Generation and characterization of the humoral immune response to DNA immunization with a chimeric β-amyloid-interleukin-4 minigene

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Active immunization with fibrillar β–amyloid peptide (Aβ$_{42}$) as well as passive transfer of anti-Aβ antibodies significantly reduces Aβ plaque deposition, neuritic dystrophy, and astrogliosis in the brain of mutant amyloid precursor protein (APP)-transgenic mice. Although the mechanism(s) of clearance of Aβ from the brain following active or passive immunization remains to be determined, it is clear that anti-Aβ antibodies are critical for clearance. DNA immunization provides an attractive alternative to direct peptide and adjuvant approaches for inducing a humoral response to Aβ. We constructed a DNA minigene with Aβ fused to mouse interleukin-4 (pAβ$_{42}$-IL-4) as a molecular adjuvant to generate anti-Aβ antibodies and enhance the Th2-type of immune responses. Gene gun immunizations induced primarily IgG1 and IgG2b anti-Aβ antibodies. Fine epitope analysis with overlapping peptides of the Aβ$_{42}$ sequence identified the 1–15 region as a dominant B cell epitope. The DNA minigene-induced anti-Aβ antibodies bound to Aβ plaques in brain tissue from an Alzheimer's disease patient demonstrating functional activity of the antibodies and the potential for therapeutic efficacy.

1 Introduction

Alzheimer's disease (AD) 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 β-amyloid peptides (Aβ$_{40}$ or Aβ$_{42}$) in plaques, and neuronal loss in affected brain regions [1]. The Aβ peptides cleaved from the amyloid precursor protein (APP) by the β- and γ-secretases [2–5] are believed to play a central role in the onset and progression of AD [6]. Recently, Schenk et al. [7] demonstrated that immunization of APP-transgenic (APP/Tg) mice with Aβ$_{42}$ induced the generation of antibodies, which dramatically reduced the deposition of amyloid plaques and promoted the clearance of plaques from the brain. Other groups have reported similar observations and have extended these studies by showing that active immunization protected mice from developing functional memory deficits [8–10]. Remarkably, passive administration of an anti-Aβ monoclonal antibody to APP/Tg mice also resulted in decreased Aβ levels in the brain, significantly reducing the AD-like neuropathology [11, 12], and reversing memory deficits [13]. These results suggest that the generation of antibodies against Aβ in humans might provide protection against the onset of AD [14].

Based on these data, Aβ immunotherapy has been moved to the clinical trials. The AN1792 vaccine was comprised of Aβ$_{42}$ as the antigen and QS21 as the adjuvant. Unfortunately, the vaccine trial was halted because approximately 5% of the participants receiving the vaccine developed some degree of meningoencephalitis [14, 15]. The failure of this clinical trial is disappointing; however, this does not mean that other Aβ immunother-

apy strategies will not eventually be successful in treating AD. Because Th1-mediated responses have been implicated in many autoimmune diseases [16], there has been speculation that the adjuvant may have contributed to the adverse response in a subset of the patients receiving the AN1792 vaccine [17, 18]. Alternative, immunotherapy approaches that polarize the immune response to Aβ towards a Th2 phenotype, that in some cases have been shown to inhibit autoimmune diseases, may provide significant advantages [19, 20, 21]. An example of this type of approach is the intranasal immunization of APP/Tg mice with Aβ$_{42}$ but without a conventional adjuvant, which was shown to induce a Th2-mediated humoral response to Aβ [18, 22].

Another adjuvant-free vaccination approach is DNA immunization, which has been shown to generate potent humoral and cellular immune responses against viral, tumor, and foreign antigens [23–34]. Importantly, it has also been reported that co-administration of IL-4 cytokine gene along with a gene, encoding foreign or "self"

antigens, caused the immune response to be driven toward a more Th2-like phenotype [20, 21, 35–39]. Here we describe a novel chimeric DNA minigene encoding Aβ fused with mouse IL-4. Immunization of wild-type mice with the pAβ$_{42}$-IL-4 generated a potent humoral response to Aβ. Analysis of the IgG isotypes showed that IgG1 and IgG2b were the predominant species, which suggests that the immune response was primarily Th2-mediated. The anti-Aβ antibodies generated were specific for the N-terminal region of Aβ$_{42}$ and recognized Aβ plaques in human AD brain tissue, which indicated that antibodies are potentially therapeutic.

