Aβ showed reduction of Aβ burden (Boutajangout et al. 2009). In this case, secreted Aβ from the bacteria may not be immunogenic. Instead, Aβ-expressing *Salmonella* phagocytosed by M-cells or dendritic cells seems to be immunogenic. If this is the case, yogurt containing recombinant Lactobacillus expressing Aβ may be useful.

Frenkel et al. made a recombinant filamentous phage expressing 10 copies of Aβ3-6 (EFRH) in protein III (pIII) or 300 copies in major coat protein VIII (pVIII). APP tg mice inoculated and repeatedly boosted intra-peritoneally or nasally with $10^{10\text{-}11}$ of the recombinant phage produced antibodies to Aβ, and showed significant reduction of SPs and improvement of the Morris water maze test (Frenkel et al. 2000; Lavie et al. 2004). The recombinant phage with high copy numbers of Aβ was much more efficient. The antibodies inhibited Aβ aggregation and Aβ-induced neurotoxicity. It is unknown whether the antibodies react with 3-pylo-glutaminyl Aβ which is the major form of aggregated Aβ in AD brain (Harigaya et al. 2000). Phage is a virus for *E. coli*, exists ubiquitously on the earth and in our body, and is said non-toxic to humans.

Recombinant viral vectors

Recombinant Adeno-associated Virus vector (AAV) carrying Aβ1-43 or Aβ1-21: We developed recombinant AAV carrying an APP signal sequence and Aβ1-43 or

Aβ1-21 cDNA (Hara et al. 2004). We used Aβ1-43 in order to differentiate it from native Aβ, because majority of native Aβ is composed of Aβ40 and Aβ42. Tg2576 mice were given orally once with 5×10^{11} viral genome of the recombinant AAV using a naso-gastric tube at the age of 15, 30 or 45 weeks old, and their brains were examined at 56 weeks old. The vaccinated mice showed elevated anti-Aβ antibodies for over 46 weeks and the subtypes were IgG1 and IgG2b. IgA antibodies were low and IgG2a antibodies were not detected. SPs were significantly reduced in all vaccinated mice compared with PBS controls, and Aβ1-43 was not increased in the brain. The antibody titers were much lower in mice vaccinated with Aβ1-21 vaccine, but the effect was the same. Splenic T cells did not proliferate when stimulated with Aβ1-42 peptide. There was no infiltration of T cells and B cells in the brain, and Iba-1^+ activated microglia were increased and $GFAP^+$ astrocytes were decreased. When serum cytokines were examined using a set of over 30 conventional cytokine microbeads and Luminex (Millipore), only transforming growth factor β-1 (TGF β-1) was significantly reduced (Hara and Tabira, 2006).

The recombinant AAV-Aβ1-43 vaccine was given similarly in Tg-2576 mice at the age of 10 months and cognitive functions were examine at 13 months. The vaccinated mice showed significant improvement in the Y-maze test, Morris water maze test, novel object recognition test and conditioned fear learning test. After the cognitive function tests, all the brains were examined. Reduction of SPs was confirmed, and SDS-soluble and formic acid (FA)-soluble Aβ40 and Aβ42 were also significantly reduced in the vaccinated mice. They also showed significant reduction of soluble 9-mers and 12-mers (Mouri et al. 2007).

Cynomolgus monkeys over 20 years old were given orally with 1×10^{13} viral genome of AAV-Aβ1-43 vaccine twice in a three months interval and the brain was examined 6 months after the first vaccination. Senile plaques were significantly reduced and there was no inflammation in the brain. There were no significant abnormalities in the laboratory tests including complete blood counts and serum chemistry, and pathological examinations of the systemic organs showed no particular changes (manuscript in preparation).

The intestine is the biggest and the most efficient immune organ in humans (Fig. 3). Therefore, immune responses were easily obtained even in the elderly. As a matter of fact, one or two oral administrations of the vaccine were enough in mice and monkeys. In addition, since the gut immune system is shifted to Th2, Th1-mediated autoimmune encephalitis is supposed to be suppressed. Further, since gut epithelial cells are renewed in a few days, majority of the transfected genes are deleted quickly. AAV is a safe vector and transfected genes are retained in the epi-

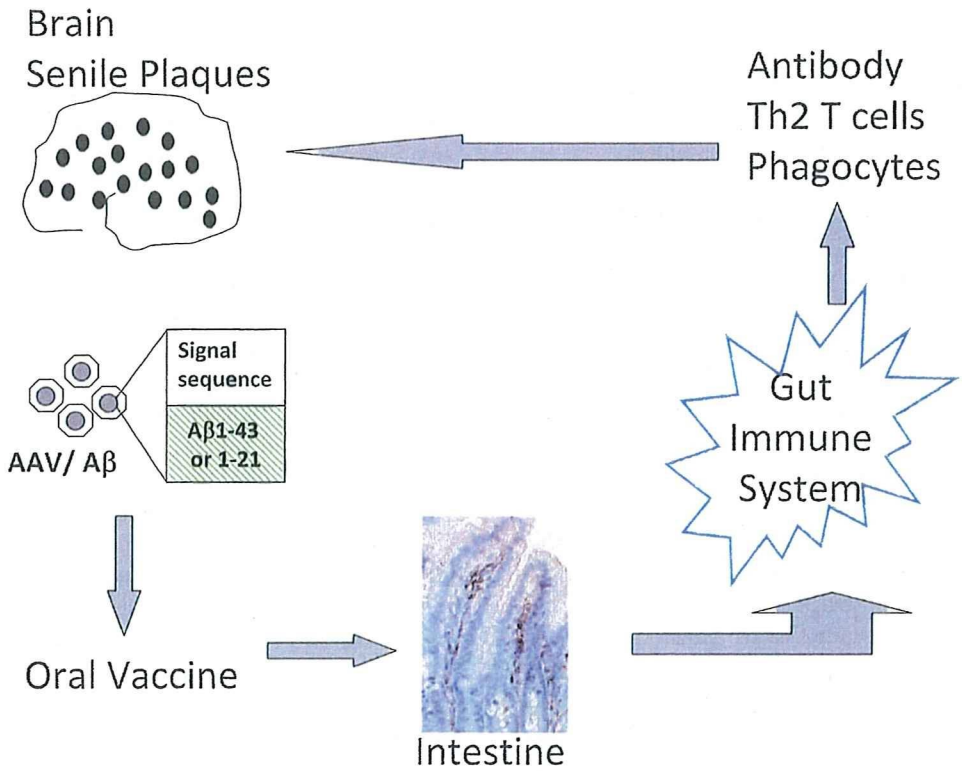

Fig. 3. Oral Vaccine using Recombinant *Adeno-associated Virus*.
Recombinant *adeno-associated virus* vector (AAV) carrying Aβ1-43 or 1-21 with an APP signal sequence was made. When this was given orally, the gut immune system responded well and produced Th2 type antibodies efficiently. It cleared senile plaque amyloid, reduced insoluble Aβ and soluble Aβ oligomers, and improved cognitive functions without inducing encephalitis or other adverse effects.

some. However, a certain amount of the transfected genes may be retained for a while, if infected in M-cells, dendritic cells and stem cells of the gut epithelium. Thus, our vaccine fulfills most of the requirement for the next generation of Aβ vaccine. In order to do a clinical trial of this vaccine, we need a company or a facility to provide an enough amount of the vaccine at the GMP level.

Recombinant AAV-Aβ1-42-CTB: A similar vaccine was reported in China (Zhang et al. 2003). However, their construct contains Aβ1-42 and cholera toxin B (CTB) as an adjuvant. They did not see antibody responses if CTB is not included. Our vaccine carries Aβ1-43 only and does not require adjuvant.

