

# Prototype Alzheimer's disease epitope vaccine induced strong Th2-type anti-A $\beta$ antibody response with Alum to Quil A adjuvant switch

Anahit Ghochikyan<sup>a</sup>, Mikayel Mkrtichyan<sup>a</sup>, Irina Petrushina<sup>b</sup>, Nina Movsesyan<sup>a</sup>,  
Adrine Karapetyan<sup>a</sup>, David H. Cribbs<sup>b,1</sup>, Michael G. Agadjanyan<sup>a,b,\*,1</sup>

<sup>a</sup> Department of Immunology, Institute for Molecular Medicine, Huntington Beach, CA 92647, USA

<sup>b</sup> Department of Neurology, Institute for Brain Aging and Dementia, University of California, Irvine, CA 92697, USA

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## Abstract

$\beta$ -Amyloid (A $\beta$ ) peptide has been proposed to be a causal factor in Alzheimer's disease (AD). Currently being investigated, active and passive A $\beta$ -immunotherapy significantly reduce A $\beta$  plaque deposition, neuritic dystrophy, and astrogliosis in the brains of APP transgenic (APP/Tg) mice. Immunization with A $\beta_{42}$  formulated in the Th1-type adjuvant QS21 was beneficial for AD patients with significant titers of anti-A $\beta$  antibodies, however, 6% of participants developed meningoencephalitis, likely due to anti-A $\beta$ -specific autoimmune Th1 cells. Thus, successful A $\beta$  vaccination requires the development of strong antibody responses without Th1-type cellular immunity. In this study, we compared the induction of humoral immune responses with Th1-type (Quil A) and Th2-type (Alum) adjuvants singly and in combination, using our novel epitope vaccine composed of self B cell epitope A $\beta_{1-15}$  and foreign T cell epitope PADRE (PADRE-A $\beta_{1-15}$ -MAP). Formulated in Quil A, this vaccine resulted in significantly higher anti-A $\beta$  antibody responses in both BALB/c (H-2<sup>d</sup>) and C57BL/6 (H-2<sup>b</sup>) mice, compared with Alum. Anti-A $\beta$  antibodies induced by Alum were predominantly IgG1 type accompanied by lower levels of IgG2a and IgG2b. Quil A induced robust and almost equal titers of anti-A $\beta$  antibodies of IgG1 and IgG2a isotypes and slightly lower levels of IgG2b. Switching adjuvants from Alum to Quil A induced higher concentrations of antibodies than injections with Alum only, however slightly lower than Quil A only. Switching both adjuvants did not change the profile of antibody responses generated by the initial adjuvant injected. These results suggest that switching from Alum to Quil A would be beneficial for AD patients because anti-A $\beta$  antibody production was enhanced without changing the initially generated and likely beneficial Th2-type humoral response.

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**Keywords:** Alzheimer's disease (AD); Epitope vaccine immunization; Th1 and Th2 adjuvants

## 1. Introduction

The cause of Alzheimer's disease (AD) remains unclear, greatly hindering the development of successful treatments for this devastating disease. The neuropathological features

of AD include neurofibrillary tangles (NFT), deposition of  $\beta$ -amyloid (A $\beta$ ) in senile plaques, and neuronal loss in affected brain regions [1]. The A $\beta$  peptide is thought to have a central role in the onset and progression of AD [2,3]. This is supported by results of studies using different transgenic mouse models of AD that express mutated forms of human amyloid precursor protein (APP), from which A $\beta$  is cleaved [4–6]. Such expression of APP induces cerebral deposition of A $\beta$  in the form of monomers, oligomers, protofibrils, and plaques, and mimics different aspects of AD in mice [7–9]. It is believed that accumulation of toxic forms of A $\beta$  deposits

\* Corresponding author at: The Institute for Molecular Medicine, 16371 Gothard Street, H, Huntington Beach, CA 92647-3652, USA.  
Tel.: +1 714 596 7821; fax: +1 714 596 3791.