2 Results

2.1 Expression of chimeric DNA minigenes *in vitro*

Three different plasmids were constructed (Fig. 1a, b), and expression of all plasmids has been confirmed by analyzing the lysate and supernatants of transiently
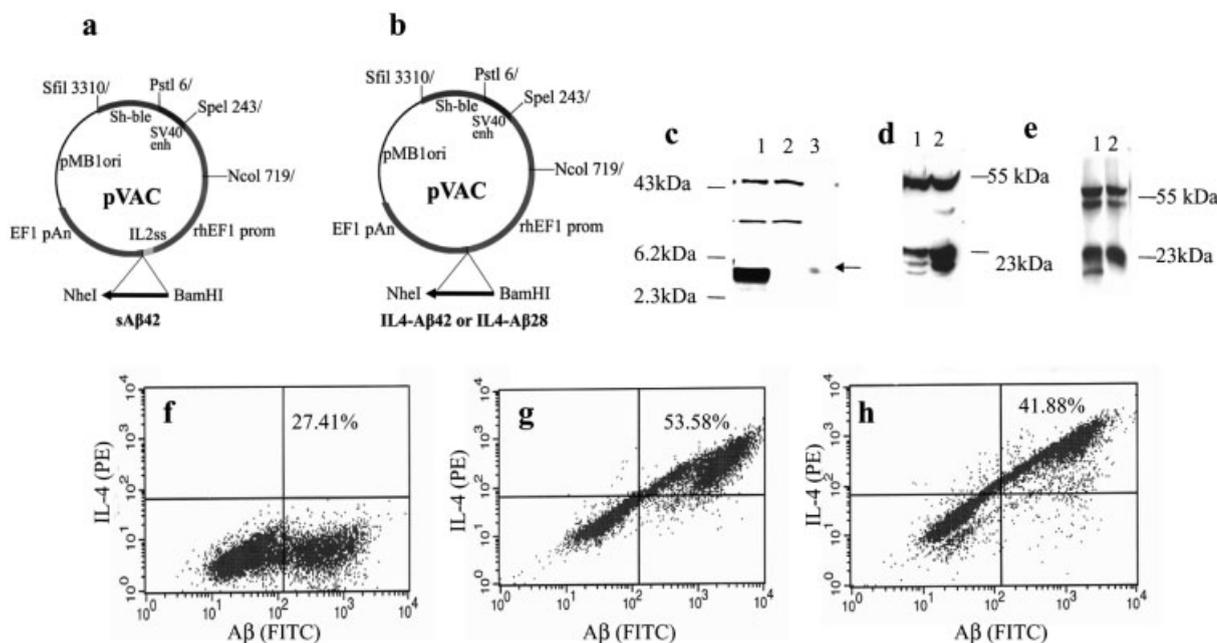

Fig. 1. (a, b) Schematic representation of the constructs. (a) DNA sequence encoding Aβ$_{42}$ peptide was cloned in frame with the IL-2 signal sequence using BamHI and NheI restriction sites; (b) DNA sequences encoding Aβ$_{42}$ or Aβ$_{28}$ peptides fused with IL-4 were generated by PCR and cloned using BamHI and NheI restriction sites. (c–e) Expression of Aβ$_{42}$ (c) and Aβ$_{42/28}$-IL-4 (d, e) minigene cassettes in CHO cells transfected with appropriate plasmid. Protein was recovered from supernatant or cell lysate by IP and analyzed on 16.5% Tricine-SDS-PAGE (c) or 15% Tris-SDS-PAGE (d, e) followed by WB. For IP and WB the monoclonal anti-Aβ antibody 6E10 was used. (c) Lane 1: supernatant of cells transfected with pAβ$_{42}$; lane 2: supernatant of cells transfected with vector; lane 3: 5 ng of Aβ$_{42}$ peptide (HPLC grade, >95% purity). (d) Lane 1: lysate of cells transfected with pAβ$_{42}$-IL-4; lane 2: lysate of cells transfected with Aβ$_{28}$-IL-4. (e) Lane 1: supernatant of cells transfected with pAβ$_{42}$-IL-4; lane 2, supernatant of cells transfected with pAβ$_{28}$-IL-4. (f–h) Flow cytometric analysis of the expression of Aβ$_{42}$ (f), Aβ$_{42}$-IL-4 (g) and Aβ$_{28}$-IL-4 (h) proteins in CHO cells transfected with appropriate plasmids. Transfected CHO cells were treated with brefeldin A overnight, incubated with 6E10 antibody alone (f) or 6E10 and rat anti-mouse IL-4-PE (g, h). To detect Aβ expression, anti-mouse Ig-FITC antibodies were added.

