

# Prototype Alzheimer's Disease Vaccine Using the Immunodominant B Cell Epitope from $\beta$ -Amyloid and Promiscuous T Cell Epitope Pan HLA DR-Binding Peptide<sup>1</sup>

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**Immunization of amyloid precursor protein transgenic mice with fibrillar  $\beta$ -amyloid ( $A\beta$ ) prevents Alzheimer's disease (AD)-like neuropathology. The first immunotherapy clinical trial used fibrillar  $A\beta$ , containing the B and T cell self epitopes of  $A\beta$ , as the immunogen formulated with QS21 as the adjuvant in the vaccine. Unfortunately, the clinical trial was halted during the phase II stage when 6% of the participants developed meningoencephalitis. The cause of the meningoencephalitis in the patients that received the vaccine has not been definitively determined; however, analysis of two case reports from the AN-1792 vaccine trial suggest that the meningoencephalitis may have been caused by a T cell-mediated autoimmune response, whereas production of anti- $A\beta$  Abs may have been therapeutic to the AD patients. Therefore, to reduce the risk of an adverse T cell-mediated immune response to  $A\beta$  immunotherapy we have designed a prototype epitope vaccine that contains the immunodominant B cell epitope of  $A\beta$  in tandem with the synthetic universal Th cell pan HLA DR epitope, pan HLA DR-binding peptide (PADRE). Importantly, the PADRE- $A\beta_{1-15}$  sequence lacks the T cell epitope of  $A\beta$ . Immunization of BALB/c mice with the PADRE- $A\beta_{1-15}$  epitope vaccine produced high titers of anti- $A\beta$  Abs. Splenocytes from immunized mice showed robust T cell stimulation in response to peptides containing PADRE. However, splenocytes from immunized mice were not reactivated by the  $A\beta$  peptide. New preclinical trials in amyloid precursor protein transgenic mouse models may help to develop novel immunogen-adjuvant configurations with the potential to avoid the adverse events that occurred in the first clinical trial. *The Journal of Immunology*, 2005, 174: 1580–1586.**

Alzheimer's disease (AD)<sup>4</sup> is the most common form of dementia in the elderly and is characterized by a progressive loss of memory and a general cognitive decline. The neuropathological features of the disease include neurofibrillary tangles, deposition of  $\beta$ -amyloid ( $A\beta$ ) in senile plaques, and neuronal loss in affected brain regions (1). The  $A\beta$  peptide is cleaved from the amyloid precursor protein (APP) by  $\beta$ - and  $\gamma$ -secretases (2–4) and is believed to play an important role in the onset and progression of AD (5, 6). Many strategies currently being 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 (7).

One potentially powerful strategy for reducing the level of  $A\beta$  in the brain is immunotherapy, where  $A\beta$ -specific Abs facilitate

the clearance of  $A\beta$ . Active immunization of APP transgenic mice (APP/Tg) with fibrillar  $A\beta$  peptide blocked the deposition of  $A\beta$  in plaques, prevented the development of dystrophic neurites, and reduced astrogliosis in the mouse brain (8, 9). In addition, when older mice with established  $A\beta$  deposits were immunized with  $A\beta$ , they were able to clear  $A\beta$  plaques from the brain. Other researchers have reported that active immunization protected mice from developing functional memory deficits (10–12), and that passive administration of anti- $A\beta$  mAbs to APP/Tg mice also reduced  $A\beta$  levels in the brain (13, 14) and reversed memory deficits (15). These results suggest that the generation of Abs against  $A\beta$  in humans might provide similar benefits to patients with AD. There were no reported adverse inflammatory events after  $A\beta$  immunotherapy in several animal models, including rabbits, guinea pigs, and monkeys. The only documented adverse incidence to  $A\beta$  immunotherapy was an increase in cerebral hemorrhages in very old, hemorrhage-prone, APP/Tg-23 mice injected with multiple high doses of anti- $A\beta$  mAb (16).

Based on the impressive preclinical results, Elan Corp. in collaboration with Wyeth-Ayerst began the first  $A\beta$  immunotherapy clinical trial with their AN-1792 vaccine, a mixture of fibrillar  $A\beta_{42}$  and the saponin adjuvant QS-21, on AD patients. Unfortunately, the phase IIa trial was halted when ~6% of the participants in the clinical trial developed aseptic meningoencephalitis (9, 17–20). The cause of the meningoencephalitis in a subset of the patients has not been definitively determined. However, postmortem examination of brains from two patients who suffered an adverse event to the vaccine revealed the presence of T lymphocyte infiltration in the leptomeninges, cerebrovasculature, and cerebral neocortex (21, 22). This has provided support for the theory that the adverse response to the vaccine was due to a T cell-mediated autoimmune response (9, 21–23).

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<sup>4</sup> Abbreviations used in this paper: AD, Alzheimer's disease;  $A\beta$ ,  $\beta$ -amyloid; APP, amyloid precursor protein; APP/Tg, APP transgenic mice; PADRE, Pan HLA DR-binding peptide;  $A\beta_{42}$ ,  $A\beta$  peptides spanning aa 1–42, 1–40 ( $A\beta_{40}$ ), 1–33 ( $A\beta_{1-33}$ ), 1–15 ( $A\beta_{1-15}$ ), and 20–31 ( $A\beta_{20-31}$ ); MAP, multiple antigenic peptides; PADRE- $A\beta_{1-15}$ -MAP;  $A\beta_{1-33}$ -MAP;  $A\beta_{1-15}$ -MAP;  $A\beta_{20-31}$ -MAP, peptides attached to PADRE and/or MAP.