E-mail address: [magadjanyan@immed.org](mailto:magadjanyan@immed.org) (M.G. Agadjanyan).

<sup>1</sup> Both D.H.C. and M.G.A. are senior authors.

in the brain have a significant, possibly central role in the onset and progression of AD.

Various strategies currently proposed as therapies for AD are aimed at reducing the level of A $\beta$  in the brain or blocking the assembly of the peptide into pathological forms. One potentially powerful strategy for reducing the level of A $\beta$  in the brain is immunotherapy, in which antibodies specific to A $\beta$  facilitate the clearance of amyloid deposits [10–18]. The first human AD vaccine consisted of fibrillar A $\beta$ <sub>42</sub> formulated in QS21 adjuvant (AN-1792 trial) that induced strong Th1-type anti-A $\beta$  immune responses [19], even in Th2-prone BALB/c mice [20]. This AN-1792 clinical trial was halted due to the development of meningoencephalitis in a small proportion of the subjects [21–26], but follow up studies have demonstrated that strong anti-A $\beta$  antibody responses specific to the linear A $\beta$ <sub>1–8</sub> peptide [27] in some patients reduced AD pathology and diminished the cognitive decline associated with the disease [24,27–33]. These data along with preclinical studies demonstrated that anti-A $\beta$  antibodies directed to N-terminal region of A $\beta$ <sub>42</sub> are effective in clearance of amyloid plaques [16,34,35]. On the contrary, the strong autoreactive Th1-type response specific to A $\beta$  is thought to underlie the development of meningoencephalitis observed in the AN-1792 trial [30,31,33]. It is known that Th1-type pro-inflammatory immune responses that are important for the generation of protection against viral infections and cancer are also implicated in autoimmune disorders, whereas Th2-type anti-inflammatory responses have generally been shown to inhibit autoimmune disease. It is important, therefore, that future AD immunotherapy results in the clearing of A $\beta$  peptides by specific anti-A $\beta$  antibodies while limiting the Th1-type immune response [28–33]. To reduce the risk of an adverse T cell-mediated immune response to A $\beta$ -immunotherapy, we developed an epitope vaccine, PADRE-A $\beta$ <sub>1–15</sub>-MAP, and demonstrated that immunizations with this vaccine induced high titers of anti-A $\beta$  antibodies, but not anti-PADRE antibodies. Formulation of our immunogen in the Th2-type adjuvant, Alum, was performed to direct the antibody response toward a Th2 phenotype in an attempt to avoid a strong Th1 humoral response, while maintaining potentially beneficial anti-A $\beta$  antibody response [36]. Alum, however, is much less potent than the majority of Th1-type adjuvants including saponin (QS21) [19,37–40], sometimes as much as 200-fold [41]. Therefore, we suggested that the combined use of both Th1- and Th2-type adjuvants may be more effective and safe immunotherapy for AD.

In the present study, we compared the immunogenicity of our novel prototype epitope vaccine that contains the immunodominant B cell epitope of A $\beta$ <sub>1–15</sub> in tandem with the synthetic universal helper T cell epitope, PADRE (PADRE-A $\beta$ <sub>1–15</sub>-MAP) [36] formulated in Th1-type adjuvant (Quil A) or Th2-type adjuvant (Alum). To identify an acceptable adjuvant formulation for use in future pre-clinical and clinical trials, we tested the use of different combinations of Quil A and Alum in BALB/c and C57BL/6 mice of two different haplotypes H-2<sup>d</sup> and H-2<sup>b</sup>, respectively.

## 2. Materials and methods

### 2.1. Animals

Eight or 10-week-old female C57BL/6 or BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animals were housed in a temperature- and light-cycle-controlled facility, and their care was according to the guidelines of the National Institutes of Health and an approved IACUC protocol at the University of California, Irvine.