transfected Chinese hamster ovary (CHO) cells (Fig. 1c–e). The additional bands in the region between 19–24 kDa (lanes 1–2) likely represent different glycosylation states [40] of $A\beta_{42}$-IL-4 or $A\beta_{28}$-IL-4 fusion proteins. Importantly, we did not detect any $A\beta$ peptides in the supernatant or lysate of cells transfected with vector DNA (Fig. 1c, lane 2, and data not shown). In addition to Western blot (WB), the expression of $A\beta_{42}$, $A\beta_{42}$-IL-4, and $A\beta_{28}$-IL-4 were confirmed by flow cytometry analysis using immunostaining with monoclonal anti-$A\beta_{42}$ 6E10 and anti-IL-4 antibodies (Fig. 1f–h). Transiently transfected CHO cells expressed $A\beta_{42}$, $A\beta_{42}$-IL-4 and $A\beta_{28}$-IL-4 molecules.

2.2 Generation of anti-$A\beta$ antibodies

We immunized B6SJLF1 mice by bombardment of the skin with microscopic gold particles coated with the various DNA minigenes using the Helios gene gun. Mice immunized with $pA\beta_{42}$ did not generate anti-$A\beta_{42}$ antibodies (data not shown). In contrast, conjugation of $A\beta_{42}$
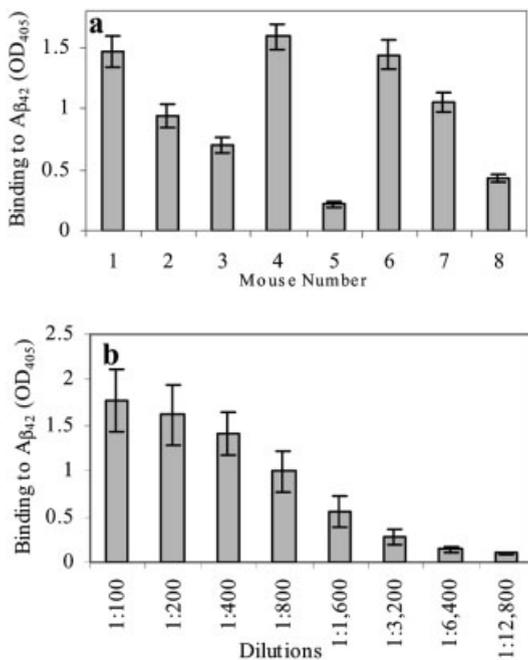

Fig. 2. Generation of anti-$A\beta_{42}$ antibodies after gene gun immunization of eight B6SJLF1 mice with plasmids, encoding $A\beta_{42}$-IL-4 fusion protein. (a) Serum of each mouse (No. 1–8) was diluted 1:250 and used in ELISA for detection of total Ig (SD represents results from three separate assays with the same sera). (b) Titer of anti-$A\beta_{42}$ antibodies was detected in the pooled sera from immunized mice (SD represent two separate experiments with sera of mice No. 1–4 and 5–8).
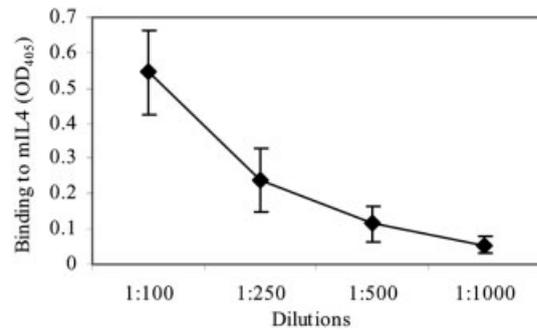
Fig. 3. Titer of anti-IL-4 antibodies detected in the sera of individual mice vaccinated with $A\beta_{42}$ DNA immunogen fused with IL-4 molecular adjuvant (*n*=8).