The development of a safe and effective AD vaccine will require a delicate balance between providing specific and adequate anti-A $\beta$  Ab responses sufficient for therapeutic benefit while eliminating adverse T cell-mediated autoimmune responses. To reduce the risk of an adverse T cell-mediated immune response to A $\beta$  immunotherapy we have designed a prototype vaccine that will target the immunogenic B cell epitope of A $\beta$  that is critical for clearance of A $\beta$  plaques, but that will not stimulate anti-A $\beta$  T cells. We have engineered an epitope vaccine composed of the B cell epitope from the immunodominant region of A $\beta_{42}$ , A $\beta_{1-15}$ , in tandem with a universal synthetic T cell epitope, pan HLA DR-binding peptide (PADRE), which consists of a pan HLA DR-binding epitope. We have demonstrated that immunization of BALB/c mice with this epitope vaccine (PADRE-A $\beta_{1-15}$ ), synthesized as a multiple antigenic peptide (MAP) and formulated in a Th2 type adjuvant (Alum), generated anti-PADRE Th cells (CD4<sup>+</sup>T1/ST2<sup>+</sup>) that support the development of potentially therapeutic anti-A $\beta$  Abs.

## Materials and Methods

### Mice

Eight- or 10-wk-old female BALB/c mice were purchased from The Jackson Laboratory. All animals were housed in a temperature- and light cycle-controlled facility, and their care was under the guidelines of the National Institutes of Health and an approved institutional animal care and use committee protocol at University of California (Irvine, CA).

### Epitope vaccine, peptide immunogens, and immunizations

To prepare a prototype epitope AD vaccine, we synthesized the N terminus of the immunodominant B cell epitope of A $\beta_{1-15}$  (24–35) in tandem with a promiscuous foreign T cell epitope, PADRE. PADRE (aK-Cha-VAAW TLKAAa, where a is D alanine, and Cha is L-cyclohexylalanine) is a small 13-mer nonnatural pan HLA DR-binding sequence that is a potent T cell epitope (36–43). In addition, we generated A $\beta$  peptides spanning aa 1–42 (A $\beta_{42}$ ), 1–40 (A $\beta_{40}$ ), 1–33 (A $\beta_{1-33}$ ), 1–15 (A $\beta_{1-15}$ ), and 20–31 (A $\beta_{20-31}$ ). All linear peptides were synthesized by Multiple Peptide Systems. In addition, peptides PADRE-A $\beta_{1-15}$ , A $\beta_{1-33}$ , A $\beta_{1-15}$ , and A $\beta_{20-31}$  were synthesized as multiple antigenic peptides (MAPs), which contain a core matrix of four branching lysines (44, 45), to generate PADRE-A $\beta_{1-15}$ -MAP, A $\beta_{1-33}$ -MAP, A $\beta_{1-15}$ -MAP, and A $\beta_{20-31}$ -MAP molecules (Invitrogen Life Technologies).

Mice were immunized with PADRE-A $\beta_{1-15}$ -MAP, A $\beta_{1-33}$ -MAP, A $\beta_{1-15}$ -MAP, or A $\beta_{42}$  as previously described (32, 33). Briefly, each peptide was resuspended in DMSO at a concentration of 10 mM, then diluted in PBS to obtain 500  $\mu$ g/ml peptide. Before immunizations, each peptide was mixed with alum, a Th2 type conventional adjuvant, and 50  $\mu$ g of Ag was injected s.c. into the mouse. Experimental mice were immunized with PADRE-A $\beta_{1-15}$ -MAP ( $n = 9$ ) or A $\beta_{1-33}$ -MAP ( $n = 9$ ), whereas control groups of BALB/c mice were immunized with linear A $\beta_{1-33}$  ( $n = 9$ ), A $\beta_{1-15}$  ( $n = 8$ ), or fibrillar A $\beta_{42}$  ( $n = 4$ ) formulated in alum. All animals were boosted five times at 3-wk intervals, and sera were collected 8–9 days after each boost.

### Detection of anti-A $\beta$ Abs by ELISA

Total anti-A $\beta_{42}$  Abs were detected as described previously (32, 33). Briefly, wells of 96-well plates (Immulon II; Dynatech Laboratories) were coated with 2.5  $\mu$ M soluble A $\beta_{42}$  (pH 9.7, 2 h, 37°C). The wells were washed and blocked, and primary sera from experimental and control mice were added to the wells at the indicated dilutions. After incubation and washing, HRP-conjugated anti-mouse IgG was added as recommended by the manufacturer (Jackson ImmunoResearch Laboratories). Plates were incubated and washed, and the *o*-phenyldiamine substrate (Sigma-Aldrich) in 0.05 M phosphate-citrate buffer, pH 5.0, was added to develop the reaction. All plates were analyzed spectrophotometrically at 405 nm. To determine the specific isotypes, pooled sera from mice were diluted 1/500 and tested in duplicate. For detection of mouse IgG1, IgG2a, IgG2b, or IgM isotypes, we used anti-mouse Ig subclass-specific, HRP-conjugated secondary Abs (Zymed Laboratories).

### Detection of A $\beta$ plaques in human brain tissues

Sera from immunized mice were screened for the ability to bind to A $\beta$  plaques in the human brain as previously described (46). Briefly, pooled sera were added to serial 50- $\mu$ m brain sections of formalin-fixed, frontal

cortical tissue from a case with neuropathological and behavioral patterns typical of severe AD. Sections were pretreated with 90% formic acid, and exogenous peroxidases were quenched. Antisera were tested at a dilution of 1/1000. As a negative control, we used the same dilutions of preimmune sera. As a positive control, anti-human A $\beta$  mAb 6E10 (dilution 1/1500) was used to immunostain plaques in AD brain sections. Binding of Abs to the brain sections was determined via the Vectastain Elite ABC mouse IgG/3,3'-diaminobenzidine substrate biotin-avidin system (both kits from Vector Laboratories), according to the manufacturer's recommendations. A digital camera (Olympus) was used to capture images of the plaques at  $\times 20$  magnification. The binding of antisera (dilution 1/1000) to the  $\beta$ -amyloid plaques was blocked by preabsorption of the sera with 5  $\mu$ M A $\beta_{1-15}$  peptide (1 h, 37°C).