### 2.2. Epitope vaccine, peptide and immunizations

A prototype epitope AD vaccine was synthesized exactly as we have described previously [36]. Briefly, the N-terminus of the immunodominant B cell epitope of A $\beta$ <sub>1–15</sub> was synthesized in tandem with a promiscuous foreign T cell epitope, PADRE (aK-Cha-VAAWTLKAAa, where “a” is D alanine and “Cha” is L-cyclohexylalanine) as Multiple Antigenic Peptides (MAP), which contain a core matrix of 4 branching lysines [42,43] to generate PADRE-A $\beta$ <sub>1–15</sub>-MAP molecules (Invitrogen Inc., CA).

Mice were immunized with PADRE-A $\beta$ <sub>1–15</sub>-MAP formulated in Th1- (Quil A) or Th2- (Alum) types of adjuvant as we have described previously [19,36,40]. Quil A (Brenntag Biosector, Denmark) is a mixture of saponins that is prepared from an aqueous extract of *Q. saponaria* bark and contains QS21 as the adjuvant component. Briefly, peptide was re-suspended in DMSO at a concentration of 10 mM, and then diluted in PBS to 500  $\mu$ g/mL of peptide. Before immunization, peptide was mixed with either Alum or Quil A, and each mouse was injected subcutaneously with 50  $\mu$ g of antigen and 1 mg of Alum or 20  $\mu$ g of Quil A. Control groups of mice were immunized with PBS mixed with the appropriate adjuvant. After immunization, two boosts were performed at 2-week intervals. All groups of mice (total six animals per group) were bled on Day 10 after the last injection, and sera were prepared for ELISA. In switching experiments, mice were injected first with immunogen formulated in Quil A (three times biweekly) then with the same immunogen formulated in Alum (three times biweekly) or vice versa (Table 1). Again, on Day 10 after the last boost, animals were bled, and sera were prepared for ELISA.

### 2.3. Detection of anti-A $\beta$ antibodies using ELISA

Plasma from the collected sera was isolated and frozen at  $-20^{\circ}\text{C}$ . Anti-A $\beta$  antibodies were measured in plasma as we described previously [19,40]. The monoclonal antibody A20.1 was used as the standard for determining anti-A $\beta$  antibody concentrations in the sera. Briefly, wells of 96-well plates (Immulon II, Dynatech) were coated with 2.5  $\mu$ M soluble A $\beta$ <sub>42</sub> (pH 9.7, 2 h,  $37^{\circ}\text{C}$ ) followed by three washes. Primary sera from experimental and control mice and A20.1 A $\beta$  MAb were diluted in TBS and added to the

Table 1  
Summary of immunization

Groups	Mouse strain	Number of mice	Immunizations with antigen (Ag) <sup>a</sup>
1	C57BL/6	6	Formulated in Alum (six injections)
2	C57BL/6	6	Formulated in Alum (three injections) plus three injections in Quil A
3	C57BL/6	6	Formulated in Quil A (six injections)
4	C57BL/6	6	Formulated in Quil A (three injections) plus three injections in Alum
5	BALB/c	6	Formulated in Alum (six injections)
6	BALB/c	6	Formulated in Alum (three injections) plus three injections in Quil A
7	BALB/c	6	Formulated in Quil A (six injections)

<sup>a</sup> PADRE-A $\beta$ <sub>1–15</sub>-MAP was used as the Ag.

wells. HRP-conjugated anti-mouse IgG (Jackson Labs, ME) and *o*-phenyldiamine substrate (Sigma, St. Louis, MO) in 0.05 M phosphate–citrate buffer, pH 5.0, were used for detection of bound anti-A $\beta$  antibody. All plates were analyzed spectrophotometrically at 405 nm. To determine the specific isotypes present, sera were pooled, diluted 1:2500, and tested in duplicate. For detection of mouse IgG1, IgG2a, IgG2b, or IgM isotypes, we used anti-mouse Ig-subclass-specific HRP-conjugated secondary antibodies (Zymed, San Francisco, CA). In C57BL/6 mice, however, instead of IgG2a, we tested for IgG2a<sup>b</sup> isotype (*Igh-1b*), also known as IgG2c [40]. To measure the concentration of IgG1, IgG2a/IgG2a<sup>b</sup>, IgG2b, or IgM anti-A $\beta$  antibodies, separate standard curves generated with the appropriate monoclonal antibodies of the same isotype would be required. Since it is technically difficult to perform, we just measured binding of antibodies of different isotypes to wells coated with A $\beta$  peptide (OD<sub>405</sub>) using appropriate secondary antibodies [19,36,40].