with IL-4 resulted in a robust anti-$A\beta_{42}$ immune response generated by three out of eight experimental mice after only two boosts. Anti-$A\beta_{42}$ antibody responses in three mice were moderate, whereas the remaining two mice responded weakly (Fig. 2a). Antibody titers of the pooled sera were detected in two separate experiments and were equal to 1:3,200 (Fig. 2b). Mice immunized with vector did not induce anti-$A\beta_{42}$ antibody production, whereas injection of $psHA$-$mC3d_3$ generated only anti-hemagglutinin (HA)-specific antibodies (data not shown). Thus, the presence of the mouse IL-4 molecular adjuvant in the plasmid was critical for generation of anti-$A\beta$ specific antibody responses.

There is a high level of homology between human and rodent $A\beta_{42}$ peptides [41]. Accordingly, we demonstrated that immunization with $pA\beta_{42}$-IL-4 that encodes human $A\beta_{42}$ induces cross-reactivity with mouse $A\beta_{42}$. However, the antisera bound to rodent $A\beta_{42}$ two times weaker than to human $A\beta_{42}$ (data not shown). Because we demonstrated the immune response to the self-$A\beta_{42}$ peptide, it was also interesting to analyze antibody production to the self-IL-4 molecule. Our data indicate that DNA vaccination with $pA\beta_{42}$-IL-4 generated low-titer antibodies to the IL-4 molecular adjuvant (Fig. 3).

2.3 Characterization of anti-$A\beta_{42}$ antibodies by isotyping

We analyzed isotypes of anti-$A\beta_{42}$ antibodies in the sera of six mice, which represent high and moderate responders. All animals generated IgG1 antibodies, whereas the level of IgG2a[b] was negligible. The production of IgG1 antibodies is an indirect measure of the relative contribution of Th2-type cytokines, whereas IgG2a antibodies reflect the contribution of Th1 to the immune response [32]. Thus our data indicate that $pA\beta_{42}$-IL-4 minigene induces a highly Th2-polarized response. Four
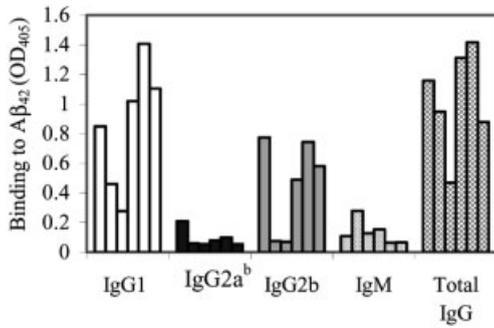
Fig. 4. Isotyping of anti-Aβ_{42} antibodies after immunization of mice with pAβ_{42}-IL-4. Each serum from six mice, which represent high and moderate responders (No. 1–4, 6, 7) were diluted 1:250 and used for detection of IgG1, IgG2a[b], IgG2b, and IgM subclasses of anti-Aβ_{42} antibodies. All mice demonstrated IgG1 (Th2)/IgG2a[b] (Th1) ratio much higher than 1.

mice with the highest production of IgG1 antibodies also generated significant levels of IgG2b (Fig. 4). There was no IgE or IgA antibody production detected in immunized mice (data not shown).

2.4 Mapping of B cell epitopes and binding to human β-amyloid plaques

In order to identify epitopes recognized by antibodies induced after DNA immunization, we analyzed binding of immune sera to the six overlapping peptides by competition ELISA. Pre-incubation of sera from high and moderate responders with the full-length Aβ_{42} peptide resulted
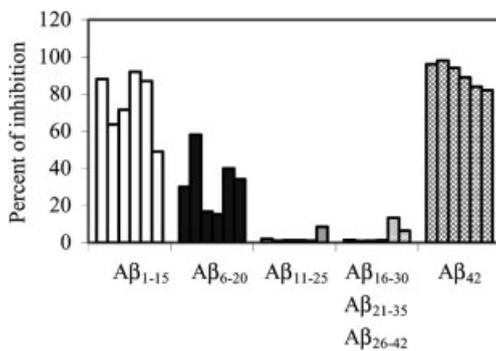