### T cell proliferation

Analyses of T cell proliferation were performed in splenocyte cultures from individual animals as we previously described (32). However, to decrease nonspecific activation of splenocytes, we used HL-1 serum-free synthetic medium (Cambrex) without FBS. Splenocytes from experimental and control mice ( $5 \times 10^5$  in 100  $\mu$ l) were restimulated in vitro with different peptides at a concentration of 10  $\mu$ g/ml. Cells were first incubated for 72 h, then 1  $\mu$ Ci of [<sup>3</sup>H]thymidine (Amersham Biosciences) was added to each well for 16–18 h. Cells were harvested using a Tomtec Mach III harvester, and [<sup>3</sup>H]thymidine uptake (cpm) was counted on a MicroBeta 1450 Trilux scintillation counter (Wallac Oy). The stimulation index was calculated as previously described (47).

### Production of cytokines by immune splenocytes

The same splenocytes used for T cell proliferation were used for detection of Th1 (IFN- $\gamma$ ) or Th2 (IL-4) lymphokines as well as for TNF- $\alpha$  using the ELISPOT technique. Experiments were conducted as recommended by the manufacturer (BD Pharmingen) and as previously described (32). The colored spots were counted, and the differences between stimulated and non-stimulated conditions for each experiment were analyzed statistically as described below.

### Detection of CD4<sup>+</sup> T cells expressing IL-18R (Th1) or T1/ST2 (Th2) molecules

Spleens from mice immunized with different immunogens or control animals were depleted of CD8<sup>+</sup> cells using MACS depletion kit (Miltenyi Biotec), and the remaining splenocytes were restimulated in HL-1 medium with the same peptide that was used for in vivo immunization. On day 0 (baseline) and after 7 days of stimulation, CD4<sup>+</sup> T cells were analyzed for the expression of IL-18R or T1/ST2-selective surface markers using a FACScan flow cytometer (BD Biosciences). The following Abs were used: FITC-labeled anti-mouse T1/ST2 (BD Biosciences), anti-mouse IL-18R $\beta$  followed by staining with PE-labeled goat anti-rat Ab (BD Pharmingen), and PerCP-labeled anti-mouse CD4 mAb (BD Pharmingen). Data were presented as the percentage of CD4<sup>+</sup> T cells expressing the appropriate marker Ag after specific activation with the indicated peptide minus background activation using an irrelevant peptide.

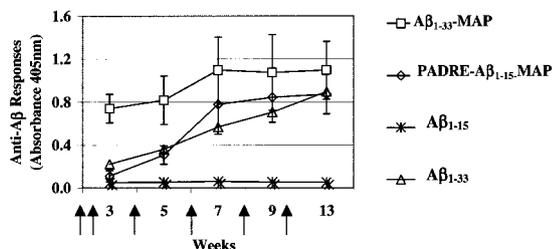
### Statistical analysis

The data for Ab production between groups were analyzed by one-way ANOVA. The results of ELISPOT were examined for differences between groups immunized and restimulated with the indicated peptides using one-way ANOVA and Tukey's pairs comparison post-test (PRISM 3.03; GraphPad).

## Results

### PADRE-A $\beta_{1-15}$ epitope vaccine induces high titers of anti-A $\beta$ Abs

Differences in the anti-A $\beta$  titers produced in response to PADRE-A $\beta_{1-15}$ -MAP, A $\beta_{1-33}$ -MAP, A $\beta_{1-15}$ , and A $\beta_{42}$  immunogens were analyzed in sera from immunized animals after each boost. Surprisingly, the best immunogen initially was A $\beta_{1-33}$ -MAP, which contains both the B and T cell epitopes of A $\beta_{42}$  (Fig. 1). As expected, linear A $\beta_{1-15}$  was not immunogenic because it lacks a T cell epitope for mice of the H-2<sup>d</sup> haplotype (32). The profile of Ab production in the group of mice immunized with PADRE-A $\beta_{1-15}$ -MAP epitope vaccine was similar to that of mice immunized with linear A $\beta_{1-33}$  peptide (Fig. 1). After the second boost, all mice from these two groups generated significant titers of anti-A $\beta$  Abs,