Aβ1-43 - IL-10 in SeV: We have also developed recombinant *Sendai virus* vector (SeV) carrying Aβ1-43 and IL-10. Sendai virus induces common cold-like symptoms in murines, but it is not pathogenic in humans. Nasal administration of this vaccine induced significant reduction of senile plaques without inducing inflammatory changes in the brain and improved cognitive functions (manuscript in preparation).

pCA-PEDI-11 × (Aβ1-16) boosted with recombinant Adenovirus carrying PEDI-11 × (Aβ1-16): Kim et al. immunized APP tg × PS1 tg mice intra-nasally twice with a plasmid DNA vaccine containing 11 tandem repeats of Aβ 1-16 cDNA and the receptor binding domain of *Pseudomonas* exotoxin A (PEDI), and then the mice were boosted nasally every 3 seeks for 10 months with recombinant *Adenovirus* containing the same gene construct (Kim et al. 2007a, 2007b). The mice produced high titers of IgG1 and IgG2b antibodies to Aβ and SPs were significantly reduced. Splenocytes from the vaccinated mice produced a significant amount of IL-10, when stimulated with Aβ. However, IgG2a antibodies were also produced, probably because PEDI contains a lot of CpG motif. CpG binds to a Toll-like receptor and activates the innate immune system, which in turn activates the acquired immune system. Th1 immune responses are induced by CpG in a certain condition.

$pHSV_{IE}$ Aβ1-42-$_{CMV}$IL-4: Frazer et al. produced a recombinant *Herpes simplex virus* (HSV) amplicon carrying Aβ1-42 gene under the control of HSV immediate early promoter (IE) and IL-4 gene under the control of Cytomegalovirus (CMV) immediate early promoter. 3X-Tg-AD mice were inoculated subcutaneously with 1×10^6 transduction units of the vaccine and were boosted with the same vaccine 1 month and 6 months after. They described that the vaccinated mice developed antibodies to Aβ42 and showed a significant reduction of SPs and phosphorylated tau, and improvement in the Barnes maze test (Frazer et al. 2008). They described that the antibody pattern was a Th2 type. However, antibody subtypes were IgG1, IgG2a, IgG2b and IgG3, and no specific pattern was shown.

Miscellaneous

Adjuvant alone: Nasal administration of Protollin, a proteosome-based adjuvant activated brain microglia and reduced SPs (Frenkel et al. 2005).

Juzen-taiho-to, an herbal medicine: Plaque amyloid is suggested to be phagocytosed mainly by bone marrow-derived microglia/macrophages (Simard et al. 2006). Brain derived microglia and bone marrow derived macrophages were activated in vitro and in vivo by Juzen-taiho-to, an herbal medicine and enhanced phagocytosis of β amyloid (Liu et al. 2008). When Tg-2576 mice were treated with Juzen-taiho-to in drinking water, amyloid burden was significantly reduced (Hara et al. 2010). This is not a vaccine, but it seems to have an vaccine-like effect in activating bone marrow-derived phagocytes in the brain.

Memapsin 2: Tg2576 mice were immunized with memapsin 2 (β-secretase) and CFA, then with the antigen and IFA, and later with the antigen alone. The actively immunized mice and mice treated with anti-memapsin 2 antibodies showed reduced memapsin 2 activity, reduced SPs and plasma Aβ, and improvement of cognitive functions (Chang et al. 2007).

APP beta-cleaving site: Rakover et al. immunized mice with APP cleaving site peptide and obtained antibodies that inhibit β-secretase cleavage. Tg2576 repeatedly injected with the antibodies showed reduction of brain inflammation, reduction of brain hemorrhage, and improvement of cognitive functions without changing brain Aβ (Rakover et al. 2007).

HA-KLH: Homocysteic acid (HA) is a neurotoxin binding to the NMDA receptor. Animals fed with vitamin B6 deficient food showed cognitive dysfunctions in association with an increase of intracellular Aβ. Active and passive immunization targeting HA improved cognitive functions in Vitamin B6 deficient animals and 3X-Tg-AD mice (Hasegawa et al. 2010).

In conclusion, there are numerous ideas and strategies in immunotherapy for AD. Some of them have been proven safe and effective in animals, and will be applied for AD patients in near future. As we learned from the study of AN-1792, only the result in a human trial can tell the efficacy and safety, unless good monkey models are established.

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Decorated Plaques in Alzheimer's Disease

It is widely accepted that amyloid-β protein (Aβ) has a central pathogenic role in Alzheimer's disease (AD). Aβ is cleaved from amyloid precursor protein as two major species: Aβ40, which is composed of 40 amino acids, and Aβ42, which has an additional 2 amino acids in the C-terminal end of Aβ40. At younger ages, Aβ is quickly degraded by enzymes, such as neprilysin, but along with age, it begins to deposit in the brain parenchyma and form senile plaques, a hallmark of AD.

Active and passive immunizations with Aβ were found to clear amyloid plaques and to prevent amyloid plaque formation, and amyloid precursor protein transgenic mice treated with certain monoclonal antibodies to Aβ showed improvement of cognitive function.[1,2] These findings prompted investigators to search for naturally-occurring autoantibodies to Aβ. Indeed, such antibodies were found in both AD patients and healthy individuals, and the levels of such antibodies were variously reported to be lower or the same in AD patients compared with control subjects.[3–5] Currently, the role of anti-Aβ autoantibodies remains unknown.

In this issue of *Annals,* Kellner and colleagues[6] clearly demonstrate that IgG antibodies against β-amyloid are common in AD and help control plaque burden. They used a tissue microarray system constructed by semiautomatic robotic punching of tissue cylinders, each with a diameter of 0.6mm, from paraffin-embedded brains. These were then transferred into a new paraffin block containing hundreds of cylindrical samples of both AD patients and control subjects. The tissue microarray systems made it possible to examine a large number of tissue preparations at once and overcome variations among tissue stainings. Kellner and colleagues[6] found that the majority of neuritic plaques were decorated with IgG autoantibodies, and that AD patients with prominent IgG-labeled neuritic plaques had increased $CD68^+$ phagocytic microglia and reduced amyloid burden. To confirm this, they stained amyloid precursor protein transgenic mice with autoantibody-positive human sera, and they could demonstrate so-called tissue amyloid plaque immunoreactive (TAPIR) antibodies. TAPIR antibodies had been previously correlated with clinical benefit to the AN-1792 vaccine.[7]

It is interesting to note that there are two patterns in the TAPIR antibody staining. In the first, IgG is mainly localized in the plaque core (core pattern), and in the second, IgG is localized in the peripheral part (corona) of neuritic plaques (doughnut pattern); some antibodies may be located in both areas. Because Aβ40 is mainly deposited in the plaque core, the core pattern appears to be derived from anti-Aβ40 IgG decoration, whereas the doughnut pattern appears to be derived from anti-Aβ42 IgG decoration because Aβ42 is mainly localized in the peripheral part of plaques.[8] Because Aβ42 is the main deposit in the early phase of AD, autoantibodies that show a doughnut pattern may be more important in modifying AD onset and course.

A previous study described a TAPIR-like mouse monoclonal antibody 3.4A10, which is a IgG2b antibody and recognized in the N-terminal portion of Aβ.[9] It had much higher affinity to Aβ42 than Aβ40 and had higher affinity to an aggregated form of Aβ42 than its monomer. Indeed, tissue immunostaining with 3.4A10 showed mainly the doughnut pattern (Fig). Repeated intraperitoneal injections of the antibody significantly reduced amyloid burden without increasing cerebral microhemorrhage, probably because Aβ40 is mainly deposited in cerebral amyloid angiopathy. Notably, decorated plaques with mouse IgG were present in the treated mice, and the number of plaque-associated microglia was significantly greater in the decorated plaques than in the nondecorated plaques. Furthermore, this monoclonal antibody reduced Aβ oligomers such as highly toxic Aβ 12-mers.[10] Thus, 3.4A10 appears to have the potential to reduce amyloid burden and modify the clinical course of AD.