#### 2.4. Immunohistochemistry

Mice were sacrificed, and brains were removed, fixed in 4% paraformaldehyde for 24 h at 4 °C, and subsequently cut in serial 50  $\mu$ m-thick sections. Sections were pre-treated with 0.3% H<sub>2</sub>O<sub>2</sub> for 30 min to eliminate endogenous peroxidase activity. The following primary antibodies were used after blocking of sections: CD45 (30-F11), CD11b (M1/70), CD4 (GK1.5), CD8a (53–6.7) (all from BD Pharmingen, San Diego, CA). All antibodies were optimally diluted in 0.1 M Tris, 0.1% Triton X-100 and 2% BSA as recommended by

manufacturer. Bound antibodies were detected using biotinylated anti-hamster (Vector Laboratories, CA) or pre-absorbed biotinylated anti-rat (Jackson Immunoresearch, PA) antibodies followed by incubation with avidin:biotin peroxidase complex (ABC) using the Vectastain Elite kit (Vector Laboratories, CA). Staining reactions were performed with 3,3-diaminobenzidine (DAB, Sigma, MO) according to manufacturers' protocols. For negative control, the primary antibody was omitted from the diluents.

#### 2.5. Statistical analysis

All statistical data (mean, standard deviation, significant differences) were calculated using GraphPad Prism software. Significant differences in antibody production between groups were analyzed by one-way analysis of variance (ANOVA) and Tukey's multiple comparison post-test.

### 3. Results

#### 3.1. Anti-A $\beta$ antibody responses in BALB/c and C57BL/6 mice immunized with PADRE-A $\beta$ <sub>1–15</sub>-MAP formulated in Th1- (Quil A) or Th2- (Alum) type adjuvants

After three injections with immunogen formulated in Alum, the average concentration of anti-A $\beta$  antibody in C57BL/6 mice was higher than that in BALB/c mice (Fig. 1A). An additional three injections dramatically

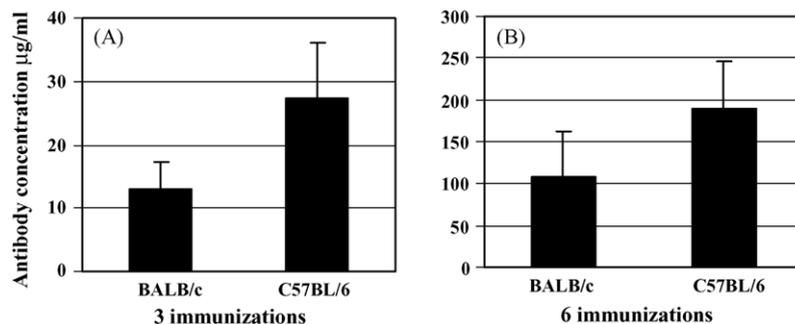


Fig. 1. Epitope vaccine PADRE-A $\beta$ <sub>1–15</sub>-MAP formulated in Alum induced a more robust humoral response in C57BL/6 mice than in BALB/c mice after three (A) or six (B) injections, however, these differences were not statistically significant ( $P > .05$ ).