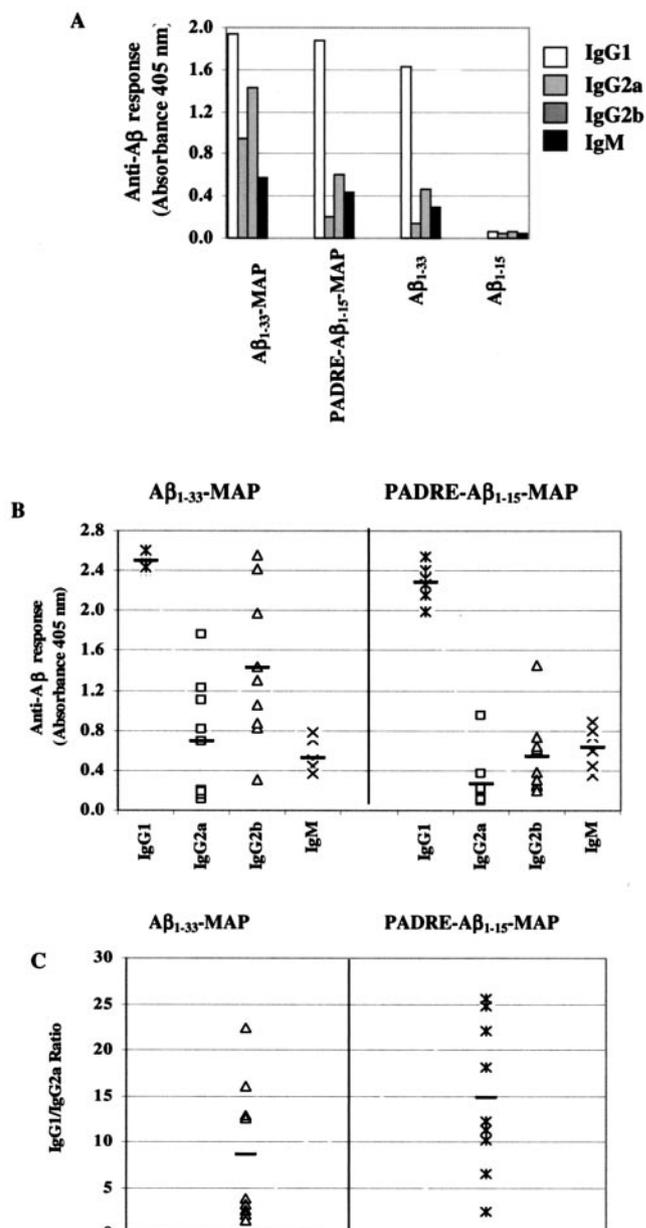


**FIGURE 1.** Epitope vaccine composed of B cell antigenic determinant ( $A\beta_{1-15}$ ) and T cell antigenic determinant (PADRE)-induced potent anti- $A\beta$  Abs in BALB/c mice of H2<sup>d</sup> haplotype. The arrows indicate the times of immunization/boosting of the mice. Three groups of mice ( $n = 9$ /group) immunized with  $A\beta_{1-33}$ -MAP, PADRE- $A\beta_{1-15}$ -MAP, and  $A\beta_{1-33}$  induced anti- $A\beta$  Abs. At 13 wk, the range of concentrations was 154–311  $\mu\text{g/ml}$  (average titer, 1/32,000 for PADRE- $A\beta_{1-15}$ -MAP or  $A\beta_{1-33}$ , and 1/64,000 for  $A\beta_{1-33}$ -MAP). The  $A\beta_{1-33}$ -MAP induces significantly higher anti- $A\beta$  Ab production at wk 3, 5, and 7 compared with PADRE- $A\beta_{1-15}$ -MAP and  $A\beta_{1-33}$  ( $p < 0.001$  to  $p < 0.05$ ). At wk 9, the data are significant only between groups of mice immunized with  $A\beta_{1-33}$ -MAP and  $A\beta_{1-33}$  ( $p < 0.05$ ), whereas at wk 13, no significant differences among these three groups were observed. Mice immunized with  $A\beta_{1-15}$  ( $n = 8$ ) did not induce anti- $A\beta$  Abs, and sera from all immunized animals did not recognize wells of ELISA plates coated with PADRE (OD,  $0.046 \pm 0.003$ ).

which continued to rise after each boost and eventually reached the level of Abs in the group injected with  $A\beta_{1-33}$ -MAP ( $p > 0.05$ ). The Abs generated by PADRE- $A\beta_{1-15}$ -MAP epitope vaccine were specific only to  $A\beta$ , because they did not bind to PADRE or the MAP backbone (data not shown). Thus, the T cell epitope PADRE (48) promoted the production of Abs against the  $A\beta_{1-15}$  B cell antigenic determinant of  $A\beta$  peptide.

#### *Ab isotypes produced in response to immunization with $A\beta_{1-33}$ , $A\beta_{1-33}$ -MAP, and PADRE- $A\beta_{1-15}$ -MAP*

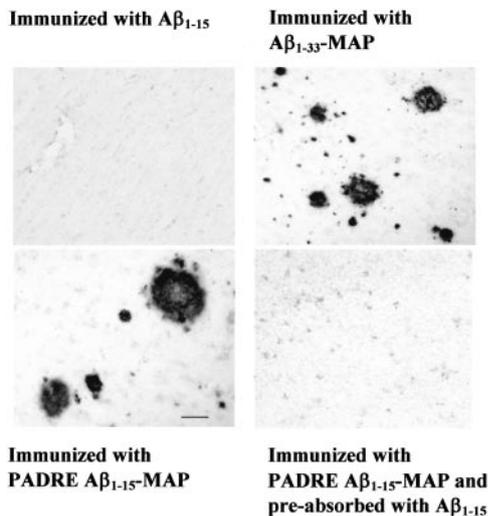
We measured the production of IgG1, IgG2a, IgG2b, and IgM anti- $A\beta$  Abs in our experiments using pooled sera and sera collected from individual animals at wk 13 after a total of six injections of vaccine. The data obtained with pooled sera demonstrated that immunization with  $A\beta_{1-33}$ -MAP induced a broad spectrum of anti- $A\beta$  Ab isotypes. More specifically,  $A\beta_{1-33}$ -MAP generated high levels of IgG1, IgG2a, and IgG2b and moderate amounts of IgM Abs (Fig. 2A). However, mice from groups immunized with  $A\beta_{1-33}$  and PADRE- $A\beta_{1-15}$ -MAP induced anti- $A\beta$  Abs predominantly of the IgG1 isotype. To investigate variability within groups, we analyzed the IgG1, IgG2a, IgG2b, and IgM isotypes of anti- $A\beta$  Abs generated in individual mice immunized with  $A\beta_{1-33}$ -MAP or PADRE- $A\beta_{1-15}$ -MAP (Fig. 2, B and C). Interestingly, in mice immunized with  $A\beta_{1-33}$ -MAP, there were five mice within the group that generated significant levels of IgG2a Abs (Fig. 2, B and C). Only one mouse in the PADRE- $A\beta_{1-15}$ -MAP group produced significant levels of IgG2a Abs. The subclass of IgG that is induced after immunization can be used as an indirect measure of the relative contributions of Th2 cytokines vs Th1 cytokines to the immune response (49). In mice, the production of IgG1 Ab is primarily induced by Th2 cytokines, whereas the production of IgG2a Ab reflects the involvement of Th1 cytokines. Therefore, we determined the IgG1/IgG2a ratios for  $A\beta_{1-33}$ -MAP and PADRE- $A\beta_{1-15}$ -MAP. The IgG1/IgG2a ratio for PADRE- $A\beta_{1-15}$ -MAP was  $\sim 2$  times greater than that for  $A\beta_{1-33}$ -MAP (Fig. 2C). These data suggest that our PADRE- $A\beta_{1-15}$ -MAP epitope vaccine formulated in alum induced a highly Th2-polarized immune responses in H-2<sup>d</sup> mice.