It is unclear how such autoantibodies are produced in humans. It is well known that self-reactive T cells are deleted during development; however, Aβ-reactive T cells remain in the peripheral blood, and the frequency of T cells is higher in older persons and AD patients than in younger individuals.[11] It is possible that self-reactive antibodies are produced because of age-related loss of normal immune regulation. Infection or immunization with microorganisms that contain proteins homologous to Aβ, such as potato virus, could also elicit antibodies cross-reactive with Aβ.[12] Such cross-reactive antibodies may be beneficial in AD in the same way that certain immune responses to central nervous system antigens are beneficial in demyelination and neurodegenerative diseases, facilitating regeneration.[13] A beneficial effect may also be acquired by the catalytic activity of certain antibodies.[14]

Although the decoration of amyloid plaques with autoantibodies to Aβ appears to control senile plaque formation, its role in controlling the pathological mechanism of AD appears to be limited because 6-year follow-up of patients immunized with Aβ1-42 (the AN-1792 vaccine) showed limited clinical benefit.[15] The AN-1792 vaccine did induce antibodies to Aβ and reduced amyloid burden; however, it provided only

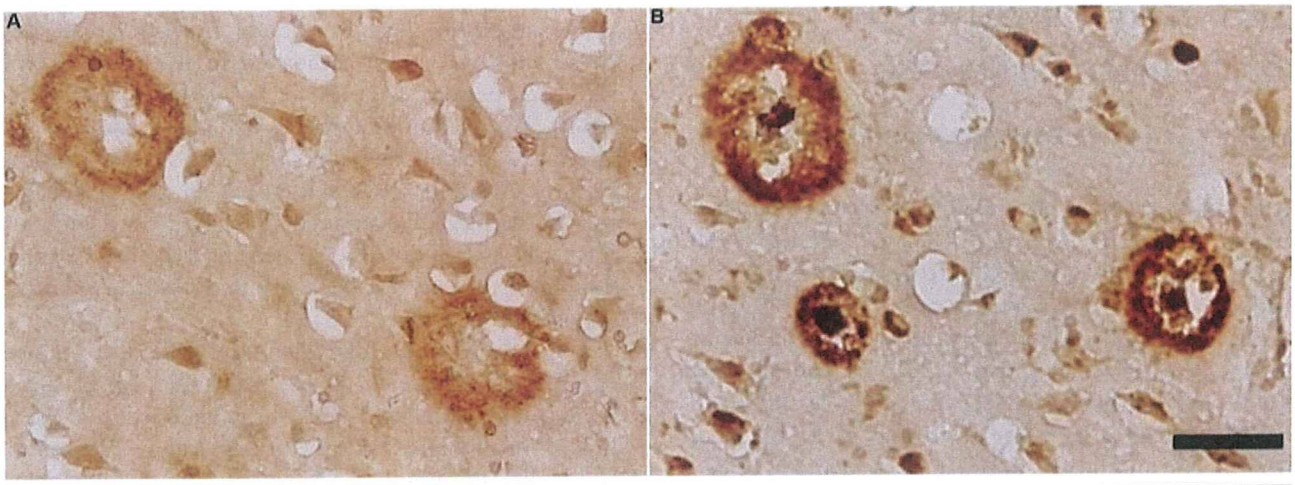

Fig. Doughnut pattern demonstrated by a monoclonal amyloid β protein antibody: 3.4A10, a monoclonal antibody to Aβ42, is a recognized amyloid existing mainly in the periphery of neuritic plaques (left), whereas 4G8 is recognized in both the plaque core and the corona (right). A similar pattern was demonstrated in the plaques decorated by autoantibodies in Alzheimer's disease.[6] Bar = 100μm.

minimal clinical benefit, even though senile plaques were almost completely eliminated in some patients. The vaccinated patients and placebo control subjects declined equally, and the survival rate was also not different. Therefore, AN-1792 did not appear to induce an immune response strong enough to modify disease progression. Alternatively, perhaps once Aβ triggers progressive neurodegeneration, removal of senile plaques might not modify the progression mechanism. Immune responses to other molecules, such as Aβ oligomers, intracellular Aβ, phosphorylated tau, or others, may also be required for a clinical benefit to occur. It is also possible that these and related approaches may, at least in theory, be more effective in prevention of AD than in treatment of established disease. Finally, other active immunization strategies, such as with Aβ vaccine using viral vectors[16,17] or Aβ complementary DNA vaccine,[18] if proved safe, might represent more effective strategies for inducing immune responses against pathological substrates.

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Development and Characterization of a TAPIR-Like Mouse Monoclonal Antibody to Amyloid-β

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Abstract. Tissue amyloid plaque immuno-reactive (TAPIR) antibody was better related to the effect of immunotherapy in Alzheimer's disease (AD) than ELISA antibody. Here we used a hybridoma technique to develop a TAPIR-like anti-human amyloid-β (Aβ) mouse monoclonal antibody. The obtained monoclonal antibody, 3.4A10, was an IgG2b isotype and recognized N-terminal portion of Aβ_{1-42} without binding denatured or native amyloid-β protein precursor. It had higher affinity to Aβ_{1-42} than to Aβ_{1-40} by Biacore affinity analysis and stained preferably the peripheral part of senile plaques and recognized the plaque core less than 4G8. It inhibited the Aβ_{1-42} fibril formation as well as degraded pre-aggregated Aβ_{1-42} peptide in a thioflavin T fluorescence spectrophotometry assay. The *in vivo* studies showed that 3.4A10 treatment decreased amyloid burden compared to the control group and significantly reduced Aβ_{42} levels rather than Aβ_{40} levels in brain lysates as well as the Aβ*56 oligomer (12mer) in TBS fraction of the brain lysates. 3.4A10 entered brain and decorated some plaques, which is surrounded by more Iba1-positive microglia. 3.4A10 therapy did not induce lymphocytic infiltration and obvious increase in microhemorrhage. We conclude that 3.4A10 is a TAPIR-like anti-human amyloid monoclonal antibody, and has a potential of therapeutic application for AD.

Keywords: Alzheimer's disease, immunotherapy, TAPIR-like anti-amyloid antibody

INTRODUCTION

Alzheimer's disease (AD) is the most common dementia in the elderly population. It has been estimated that up to 5% of the population older than 65 years is affected by AD [3] and the prevalence doubles approximately every 5 years beyond age 65 [4]. AD is a chronic neurodegenerative disorder that is characterized by progressive disturbances of cognitive functions including memory, judgment, language and so on clinically, and selective neuron and synapse losses, extracellular senile plaques containing amyloid-β peptide (Aβ) deposits as well as intraneuronal neurofibrillary tangles pathologically [4,22,28]. Although there is no curable treatment for AD, based on the growing understanding of the pathogenesis, several novel therapeutic strategies are being developed and Aβ immunotherapy is one of the potential methods [13]. Active and passive Aβ immunotherapies can reduce AD-like pathology and improve cognitive performance in animal models of the

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disease [1,5,8,11,14,19,20,27] and potentially in AD patients [7,10]. Due to the side effects (meningoencephalitis) of the active immunization in a human trial and reduced response to active vaccination in the elderly [23,24], passive administration of anti-Aβ antibodies might be a more plausible approach. Hock and colleagues suggested that tissue amyloid plaque immunoreactive (TAPIR) antibodies were corresponding to the slower cognitive decline in AD patients who received active vaccination [10]. In this study, we developed a TAPIR-like anti-human Aβ monoclonal mouse antibody by a hybridoma technique and characterized it in vitro and also evaluated its therapeutic effects in 18 months old Tg2576 mice.

MATERIALS AND METHODS

Animals

Female BALB/c mice were purchased from Central Science Company (Nagoya, Japan) and amyloid-β protein precursor (AβPP) transgenic (Tg2576) mice were purchased from Taconic Farms (Germantown, NY, USA). They were housed in plastic cages and received food (CE2, Clea Japan Inc., Tokyo, Japan) and water *ad libitum*, and were maintained on a 12/12 hours light-dark cycle. All experiments were performed under the guidelines for Animal Experiments of National Center for Geriatrics and Gerontology and approval of the institute's ethical committee for animal experiment.