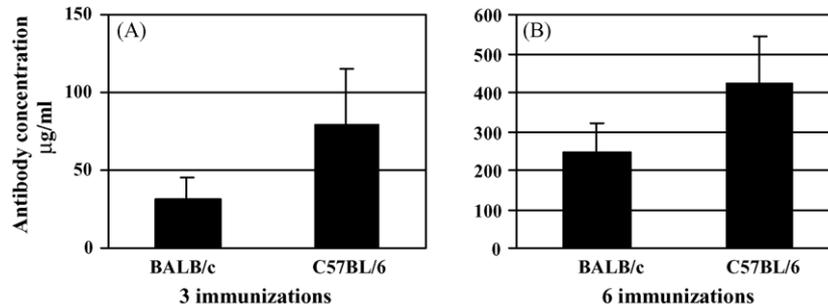


Fig. 2. Epitope vaccine PADRE-A $\beta_{1-15}$ -MAP formulated in the Quil A induced a more robust humoral response in C57BL/6 mice than in BALB/c mice after three (A) ( $P > .05$ ) or six (B) ( $P < .001$ ) injections. After six injections, the anti-A $\beta$  antibody response in both strains of mice vaccinated with PADRE-A $\beta_{1-15}$ -MAP formulated in Quil A was significantly higher than that in mice vaccinated with the same epitope vaccine mixed with Alum (compare Figs. 1 and 2,  $P < .01$  for BALB/c mice,  $P < .001$  for C57BL/6 mice).

enhanced the level of anti-A $\beta$  antibodies in both strains of mice. Boosting was more effective in BALB/c mice, however, total concentrations of anti-A $\beta$  antibodies were higher in C57BL/6 mice (Fig. 1B). Similar results were observed after immunization of these mice with PADRE-A $\beta_{1-15}$ -MAP formulated in Quil A (Fig. 2). Three injections with this vaccine induced higher concentrations of anti-A $\beta$  antibodies in C57BL/6 mice than BALB/c mice, and three additional injections considerably increased the levels in both strains of mice, although this enhancement was more profound in BALB/c mice (Fig. 2). Thus, consistent with previous results with fibrillar A $\beta_{42}$  immunogen [19,40], Quil A was significantly more potent and induced 269 and 425  $\mu\text{g}$  (average concentrations) of anti-A $\beta$  antibodies in BALB/c and C57BL/6 mice, respectively (Figs. 1 and 2,  $P < .01$  for BALB/c and  $P < .001$  for C57BL/6 mice).

### 3.2. Anti-A $\beta$ antibody responses in BALB/c and C57BL/6 mice after switching from Alum to Quil A

We measured concentrations of anti-A $\beta$  antibodies of all isotypes in BALB/c and C57BL/6 mice injected first three times with epitope vaccine formulated in Alum and then three more times with the same immunogen mixed with Quil A. Switching from Alum to Quil A increased the anti-A $\beta$

immune responses generated in BALB/c and C57BL/6 mice (Fig. 3). Both strains of mice previously injected with epitope vaccine plus Alum responded better to three additional injections with PADRE-A $\beta_{1-15}$ -MAP formulated in Quil A, than in Alum. In terms of total immune response, however, in both groups of mice, six injections with epitope vaccine formulated in Quil A were more potent (Fig. 2B versus Fig. 3).

In addition to the magnitude of humoral immune responses we also analyzed the isotype of anti-A $\beta_{42}$  antibodies, since this characteristic has previously been used as an indirect measure of the contribution of Th1 (IgG2a) and Th2 (IgG1) cytokines to the immune response [44–46]. Three to six injections with PADRE-A $\beta_{1-15}$ -MAP epitope vaccine formulated in Alum generated predominantly IgG1 anti-A $\beta$  antibodies in BALB/c mice (Fig. 4A), consistent with previous results [36]. Switching from Alum to Quil A slightly decreased IgG1 production and induced significant increase of IgG2b isotypes. Importantly, expression of IgG2a isotype was negligible. Despite the fact that the IgG1/IgG2a ratio was still above 3 in these mice, after switching, this ratio declined for approximately two times, primarily due to suppression of the IgG1 response (Fig. 4B). In other words, switching from Alum to Quil A decreased the IgG1/IgG2a ratio in Th2-prone BALB/c mice [20].