**FIGURE 2.** Detection of IgG1, IgG2a, IgG2b, and IgM subclasses of anti- $A\beta$  Abs. A, Isotypes in pooled sera from immunized mice. B, Isotypes from sera collected from individual mice immunized with  $A\beta_{1-33}$ -MAP or PADRE- $A\beta_{1-15}$ -MAP. All sera were collected after the last boost at wk 13 and used in ELISA at a dilution 1/500. C, IgG1/IgG2a ratios were calculated based on the data presented in B.

#### *Potential therapeutic efficacy of anti- $A\beta$ Abs generated in response to PADRE- $A\beta_{1-15}$ epitope vaccine*

To demonstrate the potential therapeutic efficacy of anti- $A\beta$  Abs generated in mice immunized with our PADRE- $A\beta_{1-15}$ -MAP epitope vaccine, we analyzed binding of antisera to  $A\beta$  plaques in brain tissue from an AD case. The results demonstrate that the antisera from mice immunized with  $A\beta_{1-33}$ -MAP and PADRE- $A\beta_{1-15}$ -MAP bound equally well to  $A\beta$  plaques even at a dilution of 1/1000, the end point dilution used in these experiments, which was similar in intensity to the immunostaining with mAb 6E10 (dilution 1/1500). Sera collected from mice immunized with linear  $A\beta_{1-15}$  did not bind to plaques (Fig. 3). These data suggest that anti- $A\beta$  Abs raised in mice immunized with the PADRE- $A\beta_{1-15}$ -MAP



**FIGURE 3.** Linear peptide  $A\beta_{1-15}$  did not generate anti- $A\beta$  Abs specific to amyloid plaques. On the contrary, antisera generated against PADRE- $A\beta_{1-15}$ -MAP epitope vaccine as well as  $A\beta_{1-33}$ -MAP at a dilution 1/1000 bound to amyloid plaques on the brain section of cortical tissues from AD case. The binding of antisera collected from mice immunized with PADRE- $A\beta_{1-15}$ -MAP to the  $\beta$ -amyloid plaques was specific, because it was blocked by preabsorption of the sera with  $A\beta_{1-15}$  peptide (5  $\mu$ M). Original magnification,  $\times 20$ ; scale bar, 50  $\mu$ m.

epitope vaccine are potentially therapeutic. In addition, affinity-purified anti- $A\beta$  Abs from mice immunized with the epitope vaccine blocked the assembly of  $A\beta_{42}$  into fibrils in vitro (data not shown).

#### *PADRE- and $A\beta$ -specific T cell responses in splenocytes from immunized mice*

One challenge associated with the clinical use of the  $A\beta$  self T cell epitope as part of a vaccine to treat AD patients is the potential for the development of unwanted anti- $A\beta$  or anti-APP Th1 immune responses (20–22, 32). Thus, an important test of our PADRE- $A\beta_{1-15}$ -MAP epitope vaccine was to demonstrate that T cell responses against PADRE- $A\beta_{1-15}$ -MAP immunogen were directed against PADRE and not  $A\beta$ . Immunization with fibrillar  $A\beta_{42}$  formulated in alum induced robust in vitro T cell proliferation after restimulation of the cultures with  $A\beta_{40}$  (Fig. 4C). Splenocytes from mice immunized with monomeric  $A\beta_{1-33}$  also induced T cell proliferation in vitro after restimulation with  $A\beta_{40}$  peptide. As

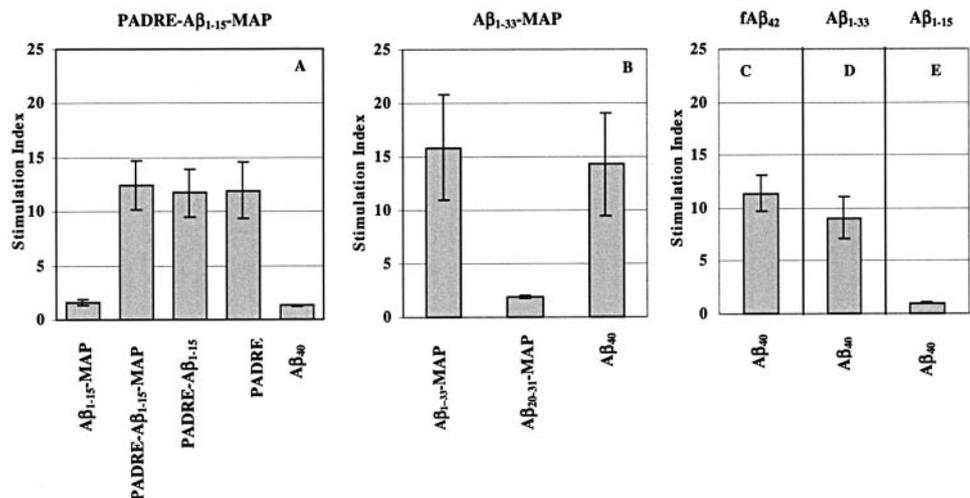
expected, splenocytes from mice immunized with monomeric  $A\beta_{1-15}$  did not induce anti- $A\beta$ -specific T cell proliferation in response to  $A\beta_{40}$  peptide. Interestingly, both  $A\beta_{40}$  and  $A\beta_{1-33}$ -MAP Ags were equally potent in anti- $A\beta$  T cell activation, whereas  $A\beta_{20-31}$ -MAP did not induce T cell proliferation. These data demonstrate that  $A\beta_{1-33}$ , but not  $A\beta_{20-31}$ , possesses the T cell epitope of  $A\beta$ . Importantly, splenocytes isolated from mice immunized with PADRE- $A\beta_{1-15}$ -MAP induced equally strong T cell proliferation after stimulation with PADRE, PADRE- $A\beta_{1-15}$ , or PADRE- $A\beta_{1-15}$ -MAP, but not with  $A\beta_{40}$  or  $A\beta_{1-15}$ -MAP (Fig. 4A). These results clearly demonstrate that PADRE, but not  $A\beta$  or MAP peptides, provides the Th lymphocyte support necessary for a strong anti- $A\beta$  Ab response to the PADRE- $A\beta_{1-15}$ -MAP epitope vaccine.