Antibodies

The antibodies used in this study were listed as follows: mouse anti-human Aβ monoclonal antibodies: 6E10 (recognizes 1–17 amino acid residues of Aβ, Chemicon International, Temecula, CA, USA), 4G8 (recognizes 17–24 amino acid residues of Aβ, Signet, Dedham, MA, USA) and 12F4 (recognizes C-terminus of $A\beta_{1-42}$, Signet); mouse anti-AβPP A4 monoclonal antibody 22C11 (Chemicon International); hamster anti-mouse CD3e monoclonal antibody and rat anti-mouse CD19 monoclonal antibody (BD Bioscience Pharmingen, San Jose, CA, USA); rabbit polyclonal anti-pan Aβ antibody (Biosource, Camarillo, CA, USA); rabbit anti-Iba1 polyclonal antibody (a gift from Dr. Imai at the National Institute of Neuroscience, NCNP); Cy3 conjugated mouse anti-glial fibrillary acidic protein (GFAP) monoclonal antibody, clone G-A-5 (Sigma-Aldrich, Saint Louis, MO, USA).

Immunization and establishment of hybridoma cell lines

The 6–8 weeks old female BALB/c mice were immunized with emulsion of 100 μl complete Freund's adjuvant (containing 1 mg/ml mycobacterium tuberculosis H37Ra, Difco Laboratories, Deteriot, MI, USA) and 100 μg $A\beta_{1-42}$ peptide (Peptide Institute, Inc., Osaka, Japan) in 100 μl PBS as previously described [27] and boosted with emulsion of 100 μl incomplete Freund's adjuvant (Difco Laboratories) and 100 μg $A\beta_{1-42}$ peptide in 100 μl PBS 2 weeks after the immunization. Just the day before the fusion, the mice received 100 μg $A\beta_{1-42}$ peptide in 100 μl PBS intraperitoneally. The spleens were harvested aseptically 4 weeks after the first immunization and the isolated splenocytes were fused to a myeloma cell line X63-Ag8.653 at 5:1 ratio in 50% w/v PEG (Hybri-Max®, MW1450, Sigma-Aldrich). The cell suspension was added into the 96-well plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at 100 μl/well. The hybridoma cells were selected by adding 100 μl culture medium containing 2×HAT (Hybri-Max®, Sigma-Aldrich) to each well two days after fusion. When the most growing wells demonstrated 10 to 25% confluence, 100 μl of culture medium from each well to be tested was used to detect positive clones by an ELISA screening assay. To get monoclonal antibodies, limiting dilution at 5 cells/ml in concentration was performed twice. To keep the cells growing in the low concentration, 10% fetal bovine serum (ICN Biomedicals, Aurora, OH, USA) and 10% hybridoma cloning factor (BioVeris, Gaithersburg, MD, USA) were added into the culture medium (RPMI medium 1640, Gibco®, Invitrogen, Grand Island, NY, USA). The cells were cultured in a humidified 37°C, 5% CO_2 incubator.

Hybridoma screening and antibody isotyping

The 96 well ELISA plates (Nunc-Immuno plate, Maxisorb surface, Roskilde, Denmark) were coated with 100 μl of 4 μg/ml $A\beta_{1-42}$ peptide in 55 mM $NaHCO_3$ solution (PH 9.0) overnight at 4°C. Plates were washed with 20 mM Tris-HCl, pH 7.4 containing 150 mM NaCl and 0.05% Tween20 (TBS-T) 2 times and blocked with 1% BSA and 2% normal goat serum in TBS-T (blocking buffer) for 1 hour at room temperature (RT). Plates were washed 2 times and incubated with 100 μl hybridoma culture medium for 2 hours at RT. Plates were washed 4 times and incubated with 100 μl goat anti-mouse IgG+IgM (H&L)-HRPO (American

Qualex, San Clemente, CA, USA) diluted 1:2000 in blocking buffer for 2 hours at RT. Plates were washed 4 times and added 100 μl SureBlue Reserve™ TMB Microwell Peroxidase substrate (KPL, Baltimore, MD, USA) and allowed to develop color in dark for 30 minutes at RT. The plates were read at 450 nm wave length by a microplate reader (model 550, Bio-Rad Laboratories, Hercules, CA, USA) after adding 100 μl TMB stop solution (KPL). The isotypes of the monoclonal antibodies were detected by a mouse immunoglobulin isotyping ELISA kit (BD Biosciences Pharmingen) according to the assay procedure of the product instruction.

Ascites development and antibody purification

0.5 ml pristane (Sigma-Aldrich) was administrated to 6–8 weeks old female BALB/c mice intraperitoneally one week before hybridoma injection. The hybridoma cells were washed with PBS twice to eliminate fetal bovine serum and suspended with PBS at 5×10^6 cells/ml. Each mouse received 2 ml hybridoma cell suspension intraperitoneally. The ascites was allowed to develop for 2 weeks. If there was no ascites developed, hybridoma cell suspension was administrated to the mice again. The ascites was drained every 3 days by a 16 G needle till no ascites was drained or the mice died. The ascites was centrifuged for 10 minutes at 1500 × g, RT. The supernatants were harvested and kept at 4°C till purification. The monoclonal antibody was purified by an Affi-Gel® Protein A MAPS® kit (Bio-Rad Laboratories) according to the product manual and under the monitor of an ÄKTA FPLC system (Amersham Biosciences AB, Uppsala, Sweden). The purified monoclonal antibody was dialyzed against PBS. After 4 times dialysis, the antibody was filtrated by 0.22 μm filter (MILLEX®-GV PVDF syringe driven filter unit, Millipore, Cork, Ireland) and the concentration was measured by a BCA™ protein assay kit (Pierce Biotechnology, Rockford, IL, USA) with pre-diluted bovine gamma globulin (Pierce Biotechnology) as the standard. For *in vivo* study, the endotoxin was removed by a ProteoSpin™ endotoxin removal kit (Norgen Biotek, Ontario, Canada) following the product manual.

In vitro characterization of the monoclonal antibody

Dot blot and western blot for detection of Aβ peptide fragments and AβPP fragments

Several Aβ peptide fragments were used. $A\beta_{1-28}$ and $A\beta_{34-42}$ were purchased from Sigma-Aldrich. $A\beta_{25-35}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ were purchased from Peptide Institute. All the peptides were dissolved in DMSO at the concentration of 1mM. Then an aliquot of each peptide was diluted with PBS to the final concentration at 4 μg/ml. 100 μl of each peptide solution was added to 0.2 μm nitrocellulose transfer membrane (Whatman GmbH, Dassel, Germany) in a dot blot device (Bio-Rad Laboratories). Then the membrane was blocked by 5% skim milk in TBS for 30 minutes at RT. After a short wash with dH_2O, the membrane was incubated with primary antibodies (3.4A10, our anti-Aβ monoclonal antibody and 4G8) at 1 μg/ml in StartingBlocking™ T20 (TBS) blocking buffer (Pierce Biotechnology) for 1 hour at RT. The membrane was washed 3 times with TBS-T and incubated with goat anti-mouse IgG (H&L)-HRPO (American Qualex) diluted 1:2000 in blocking buffer for 1 hour at RT. After washing, the dots were visualized by a western lightning chemiluminescence reagent (PerkinElmer, Boston, MA, USA). For western blot, 100 ng of $A\beta_{1-28}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ was loaded into 16.5% peptide SDS-PAGE gels (Bio-Rad Laboratories, Tokyo, Japan) and transferred onto 0.2 μm nitrocellulose membrane at 200 mA for 1 hour. The subsequent steps were the same as dot blot. sAβPPα, immature AβPP and mature AβPP were got from the lysate of wild type human AβPP-transfected human neuroblastoma SH-SY5Y cells (generous gift from Dr. K. Takeda). 10 μl of the cell lysate was loaded into 7.5% SDS-PAGE gels (Daiichi Pure Chemicals, Tokyo, Japan) and the bands were detected by 1 μg/ml 3.4A10 or 22C11 in blocking buffer and visualized as described above.