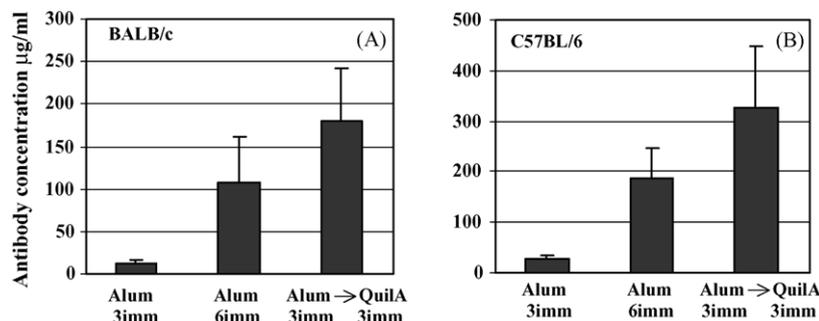


Fig. 3. Switching from epitope vaccine formulated in Alum to that mixed with Quil A after three injections enhanced the immune response in both BALB/c (A) and C57BL/6 (B) mice although the concentrations of anti-A $\beta$  antibodies were lower than that in mice injected six times with epitope vaccine formulated in Quil A (see Fig. 2B). Differences were significant ( $P < .05$ ) in C57BL6 mice, but not in BALB/c mice ( $P > .05$ ).

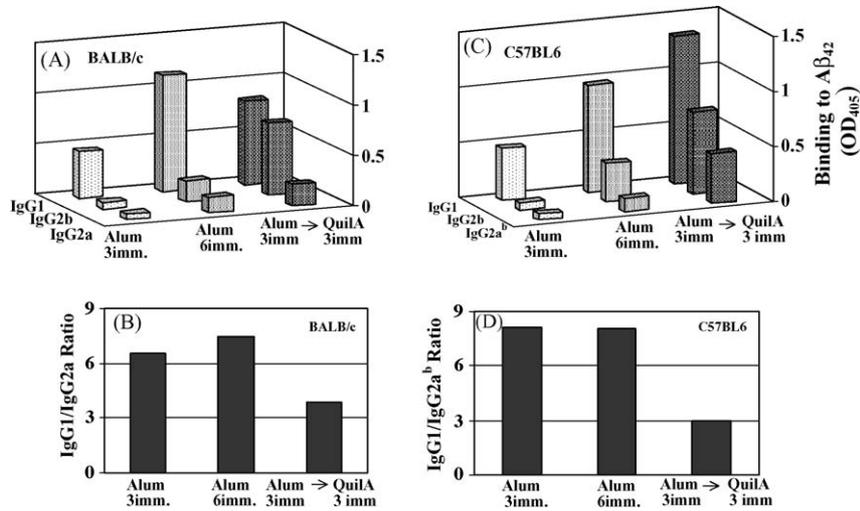


Fig. 4. Switching from Alum to Quil A did not change the pattern of anti-A $\beta$  antibody responses initially generated by the same epitope vaccine formulated in Alum. Isotyping of antibody responses in BALB/c (A) and C57BL/6 (C) mice. IgG1/IgG2a ratios in BALB/c (B) and C57BL/6 (D) mice were calculated based on data presented in A and C.

As expected, immunization of C57BL/6 mice with epitope vaccine formulated in Alum also induced anti-A $\beta$  antibodies predominantly of IgG1 isotype, although we detected low levels of IgG2b antibodies in mice that had received six injections of the vaccine (Fig. 4C). Switching from Alum to Quil A enhanced production of IgG1, IgG2b, and IgG2a<sup>b</sup> isotypes, and decreased the ratio of IgG1/IgG2a<sup>b</sup> by more than 2.5 times (Fig. 4C and D). Thus, results from BALB/c and C57BL/6 mice were similar and indicated that switching from Alum to Quil A induced strong Th2-type anti-A $\beta$  antibody production without changing the pattern of antibody responses initiated by pre-immunizations of the same mice with epitope vaccine formulated in Alum.