#### *Peptide-induced lymphokine responses in splenocytes from immunized mice*

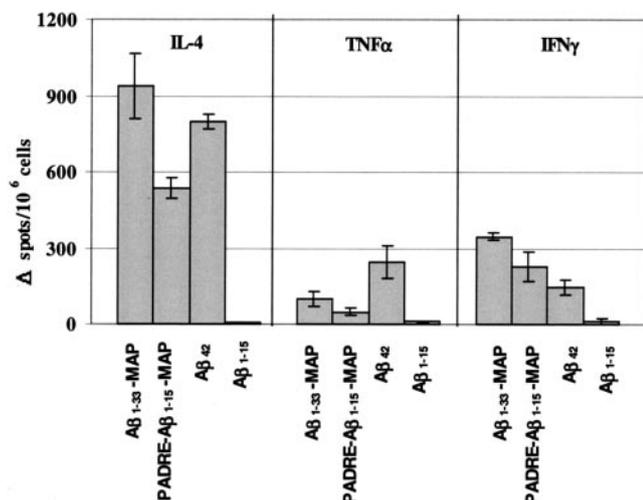
We analyzed the production of Th1 (IFN- $\gamma$ ) and Th2 (IL-4) lymphokines and TNF- $\alpha$ , a proinflammatory cytokine, in splenocyte cultures from immunized and control mice. Groups of mice injected with  $A\beta_{1-33}$ -MAP induced the highest IL-4 response, whereas mice immunized with  $A\beta_{42}$  and PADRE- $A\beta_{1-15}$ -MAP had intermediate and low responses ( $p > 0.05$  and  $p > 0.001$ ), respectively.  $A\beta_{1-33}$ -MAP induced the highest IFN- $\gamma$  response based on the number of cells producing this lymphokine, which was significantly higher than those in the other two groups ( $p < 0.001$ ). On the contrary, mice immunized with  $A\beta_{42}$  generated a significantly higher number of cells producing the proinflammatory cytokine TNF- $\alpha$  than mice immunized with  $A\beta_{1-33}$  MAP or PADRE- $A\beta_{1-15}$ -MAP ( $p < 0.05$  and  $p < 0.001$ ). As expected, splenocytes from mice immunized with nonimmunogenic  $A\beta_{1-15}$  peptide did not generate IL4, IFN- $\gamma$ , or TNF- $\alpha$  cytokines (Fig. 5).

#### *Expression of Th1 (IL-18R) and Th2 (T1/ST2)-specific markers on CD4<sup>+</sup> T cells from immunized mice*

To further characterize the contributions of Th1 and Th2 CD4<sup>+</sup> T cells to the immune response in immunized BALB/c mice, we analyzed the percentage of CD4<sup>+</sup> T cells expressing either Th1 (IL-18R) or Th2 (T1/ST2)-specific markers. Without in vitro activation, we did not detect a significant number of CD4<sup>+</sup> T cells expressing IL-18R or T1/ST2 molecules. However, restimulation of splenocytes from mice immunized with  $A\beta_{1-33}$ -MAP or PADRE- $A\beta_{1-15}$ -MAP generated 18–19% CD4<sup>+</sup> T cells that



**FIGURE 4.** T cell proliferation in mice immunized with the epitope vaccine PADRE- $A\beta_{1-15}$ -MAP and other control Ags. Splenocytes from individual mice were restimulated in vitro with the indicated peptides. Only the epitope vaccine PADRE- $A\beta_{1-15}$ -MAP (A) induced the activation of PADRE-specific, but not  $A\beta$ -specific, T cells. In contrast,  $A\beta_{1-33}$ -MAP (B), fibrillar  $A\beta_{42}$  (C), and  $A\beta_{1-33}$  (D) Ags stimulated anti- $A\beta$  T cell responses.



**FIGURE 5.** Detection of splenocytes producing IFN- $\gamma$  (Th1), IL-4 (Th2), and TNF- $\alpha$  in mice immunized with A $\beta$ <sub>1-33</sub>-MAP, PADRE-A $\beta$ <sub>1-15</sub> MAP, fibrillar A $\beta$ <sub>42</sub>, or linear A $\beta$ <sub>1-15</sub>. The ELISPOT technique was used, as described in *Materials and Methods*.

expressed T1/ST2 molecules (Fig. 6). Mice immunized with fibrillar A $\beta$ <sub>42</sub> also induced only CD4<sup>+</sup>T1/ST2<sup>+</sup> T cells, but the percentage of these cells was only 7.5%.

## Discussion

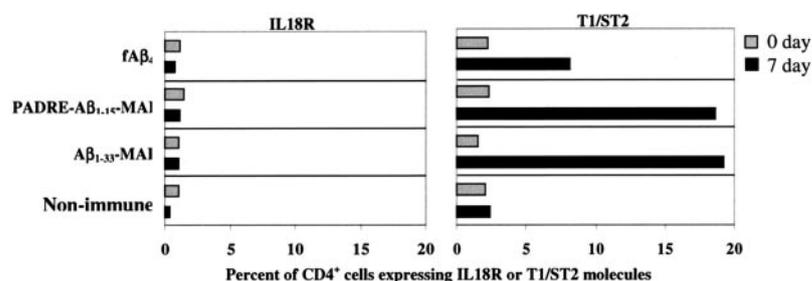
The major cause of dementia in the industrialized countries is AD, which most often strikes people 65 years of age and older. However, people with familial AD mutations can be affected at much younger ages. Ten percent of people over 65 years and up to 47% of those 85 years or older suffer from AD. Currently, there are >4 million Americans with AD. This number is projected to climb to a staggering 14 million by the middle of the 21st century. The disease claims over 100,000 lives/year in the U.S., making it the fourth leading cause of death for adults (50, 51). The A $\beta$  peptide is believed to play an important role in the onset and progression of AD (2–6). Many strategies currently being investigated as therapies for AD are based on reducing the level of A $\beta$  in the brain or blocking the assembly of this peptide into potentially pathological forms (7, 52).