Staining of AβPP-transfected SH-SY5Y living cell

Human neuroblastoma SH-SY5Y cells were transfected with an expression plasmid encoding wild type human AβPP. Staining of the cell surface AβPP ectodomains was performed as previously described [9]. Briefly, the cells were incubated with 5 μg/ml 3.4A10, 6E10 or normal mouse IgG in culture medium at 4°C for 30 minutes. After brief washing with PBS, the cells were fixed in 4% paraformaldehyde for 15 minutes, blocked, stained with Alexa-594 labeled Donkey anti-mouse IgG (Molecular Probes, Eugene, OR, USA) at 1:500 dilution and counter-stained the nuclei with Hoechst 33342 (Molecular Probes).

Biacore epitope and affinity analysis

Freshly prepared 50 μg/ml $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides in 10 mM sodium acetate buffer, pH 4.0 were immobilized to the CM5 sensor chips (Biacore AB, Uppsala, Sweden) according to the user manual. All analyses were run on a Biacore J system (Biacore AB). For the competitive epitope analysis, an $A\beta_{1-42}$ immobilized CM5 chip was firstly saturated with 200 μg/ml 3.4A10 in PBS for 5 minutes at a flow rate of 30 μl/min, then 20:1 ratio antibody mixtures (3.4A10:6E10, 4G8 or 12F4) was injected at the same flow rate for 3 minutes and the response levels of each cycle were recorded. For the affinity analysis, 3.4A10 at concentrations ranging from 100 nM to 500 nM in PBS was injected into $A\beta_{1-40}$ or $A\beta_{1-42}$ immobilized CM5 sensor chips at a flow rate of 30 μl/min for 5 minutes. Dissociation of bound antibody in PBS flow was followed for 3 minutes and the data were analyzed by BIAevaluation software (Biacore AB). The chips were regenerated with 50 mM NaOH at a flow rate of 30 μl/min for 2 minutes after each cycle.

Thioflavin T fluorescence spectrophotometry

To determine whether 3.4A10 can inhibit $A\beta_{1-42}$ aggregation and disaggregate pre-aggregated $A\beta_{1-42}$, the experiments were performed as described previously [29,30]. For the inhibition assay, the freshly prepared 25 mM $A\beta_{1-42}$ in PBS was incubated with 2.5 mM 3.4A10 in a final volume of 25 μl at 37°C for 1 week, and 1 ml of 5 mM thioflavin T (Sigma Aldrich Chemie Gmbh, Steinheim, Germany) in PBS was added to the system and fluorescence was measured using a fluorescence spectrophotometer (Model F-2500, Hitachi, Tokyo, Japan) at an excitation wavelength of 445 nm and an emission wavelength of 490 nm. For the disaggregation assay, 10 μl of 62.5 mM $A\beta_{1-42}$ was incubated in PBS at 37°C for 1 week and then 3.4A10 was added at the final concentration of 2.5 mM and the final volume is adjusted to 25 μl. The mixture was incubated for another 48 hours and 1 ml of 5 mM thioflavin T was added to the mixture and fluorescence was measured as described above. PBS without antibody was added for the control. These experiments were repeated 3 times and duplicated each time. The results were the means of three independent experiments and the percentage of the control was used as presentation.

Senile plaque staining

The frozen sections of AD patient brain (with the permission of the family for AD study) were fixed with 70% formic acid for 20 minutes at RT. After washing with TBS-T, the sections were incubated with 0.3% H_2O_2 in methanol for 30 minutes to block endogenous peroxidase. The sections were washed with TBS-T and then incubated with 1 μg/ml 3.4A10 or 4G8 in the blocking buffer (5% skim milk in TBS containing 0.4% Triton X-100 and 10% normal horse serum) for 1 hour at RT. The sections were washed with TBS-T, 1:500 biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) was added to the sections and incubated at RT for 1 hour. After washing, the sections were stained with Avidin-HRP/DAB kit (Vector Laboratories). Senile plaques with or without the plaque core were counted, and the difference between 3.4A10 and 4G8 was compared.

Passive immunization with 3.4A10

3.4A10 was administrated to 18 months old female Tg2576 mice intraperitoneally at 10 mg/kg body weight weekly for 8 weeks ($n = 4$). The control group received PBS only ($n = 4$).

Immunohistochemistry

After 8 weeks treatment, the mice were anesthetized by inhalation of diethyl ether. After blood samples were taken from heart, the mice were sacrificed by cervical dislocation. The brains were removed and cut in half sagittally. The hemisphere from each mouse was fixed in 4% paraformaldehyde at 4°C over night. The brains then were washed with PBS and dehydrated with gradient concentration of sucrose, and embedded in O.C.T. (Sakura Finetechnical, Tokyo, Japan). The frozen sections were cut with a Leica CM 1850 cryostat microtome (Leica Microsystem Nussloch GmbH, Nussloch, Germany) at 9 μm in thickness. Sections were washed with PBS and incubated with blocking buffer (5% skim milk in TBS containing 0.4% Triton X-100 with 10% normal second antibody relevant animal serum) for 30 minutes at RT. Sections were then incubated at RT for 2 hours with the antibody indicated below, washed with TBS-T, incubated with relevant biotin conjugated second antibody for 2 hours at RT, washed with TBS-T again, and stained by the avidin-biotin HRP/DAB method. The primary antibody against CD3e and CD19 were used at 1 μg/ml. $A\beta$ plaque-containing sections were stained with rabbit polyclonal anti-pan $A\beta$ antibody at 1 μg/ml. The samples were counterstained with hematoxylin.

A quantitative analysis of amyloid burden was performed by using a Winroof software (version 5.7, Mitani, Fukui, Fukui Ken, Japan) in the whole cortex and

hippocampus. Aβ burden was expressed as the percentage of brain tissue occupied by Aβ deposits. Three immunolabeled sections were analyzed per mouse, and the average of the individual measurements was used to calculate group means.

Immunofluorescence staining

To prove peripherally administrated 3.4A10 had entered the brain, the brain sections were firstly stained with 1 μg/ml rabbit anti-pan Aβ antibody in blocking buffer (10% normal donkey serum and 2% bovine serum albumin in TBS) for 1 hour at RT following 30 minutes incubation with blocking buffer. After 3 time washes with TBS-T, the sections were incubated with 1:500 Alexa 594-labeled donkey anti-mouse IgG antibody and 1:500 Alexa 488-labeled donkey anti-rabbit IgG antibody (Molecular Probes) for 1 hour at RT. After washing, the fluorescence was observed by a Olympus IX70 microscope (Olympus, Tokyo, Japan) and recorded by a Nikon digital camera DXM1200F (Nikon, Tokyo, Japan).

To evaluate activation of microglia, the sections were stained with 1 μg/ml rabbit anti-microglia polyclonal antibody (Iba-1), the microglia and senile plaques were visualized with 1:500 Alexa 488-labeled donkey anti-rabbit IgG antibody and Alexa 594-labeled donkey anti-mouse IgG antibody and the images were recorded by a Zeiss LSM510 image system (version 2.3, Carl-Zeiss Co., Ltd, Tokyo, Japan). The numbers of microglia associated with 3.4A10 positive and negative plaques were counted, and the average cell number per plaque was compared. The astrocytes were stained by 1:500 Cy3-conjugated mouse anti-GFAP monoclonal antibody. The fluorescence was observed and recorded as described above.