3.3. Anti-A $\beta$  antibody responses in C57BL/6 mice after switching from Quil A to Alum

C57BL/6 mice pre-vaccinated with PADRE-A $\beta$ <sub>1-15</sub>-MAP formulated in Quil A and then given three injections with the same immunogen mixed with Alum exhibited signif-

icantly enhanced concentrations (double) of anti-A $\beta$  antibodies compared with mice given three injections of vaccine formulated in Quil A only (Fig. 5A). However, when we did not switch from Quil A to Alum and continue immunizations with PADRE-A $\beta$ <sub>1-15</sub>-MAP formulated in Quil A, mice produced almost two times higher concentrations of anti-A $\beta$  antibodies than that induced after additional injections with immunogen plus Alum (Fig. 5A). In fact PADRE-A $\beta$ <sub>1-15</sub>-MAP formulated in Quil A was the formulation of vaccine that induced the highest concentration of antibodies specific to amyloid (Figs. 2 and 5A).

To detect the influence of switching adjuvants on Th1- and Th2-types of humoral immune responses, we analyzed IgG isotypes of antibodies. The epitope vaccine PADRE-A $\beta$ <sub>1-15</sub>-MAP formulated in Quil A generated anti-A $\beta$  antibodies of IgG1, IgG2b, and IgG2a<sup>b</sup> isotypes (Fig. 5B). After three to six injections, the IgG1/IgG2a<sup>b</sup> ratio was 0.9–1.1 (Fig. 5C), indicating predominantly Th1-type humoral responses. Switching from Quil A to Alum did not significantly change the profile of anti-A $\beta$  antibody isotypes or the IgG1/IgG2a<sup>b</sup> ratio (Fig. 5B and C).

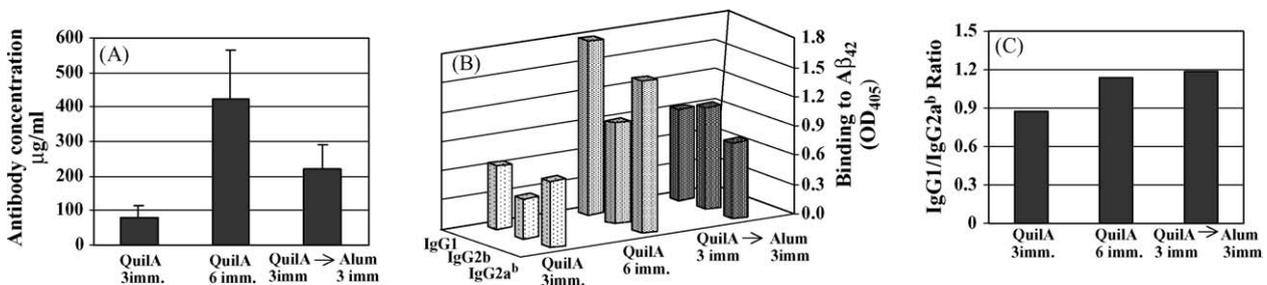


Fig. 5. Switching from Quil A to Alum lowered the magnitude of anti-A $\beta$  antibody response compared with using Quil A alone in C57BL/6 mice (A) ( $P > .05$ ). Switching from Quil A to Alum did not change the isotype profile of these antibodies (B, C).

### 3.4. Epitope vaccine formulated in Th1 or Th2 adjuvants did not induce inflammation in the brains of immunized mice

To examine inflammation-related pathology in the brains of animals immunized with epitope vaccine formulated in Alum or Quil A, we conducted immunohistochemical analysis. To identify microglia activation as well as leukocyte infiltration and accumulation in the brain, we analyzed expression of CD45, CD11b, CD4, and CD8 molecules. The immunostaining in sections from the frontoparietal cortex and hippocampus revealed no observable differences between non-immunized and immunized mice. In other words, we had not observed the lymphocyte infiltration or microglia activation in the CNS of both BALB/c and C57BL/6 mice. In addition, in order to investigate toxicity and potential negative effects of vaccination, we examined Glial Fibrillary Acidic Protein (GFAP) and had not found astrogliosis.