Fig. 5. Mapping of B cell epitopes by six overlapping peptides. Serum from each mouse (No. 1–4, 6, 7) was pre-incubated with indicated peptides or mixture of peptides before binding to Aβ_{42}-coated wells. The percent of inhibition by small peptides as well as by control full-length peptide was calculated based on binding of sera without competing peptides to Aβ_{42} as 100%.

in the most complete inhibition of antibody binding to the adsorbed Aβ_{42} peptide (82–98% inhibition). The peptide Aβ_{1-15} was also effective, inhibiting the binding of antisera by 49–92%. In addition, Aβ_{6-20} showed partial inhibition of antibody binding. Notably, a mixture of Aβ_{16-30}, Aβ_{21-35} and Aβ_{26-42} peptides (2.5 μM each) was ineffective (Fig. 5).

To analyze the capability of these antibodies to bind to Aβ plaques in human brain tissue, we used pooled sera from six immunized mice. This antiserum bound to Aβ plaques on the brain sections of cortical tissue from a severe AD case (Fig. 6). The antisera were effective at a dilution of 1:1,000, the endpoint dilution used in these experiments. The pre-immune sera from these mice did not bind to the Aβ plaques. Notably, Aβ_{42} peptide abrogated binding of antisera to the Aβ plaques in human brain tissue (Fig. 6).

To examine inflammation-related pathology in the brain of animals immunized with DNA minigene, 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, CD8, and CD54 molecules. As shown in Fig. 7, immunostaining in sections from the frontoparietal cortex revealed no observable differences between wild-type and immunized mice. CD11b-positive micro-
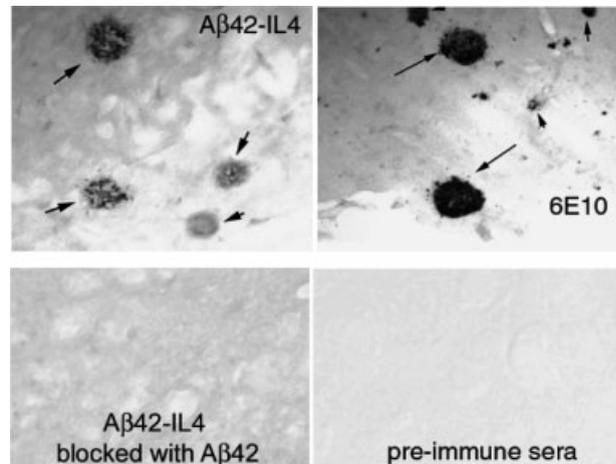
Fig. 6. Binding of immune (Aβ_{42}-IL-4), pre-immune (negative control) sera, and 6E10 monoclonal antibody (positive control) to the 50-μm brain sections of formalin-fixed cortical tissues from an 80-year-old man with neuropathological and behavioral patterns typical to severe AD. Both pre-immune and immune sera were used at a dilution of 1:1,000, whereas the monoclonal antibody 6E10 was used at a dilution of 1:1,500. Binding of antisera to the brain tissue was blocked by Aβ_{42} peptide. Arrows indicate senile plaques in frontal cortex sections.

Fig. 7. Detection of neuroinflammation in brains of mouse immunized with pAβ$_{42}$-IL-4 or vector. Markers of neuroinflammation were negative in vaccinated mice compared to control (immunized with vector) animals. The left column shows sections from the frontoparietal co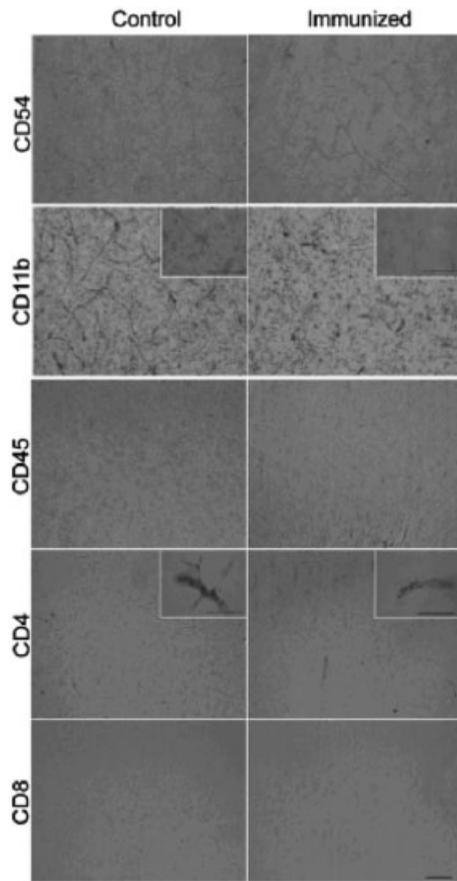rtex of a control animal and the right panel from a vaccinated mouse. Markers used are indicated in each row. No evidence for CD54-, CD45-, or CD8-positive cells was observed. CD11b-positive microglial cells exhibited a resting morphology with long fine processes (illustrated in insets). CD4-positive cells were found within blood vessels (insets), but had not infiltrated into the neuropil. Bars = 50 mm. These data were repeated with two other animals.