Berlin blue staining

To detect the hemorrhagic lesion in the mouse brain sections, Berlin blue stain was used. Briefly, brain sections were stained in potassium ferrocyanide solution (2% potassium ferrocyanide: 2% Hydrochloric acid = 1 vol:1 vol; Muto Pure Chemicals Co., Ltd, Tokyo, Japan) for 30 minutes at RT. After washing in distilled water, sections were incubated in Kernechtrot stain solution (Muto Pure Chemicals) for 2 minutes. The positive blood vessel numbers were counted in 3 sections per mouse, and average of the individual measurements was used to calculate group means.

ELISA measurements for Aβ levels in brain tissue lysate

The samples for measurement of insoluble and soluble Aβ was prepared as previously reported [8,20]. The brain hemispheres of both therapeutic and control groups were homogenized with a homogenizer in 1 ml of TBS with complete protease inhibitor plus 20 μg/ml pepstatin A (Roche Diagnostics GmbH, Mannheim, Germany), then centrifuged at 100,000 g for 1 hour at 4°C using an Optima TLX ultracentrifuge (Beckman-Coulter, Fullerton, CA, USA). The pellets were homogenized in 1 ml of 2% SDS/TBS with complete protease inhibitor, then centrifuged at 100,000 g for 1 hour at 25°C following 15 minutes incubation at 37°C. The pellets (corresponding to insoluble fraction of Aβ peptides) were homogenized in 1 ml of 70% formic acid and centrifuged at 100,000 g for 1 hour. The supernatants were neutralized with 1 M Tris-HCl, pH 8.0 at 1:20 ratio. The samples were kept in −80°C. The Aβ levels of SDS-fraction and formic acid fraction were quantified with an Aβ ELISA kit (Wako Pure Chemicals Industries) according to the product instruction. The supernatants were diluted with standard dilution buffer at 1:2000 ($Aβ_{1-40}$) or 1:400 ($Aβ_{1-42}$). The obtained values were corrected with the wet weight of each brain hemisphere samples and expressed as nmol/g brain.

Western blot for detection of Aβ oligomers in TBS fraction of brain lysate

For analysis of Aβ oligomers in the soluble fraction of mouse brain lysate, 10 μl of each TBS fraction of soluble brain lysates was loaded to 15/25 SDS-PAGE gel (Daiichi Pure Chemicals) and transferred to 0.2 μm nitrocellulose membrane. The bands were detected with 6E10 at 1: 2000 dilution and HRP-conjugated goat anti-mouse IgG (H&L) at 1: 5000 dilution and visualized as described above. The size of the bands was determined by using Alpha Ease FC software (Alpha Innoteck, San Leandro, CA, USA) based on the molecular weight of the standard.

Statistic analysis

All results were expressed as the mean ± SD except mentioned otherwise. The difference of the two groups was analyzed with Student's t test.

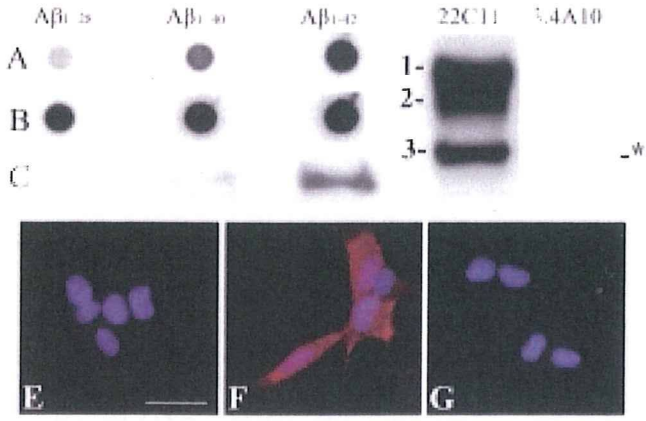

Fig. 1. 3.4A10 had higher affinity to $A\beta_{1-42}$ and did not bind native or denatured AβPP fragments. A) 400 ng of different Aβ fragments were immobilized onto the nitrocellulose membrane, 1 μg/ml 3.4A10 or 4G8 was used to detect the dots. 3.4A10 recognized $A\beta_{1-28}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ with an increasing signal density and did not bind $A\beta_{25-35}$ and $A\beta_{34-42}$ (data not shown). B) As a positive control, 4G8 recognized $A\beta_{1-28}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ with an equal signal density. C) The western blot results showed that 3.4A10 recognized $A\beta_{1-42}$ monomer better than $A\beta_{1-40}$ monomer, and did not recognized $A\beta_{1-28}$ monomer. D) 3.4A10 did not recognize the denatured sAβPPα, immature AβPP and mature AβPP separated on SDS-PAGE from cell lysate of human neuroblastoma SH-SY5Y cells transfected with wild type human AβPP, while as a positive control anti-AβPP N terminal-specific antibody 22C11 recognized these bands. 1, mature AβPP; 2, immature AβPP; 3, sAβPPα; *, 100 kD. E) 3.4A10 did not bind native AβPP that was expressed on the surface of human neuroblastoma SH-SY5Y cell line transfected with wild type human AβPP by a living cell staining assay, 6E10 as a positive control (F), normal mouse IgG as a negative control (G). Bar = 20 μm; Blue, Hochst33342; Red, AβPP.

RESULTS

One IgG2b secretary hybridoma cell line was established

Each immunized spleen was used to perform one fusion. Totally 5 fusions were performed, and 8 anti-Aβ antibody secretory hybridoma cell lines were detected by an ELISA screening assay. The monoclonal hybridoma cell line was established by twice limiting dilution. Then the isotype assay was performed. One cell line 3.4A10 was an IgG2b isotype, while others were an IgM isotype, and the clone 3.4A10 was investigated further in this study.

Results of in vitro studies

3.4A10 recognized N-terminal epitope of $A\beta_{1-42}$

To analyze the epitope of 3.4A10, the different Aβ fragments were used firstly. Even though same amount of peptides was loaded to the membrane or SDS-PAGE gel, 3.4A10 had different signal density to the different peptide. Dot blot results showed that 3.4A10 recognized $A\beta_{1-28}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ with an increasing signal density (Fig. 1A) and did not recognize $A\beta_{25-35}$ and $A\beta_{34-42}$ (data not shown), while as a positive control 4G8 recognized $A\beta_{1-28}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ with a similar signal density (Fig. 1B). 3.4A10 bound $A\beta_{1-42}$ monomer with much higher density than $A\beta_{1-40}$monomer and did not recognize the $A\beta_{1-28}$ monomer on western blot (Fig. 1C). The epitope of 3.4A10 cannot be determined according to the data as above mentioned, to elucidate the epitope further, Biacore competitive epitope analysis was used. Biacore data showed that after saturation of $A\beta_{1-42}$ immobilized CM5 chip with 3.4A10, only the antibody mixture which contained 6E10 did not increase the response level, while the antibody mixture which contained 4G8 or 12F4 could increase the response levels (>2000 IU) (Table 1). This data suggested that 3.4A10 competitively inhibited binding of 6E10 to the sensor chip and its epitope located in the N-terminal portion of $A\beta_{1-42}$ as 6E10 did.

3.4A10 had higher affinity to $A\beta_{1-42}$ than $A\beta_{1-40}$

Biacore affinity analyses were performed to measure the affinity of 3.4A10 to $A\beta_{1-40}$ and $A\beta_{1-42}$. The Biacore binding curves obtained using concentrations of 3.4A10 from 100 nM to 500 nM were analyzed assuming a one to one interaction by a BIAevaluation software. The dissociation constants (KD) of 3.4A10 to $A\beta_{1-40}$ and $A\beta_{1-42}$ were 3.77×10^{-8} M and 5.64×10^{-11} M, respectively (the χ^2 of the two analyses were 10.4 and 11.7, respectively) (Table 2). These data were consistent to the results of dot blot and western blot as well as the results of senile plaque staining as mentioned below.