## 4. Discussion

Despite the adverse events (meningoencephalitis) in the AN-1792 clinical trial, follow up studies demonstrated that strong anti-A $\beta$  antibody responses, rather than autoimmune T cell responses generated in AD patients, were capable of reducing AD pathology and diminishing the progressive cognitive decline associated with the disease [24,28–33]. More specifically, published data from AN-1792 clinical trials suggest that the aseptic meningoencephalitis may have been caused by a T cell-mediated autoimmune response, whereas production of high titers of anti-A $\beta$  antibodies may have been therapeutic to the AD patients. Recently, in order to reduce the risk of an adverse T cell-mediated immune response to A $\beta$ -immunotherapy, we developed epitope vaccine PADRE-A $\beta$ <sub>1–15</sub>-MAP and demonstrated that immunizations with this vaccine induced high titers of anti-A $\beta$ , but not anti-PADRE antibodies in Th2-prone BALB/c mice. On the contrary, splenocytes from immunized mice showed robust T cell stimulation *in vitro* in response to PADRE, but not to A $\beta$  peptides. In these experiments in addition to replacing of the self A $\beta$  T cell epitope with a foreign T cell epitope PADRE, we formulated immunogen in a Th2-type adjuvant, Alum in order to bias antibody response toward a Th2 phenotype [36]. Accordingly, we demonstrated that our epitope vaccine induced strong Th2-type of immune responses in Th2-prone BALB/c mice [36]. The results of the present study demonstrate that either a Th1-type adjuvant (Quil A) or a Th2-type adjuvant (Alum) incorporated in our epitope vaccine formulation was effective in generating of high titers of anti-A $\beta$  antibodies in both BALB/c and C57BL/6 mice (Figs. 1 and 2). Quil A was the most effective adjuvant in both strains of mice tested (Fig. 2). Using a combination of the two adjuvants was also effective, switching from vaccine formulated with Quil A to Alum (or vice versa) midway through six injections

(Fig. 3). Switching from Alum to Quil A produced an anti-A $\beta$  antibody response that was almost as strong as that produced by Quil A alone. More importantly, switching adjuvants did not change the outcome of the antibody responses generated by the adjuvant used initially. The presence of IgG1 antibody is indicative of a Th2-type immune response, whereas IgG2a is indicative of a Th1-type immune response [44–48]. The results of the present study show that, in both strains of mice, switching from Alum to Quil A did not change the Th2 phenotype of antibody responses, although it reduced the IgG1/IgG2a ratio (Fig. 4). Likewise, switching from Quil A to Alum did not change the outcome of Th1 phenotype of antibodies initially generated (Fig. 5). As mentioned above, recent analysis of data from the AN-1792 clinical trial has produced encouraging results including demonstration of a reduction in plaque load and attenuation of cognitive decline in vaccinated AD patients who produced high titers of anti-A $\beta$  antibodies [28,29,33]. On the other hand, Th1, but not Th2 type of immune response in vaccinated AD patients has been implicated in cell-mediated autoimmune response resulted in meningoencephalitis. It is important, therefore, that future AD immunotherapy results in the clearance of A $\beta$  peptides by specific anti-A $\beta$  antibodies while limiting the Th1-type immune response [28–33]. The results of the present study suggest that first using a Th2-type adjuvant (Alum) and then switching to the Th1-type saponin adjuvant (QS21) used in the AN-1792 clinical trial would be beneficial, because this strategy induced the levels of anti-A $\beta$  antibodies comparable with those achieved using the Th1-type adjuvant Quil A alone, without changing the potentially beneficial Th2-type response initiated by Alum into a pro-inflammatory Th1-type response. In other words, switching from a Th2-type adjuvant to a Th1-type adjuvant enhanced the therapeutically relevant anti-A $\beta$  antibody production without inducing the potentially harmful Th1 immune response. The data of the present study may help to develop novel immunogen-adjuvant configurations with the potential to avoid the adverse immune response observed in the first clinical trial, while maintaining the therapeutic value of the production of anti-A $\beta$  antibodies in reducing AD pathology and improving cognitive function in AD patients.

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