Immunotherapy has recently been proposed as a strategy for reducing A $\beta$  levels in the brains of AD patients (9). Preclinical studies in APP/Tg mice have shown that active immunization with fibrillar A $\beta$ <sub>42</sub> peptide or passive transfer of anti-A $\beta$ <sub>42</sub> Abs into these mice can block A $\beta$  deposition and facilitate the removal of existing A $\beta$  deposits in the brain (8, 13, 14). In addition, neither protective nor therapeutic vaccinations induced adverse autoimmune or inflammatory responses in peripheral tissues or in the brains of experimental animals immunized with fibrillar A $\beta$ <sub>42</sub> (8–15). Based on these exciting results, Elan Corp. and Wyeth-Ayerst

began an immunotherapy clinical trial on AD patients. Unfortunately, the phase II trial was halted because ~6% of the volunteers developed symptoms of an adverse inflammatory response in the brain (9, 17–20). It is unclear what was the actual cause of the adverse events in response to active immunization, but importantly, the Ab response to A $\beta$  did not correlate with the presence or severity of the adverse events. In fact, some of the patients who developed meningoencephalitis did not have detectable levels of anti-A $\beta$  Abs, suggesting that the adverse reaction to A $\beta$  immunotherapy was not due to the humoral Ab response, but, rather, to a T cell-mediated immune response to AN-1792 (9, 20, 32, 53–56). The first case report on the neuropathology from an AD patient who was immunized multiple times with the AN-1792 vaccine supports the hypothesis that an adverse T cell-mediated autoimmune response was the cause of the aseptic meningoencephalitis (22). More specifically, neuropathological examination of the brain demonstrated infiltration of predominantly CD4<sup>+</sup> T cells in the leptomeninges and the cerebrovasculature in areas enriched with amyloid angiopathy. There were also sparse CD4<sup>+</sup> T cell infiltrates in the cerebral cortex and perivascular spaces. In addition, diffuse abnormalities in the cerebral white matter were observed, with a marked reduction in the density of myelinated fibers accompanied by extensive macrophage infiltration. However, there were also a number of promising changes in the neuropathology. Extensive areas of the neocortex were largely free of A $\beta$  deposits, dystrophic neurites, and activated astrocytes, although these regions still contained neurofibrillary tangles, neuropil threads, and cerebral amyloid angiopathy. These results provide the first evidence that anti-A $\beta$  Abs may significantly reduce A $\beta$  pathology in AD patients. The second case report from the AN-1792 clinical trial (21) generally agrees with the first report, although there are some distinct differences, such as the presence of multinucleated giant cells filled with dense deposits of A $\beta$ , multiple cortical hemorrhages, and CNS infiltration of CD8<sup>+</sup> T cells.

Drawing general conclusions regarding the effectiveness of A $\beta$  immunotherapy from only two published case reports from the clinical trial is risky, because the brains of many elderly humans typically contain pathological lesions even though they may not present with clinical symptoms. However, there was additional preliminary evidence from the AN-1792 vaccine trial to suggest that Abs against A $\beta$  may be beneficial to AD patients. For example, it was reported that some AD patients from the AN-1792 clinical trial showed significantly slower rates of decline in cognitive functions and activities of daily living. The beneficial effects were also observed in two patients who suffered from transient attacks of meningoencephalitis. Importantly, the degree of protection was correlated with the titer of the Abs binding to A $\beta$  plaques (19). Thus, the prominent T cell infiltration in the two case reports currently available from the clinical trial suggest that the aseptic subacute meningoencephalitis in patients who received the vaccine may have been caused by autoreactive anti-A $\beta$  T cells, whereas the

**FIGURE 6.** Expression of IL-18R (Th1) and T1/ST2 (Th2) molecules on the surface of CD4<sup>+</sup> T cells. Splenocytes from all groups were depleted by CD8<sup>+</sup> T cells and restimulated with the same peptides that were used for in vivo injections, except splenocytes from mice immunized with A $\beta$ <sub>42</sub> were activated in vitro with A $\beta$ <sub>40</sub>. Splenocytes were analyzed before in vitro activation (0 day) and after 7 days of activation (7 day). For details, see *Materials and Methods*.



clearance of plaques was probably connected with the production of anti-A $\beta$  Abs.

We have designed a prototype AD vaccine that will induce Ab responses directed to the immunodominant B cell epitope of A $\beta$ , but that will not generate anti-A $\beta$  T cells. The immunodominant B cell epitope of A $\beta_{42}$  has been mapped to the N terminus of this peptide (aa spanning residues 1–5, 1–7, 1–8, 1–11, 1–15, 1–16, or 4–10) (27–35). Based on these data, we chose the A $\beta_{1-15}$  sequence as the B cell-immunodominant antigenic determinant for generation of our prototype epitope vaccine. Importantly, recent results clearly indicate that this A $\beta_{1-15}$  peptide does not contain a T cell epitope in BALB/c mice (32) or in humans (57). The second component required for the design of the epitope vaccine was a T cell epitope that could provide strong T cell support to promote a potent humoral response to the A $\beta_{1-15}$  B cell epitope in mice and humans. For our epitope vaccine we chose a universal synthetic, nonnatural pan HLA DR-binding epitope, PADRE, which was engineered to provide a Th cell epitope that is chemically defined, easily manufactured, and able to generate effective Th cell responses in the general human population. PADRE binds with high affinity to 15 of 16 of the most common HLA-DR types tested to date (37–43). When PADRE was tested against human T cells in a proliferation assay, it was found to be 100-fold more potent on a molar basis than a tetanus-derived universal epitope. PADRE also binds with high to intermediate affinity to mouse I-A<sup>b/d</sup> and I-E<sup>b/d</sup> MHC haplotypes (37, 39, 41, 42). In addition, PADRE has been shown to be safe and well tolerated in human clinical trials (40). We synthesized our epitope vaccine consisting of PADRE-A $\beta_{1-15}$  on a MAP platform, which contains multiple copies of the antigenic determinant attached to a branched lysine backbone (44).