Table 1
Biacore competitive epitope analysis of 3.4A10

	Cycle 1			Cycle 2			Cycle 3		
	baseline	3.4A10	with 6E10	baseline	3.4A10	with 4G8	baseline	3.4A10	with 12F4
Response level (RU)	3001.6	7937.8	7927.1	2750.5	8620.0	10684.6	2752.7	8602.4	10937.7

The $A\beta_{1-42}$ immobilized CM5 sensor chip was firstly saturated with 200 μg/ml 3.4A10 in PBS, then a 20:1 ratio antibody mixture (3.4A10: 6E10, 4G8 or 12F4) was injected and the response levels of each cycle were recorded. Results showed that 3.4A10 competed binding site with 6E10, as a result there was no increase in response levels after antibody mixture injection, while 3.4A10 did not compete binding site with 4G8 or 12F4, so after injection of antibody mixture, there was an increase in response levels (>2000 RU). The result suggests that the epitope of 3.4A10 exists in the same region as that of 6E10.

Table 2
Dissociation constant of 3.4A10 to $A\beta_{1-40}$ and $A\beta_{1-42}$

	$A\beta_{1-40}$	$A\beta_{1-42}$
Dissociation constant (KD)	3.77×10^{-8}M	5.64×10^{-11}M
χ^2	10.4	11.7

The Biacore binding curves obtained using concentrations of 3.4A10 from 100 nM to 500 nM were analyzed assuming a one to one interaction by BIAevaluation software. The data show that 3.4A10 has more affinity to $A\beta_{1-42}$ than to $A\beta_{1-40}$.

3.4A10 did not bind AβPP fragments

One of TAPIR antibody's features is that the antibody does not recognize both native and denature AβPP fragments. Western blot results showed that 3.4A10 did not recognize the denatured sAβPPα, immature AβPP and mature AβPP separated on SDS-PAGE from the lysate of human neuroblastoma SH-SY5Y cells transfected with wild type human AβPP, and mouse anti-AβPP A4 monoclonal antibody 22C11 was used as a positive control (Fig. 1D). To demonstrate that 3.4A10 did not bind native AβPP, a living cell staining was used. The results showed that 3.4A10 did not recognize the native AβPP that expressed on the surface of the cells (Fig.1E), while as a positive control 6E10 stained the cells (Fig. 1F).

3.4A10 inhibited $A\beta_{1-42}$ fibrillar aggregation and disaggregated pre-aggregated $A\beta_{1-42}$

To determine whether 3.4A10 has an effect on inhibition of $A\beta_{1-42}$ aggregation, 3.4A10/freshly prepared $A\beta_{1-42}$ was incubated at 1:10 molar ratio at 37°C for 1 week. 3.4A10 inhibited $A\beta_{1-42}$ aggregation to 36.7 ± 0.67% of the control (PBS only without antibody) by a thioflavin T fluorescence spectrophotometry assay (Fig. 2A). To elucidate the solubilization effect of 3.4A10 on $A\beta_{1-42}$, 3.4A10 was also incubated with pre-aggregated $A\beta_{1-42}$about 1:10 molar ratio at 37°C for 2 days, and the results of fluorescence spectrophotometry assay showed that 3.4A10 reduced 490 nm emission value of pre-aggregated $A\beta_{1-42}$ by 22.3 ± 3.48% compared to the control (Fig. 2B).

3.4A10 recognized less senile plaque core than 4G8

Consecutive AD brain sections were cut, and 3 pairs of the sections 100 μm apart were stained with 3.4A10 or 4G8. The 3.4A10 stained both classical and diffuse plaques in a similar pattern with 4G8, but only 7.5 ± 1.57% of the plaques had the core, while 4G8 stained the core in 14.93 ± 2.7% of the plaques ($P < 0.05$) (Fig. 3). To confirm this result, we stained another 3 pairs of the sections and got similar results (data not shown).

Therapeutic effects of 3.4A10

One mouse in the treatment group died after receiving 4 times injection due to an unknown reason. So at the end point of the experiment, 3 mice in the therapeutic group and 4 mice in the control group enrolled in further studies.

3.4A10 reduced the amyloid burden

The effect of 3.4A10 administration on development of an AD-like neuropathology in Tg2576 mice was investigated by a quantitative image analysis. Administration of 3.4A10 for 8 weeks resulted in marked reduction of amyloid burden in cortex and hippocampus compared with the control group (Fig. 4A). The mean value of amyloid burden in therapeutic group (cortex: 0.31 ± 0.09%, hippocampus: 0.59 ± 0.08%) was significantly reduced compared with the control group (cortex: 0.72 ± 0.23%, hippocampus: 0.99 ± 0.25%, $P < 0.05$) (Fig. 4B).

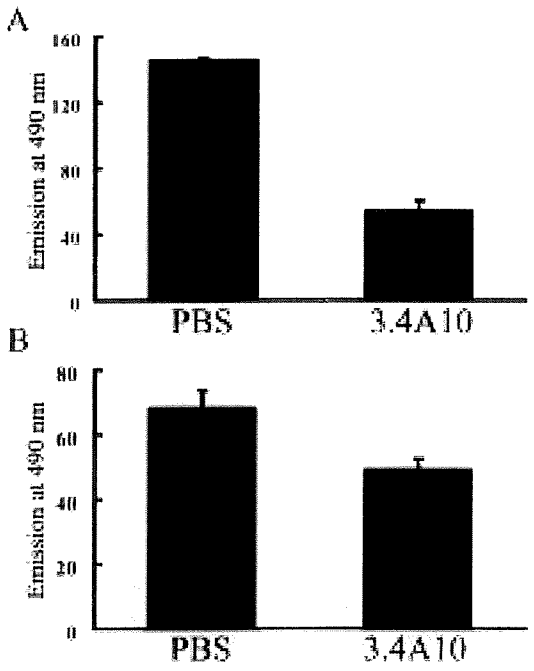

Fig. 2. 3.4A10 inhibited $A\beta_{1-42}$ fibrillation and degraded pre-aggregated $A\beta_{1-42}$ by thioflavin T-based fluorescence spectrophotometry. A) The freshly prepared 25 mM $A\beta_{1-42}$ in PBS was incubated with 2.5 mM 3.4A10 in a final volume of 25 μl at 37°C for 1 week, and 1 ml of 5 mM thioflavin T was added to the system and fluorescence was measured at an excitation wavelength of 445 nm and an emission wavelength of 490 nm. The results showed that 3.4A10 decreased the fluorescence to 36.7% ± 0.67% of the control. B) For degradation assay, 3.4A10 was incubated with pre-aggregated $A\beta_{1-42}$ about 1:10 molar ratio at 37°C for 2 days, and fluorescence spectrophotomtry assay showed that 3.4A10 reduced 490 nm emission value of pre-aggregated $A\beta_{1-42}$ by 22.3 ± 3.48% compared to the control. The figure shows the values (mean ± SD) of an example of similar results.

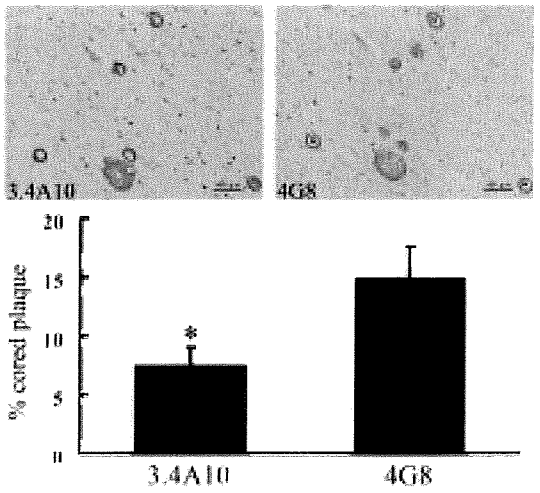

Fig. 3. 3.4A10 recognized less plaque cores than 4G8. Pairs of consecutive frozen sections of AD patient brain were stained with 1 μg/ml 3.4A10 or 4G8. 3.4A10 stained preferably the peripheral zone of senile plaques in the similar pattern with 4G8 and recognized less plaque cores compared with 4G8. The percentage of cored plaques was compared between 3.4A10 and 4G8 (7.5 ± 1.57% versus 14.93 ± 2.7%). Scale bar represents 100 μm. $^*P < 0.05$.

The reduced amyloid burden was further confirmed by a quantitative ELISA measurement for the $A\beta_{42}$ and $A\beta_{40}$ contents in brain lysates. The results showed that $A\beta_{42}$ levels of the 3.4A10 therapeutic group in both SDS-soluble fraction and formic acid (non-soluble) fraction were decreased significantly compared with the control group (41.86 ± 2.64 nmol/g and 53.06 ± 4.99 nmol/g for SDS-soluble fraction of the therapeutic and the control group, respectively, $p = 0.017$; 682.91 ± 107.17 nmol/g and 1,040.40 ± 212.21 nmol/g for non-soluble fraction of the therapeutic and the control group, respectively, $p = 0.046$). While 3.4A10 therapy less interfered with the $A\beta_{40}$ levels compared to the control group (109.26 ± 39.91 nmol/g and 118.71 ± 13.89 nmol/g for SDS soluble fraction of the therapeutic and the control group, respectively, $p = 0.67$; 3,331.29 ± 2135.03 nmol/g and 5,485.44 ± 2620.58 nmol/g for non-soluble fraction of the therapeutic and the control group, respectively, $p = 0.30$) (Fig. 4C). The $A\beta$ oligomers in TBS fraction were detected by western blot. 3.4A10 therapy resulted in a reduction in the contents of $A\beta$*56 (12mer) compared to the control, while no apparent effects on other $A\beta$ oligomers (Supplementary Fig. 1).

No lymphocytic infiltration and no obvious increase of microhemorrhage in the brain of 3.4A10-treated mice

The lymphocytic infiltration was investigated in the brain of Tg2576 mice treated with 3.4A10 by staining with hematoxylin and eosin (HE) and by immunohistochemical staining with antibodies against CD3e and CD19, the markers of mature T-lymphocyte and B-lymphocyte, respectively. There were no histological or immunohistochemical differences in the brain sections between the therapeutic and the control groups (Fig. 5A–F).

The brain microhemorrhage is reported as an adverse side effect of passive immunotherapy in $A\beta$PP transgenic mice due to the increased vascular amyloid burden [25,26]. There was slightly increased microhemarrhage in the therapeutic group by counting the Berlin blue-positive blood vessels but it was not statistically significant (1.67 ± 1.17 versus 0.42 ± 0.21, $P = 0.27$). The Berlin blue-positive blood vessels of both groups were shown in Fig. 5G-H.

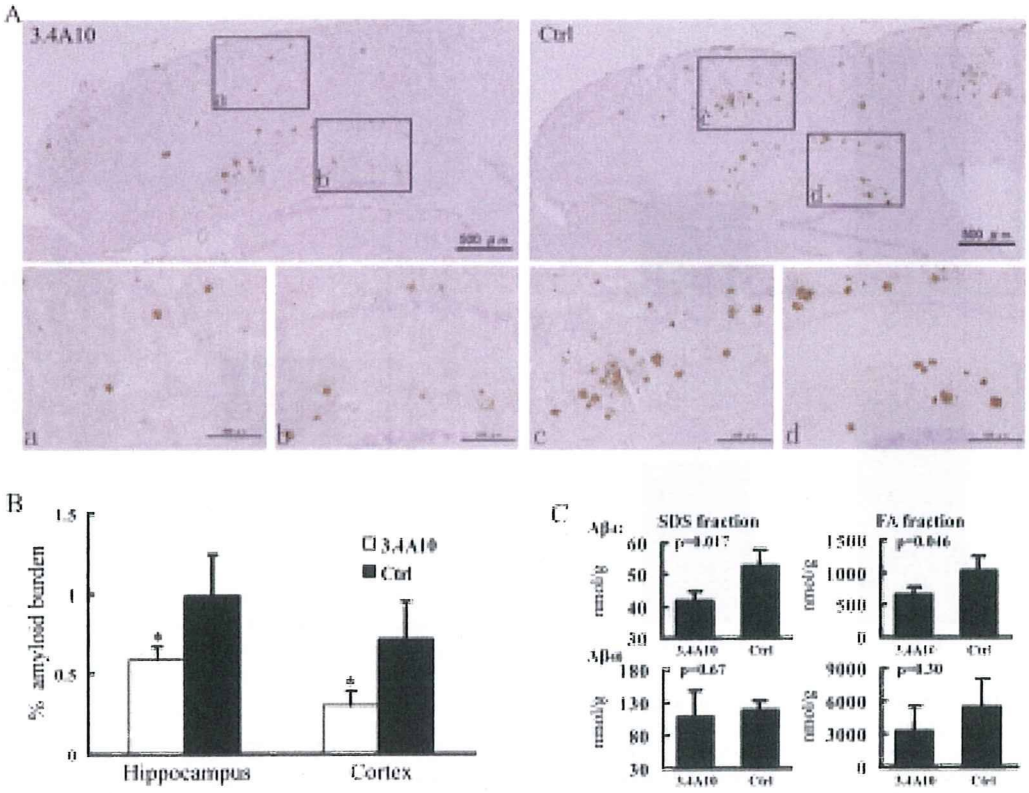

Fig. 4. 3.4A10 reduced the brain amyloid burden. A) Pan Aβ staining in sagittal brain sections showed that 3.4A10 therapy reduced the amyloid burden markly compared to the control. The upper two pictures represent the hippocampus and adjacent cortex of the 3.4A10 therapeutic and the control group in a low magnification and picture a-d represent the regions with more pronounced effect between the two groups. a, c: cortex; b, d: hippocampus, scale bar = 100 μm. B) The percentage of the area occupied by Aβ deposits in the brain section, which was determined by a quantitative image analysis. 3.4A10 therapy significantly reduced the amyloid burden in cortex and hippocampus compared to the control group. $^{*}P < 0.05$. C) The results of a quantitative ELISA for $A\beta_{40}$ and $A\beta_{42}$ contents in the SDS-soluble and formic acid fractions of the brain lysate. FA, formic acid. The 3.4A10 therapy significantly reduced $A\beta_{42}$ levels in both fraction and less interfered with $A\beta_{40}$ levels.

3.4A10 entered the brain and decorated some plaques which were surrounded with more Iba1-positive microglia

Alexa 594-labeled donkey anti-mouse IgG antibody was used to detect whether 3.4A10 could enter the brain. The immunofluorescence staining showed 3.4A10 entered the brain and the fluorescence (red) was co-localized with some of rabbit anti-pan Aβ antibody-detected senile plaques (green) (Fig. 6A upper panel). Alexa 594-labeled donkey anti-mouse IgG antibody did not stain any plaques in the control group (Fig. 6A lower panel).

Some plaques in the therapeutic group were surrounded by more Iba1-positive microglia, but the average number of microglia per plaque was not different between the two groups (data not shown). Then the average number of microglia was compared between 3.4A10-decorated and non-decorated plaques in the therapeutic group, the results showed that 3.4A10-decorated plaques were surrounded with more Iba1-positive microglia compared to the non-decorated plaques (9.28 ± 1.80 versus 5.43 ± 1.51, $p < 0.001$) (Fig. 6B–C).

Astrocytes were investigated by GFAP immunofluorescence staining. The results showed that there was no difference in GFAP-positive astrocytes between the two groups (Fig. 6D).

DISCUSSION

As one of key features of AD pathology, the extracellular amyloid deposits comprised of Aβ is a target of therapeutic approaches to AD, and the immunotherapies aimed at decreasing amyloid burden have been shown their effects on improvement of pathologic changes and cognitive functions in animals [1,5, 8,11,14,19,20,27] as well as in humans [7,10,21]. As