Mice immunized with our PADRE-A $\beta_{1-15}$ -MAP epitope vaccine formulated in alum, a Th2-type adjuvant, generated robust anti-A $\beta_{42}$  humoral immune responses. The Ab titers were equivalent to titers in mice immunized with A $\beta_{1-33}$ -MAP, which contains both B and T cell epitopes of A $\beta_{42}$  (Fig. 1). It is important to note that BALB/c mice respond very strongly to the T cell epitope of A $\beta_{42}$  (32) and only moderately to the PADRE T cell epitope (39). Thus, even though BALB/c mice are low responders to PADRE, they still generated a strong anti-A $\beta_{42}$  response. The Abs induced by PADRE-A $\beta_{1-15}$ -MAP were specific to A $\beta$ , because they did not bind to the MAP backbone or PADRE (data not shown). Thus, the non-self T cell epitope PADRE promoted production of Abs against the A $\beta_{1-15}$  B cell epitope and the anti-A $\beta$  Abs bound to amyloid plaques in brain tissue from an AD patient. Ab isotyping has previously been used as an indirect measure of the contributions of Th1 (IgG2a) and Th2 (IgG1) cytokines to the immune response (49). PADRE-A $\beta_{1-15}$ -MAP induced anti-A $\beta$  Abs of mainly the IgG1 isotype, demonstrating that the PADRE-A $\beta_{1-15}$ -MAP epitope vaccine formulated in alum generated predominantly a Th2 phenotype in H-2<sup>d</sup> mice.

To distinguish the role of each component of the epitope vaccine in T cell activation, we compared T cell proliferation in splenocytes from animals immunized with PADRE-A $\beta_{1-15}$ -MAP, A $\beta_{1-33}$ -MAP, A $\beta_{42}$ , A $\beta_{1-33}$ , and A $\beta_{1-15}$ . Only splenocytes from mice immunized with PADRE-A $\beta_{1-15}$ -MAP responded to PADRE. Importantly, these splenocytes did not respond to A $\beta$  T cell epitopes or MAP (Fig. 4). These results demonstrate that PADRE, but not A $\beta_{1-15}$  or MAP, provides the T cell antigenic determinant in the PADRE-A $\beta_{1-15}$ -MAP epitope vaccine. Mice immunized with fibrillar A $\beta_{1-33}$ -MAP generated anti-A $\beta$ -specific T cells, demonstrating that A $\beta_{1-33}$  possesses both B and T cell epitopes. Finally, immunization with monomeric A $\beta_{1-15}$  did not induce Abs, and no T cell proliferation was detected after restimulation with A $\beta_{40}$ , confirming our previous finding (32) that this peptide does not

possess a T cell epitope for mice of the H-2<sup>d</sup> haplotype (Fig. 4). We also compared cytokine production in the pooled splenocyte cultures from each group of immunized BALB/c mice. As expected for BALB/c mice immunized with alum as the adjuvant, the predominant cytokine produced by splenocytes from all immunized groups was IL-4.

Recently, Th1 (IL-18R) and Th2 (T1/ST2)-specific markers were identified on CD4<sup>+</sup> T cells isolated from TCR-transgenic or T1/ST2-deficient mice (58–60). In this study we used Abs to IL-18R and T1/ST2 to analyze the expression of these phenotypic markers on the surface of CD4<sup>+</sup> Th cells isolated from immunized mice. After 7 days of peptide restimulation, CD4<sup>+</sup> lymphocytes from mice immunized with A $\beta_{1-33}$ -MAP, PADRE-A $\beta_{1-15}$ -MAP, and fibrillar A $\beta_{42}$  expressed high levels of T1/ST2 and low levels of IL18R. These results confirm that our PADRE-A $\beta_{1-15}$ -MAP epitope vaccine formulated in alum induced a Th2-polarized immune response, which may provide useful benefits for A $\beta$  immunotherapy (32). Although these results were not surprising, because the peptide Ags were formulated in alum, a Th2-type adjuvant, additional studies are needed to confirm that these markers for Th1 and Th2 subclasses work reliably in a polyclonal population of CD4<sup>+</sup> cells isolated from genetically unmodified animals.

The failure of the first immunotherapy clinical trial was disappointing; however, a detailed analysis of the results may help to identify the factor(s) that triggered the adverse inflammatory response in a subset of patients receiving the AN-1792 vaccine. Alternative approaches that bias the immune response toward a Th2 phenotype and/or replace the A $\beta$  T cell epitope with a foreign T cell epitope may prevent the adverse events that occurred during the first clinical trial. We are currently vaccinating three different types of APP/Tg mice on the H-2<sup>b</sup> background, which are high responders to PADRE, with our new PADRE-A $\beta$ -MAP epitope vaccine. Preliminary data demonstrate that these animals produce robust levels of anti-A $\beta$  Abs in response to the epitope vaccine (D. H. Cribbs, A. Ghochikyan, V. Vasilevko, I. Petrushina, N. Movsesyan, M. Mkrtychyan, and M. G. Agadjanyan, unpublished observations). New preclinical trials in APP/Tg mouse models may help to develop novel immunogen-adjuvant configurations with the potential to avoid the adverse immune response that occurred in the first clinical trial.

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