glial cells exhibited a resting morphology with long fine processes. Importantly, CD4-positive cells were found within blood vessels but had not infiltrated into the neuropil.

3 Discussion

The development of a safe and effective vaccine requires a delicate balance between providing a specific and adequate immune response to provide protection, and reducing or eliminating unwanted adverse events that

may induce excess inflammation or an autoimmune response. While there are significant safety concerns about Aβ immunotherapy as a possible treatment for AD, there is also considerable optimism that these hurdles can be overcome with other vaccination approaches. Alternative strategies have been suggested, such as using different adjuvants, different routes of immunization and the using molecular adjuvant(s), such as cytokines in conjunction with Aβ-based vaccine to drive the Th phenotype toward the desired pathway [18, 19, 22, 42]. In this study we have employed a chimeric DNA minigene to deliver a fusion protein containing Aβ antigen together with IL-4 as a molecular adjuvant to amplify immune response to Aβ and to further polarize the response toward a Th2 phenotype *in vivo*.

Thirteen years ago, it was demonstrated that a gene could be transferred *in situ* by inoculation of a plasmid vector into muscle [43]. Now, the generation of potent humoral and cellular immune responses to a broad spectrum of pathogen antigens has been demonstrated in different animal model systems using DNA vaccination [25–30, 34, 44–49]. DNA immunization offers significant advantages over peptide/protein-based immunization. First it offers the capability to modify genes encoding desired antigen(s), to change the cellular localization of an antigen by adding or removing signal sequences or transmembrane domains, and to target the desired type of immune response using the appropriate molecular adjuvants. Previously we used these approaches to generate potent humoral and cellular immune responses against different antigens [25–28, 45, 50]. Now to generate potent antibody production to Aβ antigen we prepared plasmids, encoding antigen (Aβ$_{42}$ or Aβ$_{28}$) fused with IL-4 (Fig. 1). We demonstrated that mice immunized with pAβ$_{42}$ did not generate anti-Aβ$_{42}$ antibodies even after six boosts (data not shown), whereas plasmids, encoding immunogen and IL-4 induced anti-Aβ$_{42}$ antibodies after just two boosts (Fig. 2).

We and others have previously mapped B cell epitopes using antisera from mice immunized with fibrillar Aβ$_{42}$ and small peptides derived from Aβ$_{42}$ [11, 19, 22, 51, 52]. These results have been confirmed and the linear Aβ$_{42}$ epitope spanning residues 4–10 was identified using high-resolution mass-spectrometry technique [53]. In this report we demonstrated that antibodies generated after pAβ$_{42}$-IL-4 immunization also recognized Aβ$_{1–15}$ peptide. Binding to peptide Aβ$_{6–20}$ was detectable, but significantly weaker than binding to Aβ$_{1–15}$ and Aβ$_{42}$ (Fig. 5). Thus, like fibrillar Aβ$_{42}$ formulated in different adjuvants [11, 19, 22, 51–54], DNA immunization induced antibodies against linear epitope(s) of Aβ$_{42}$ that span the N-terminal amino acids of this peptide. Collectively these data suggest that Aβ$_{1–15}$ represents the major

B cell antigenic determinant of $A\beta_{42}$ regardless of DNA or protein immunizations. Interestingly, binding of anti-bodies to this region of $A\beta_{42}$ coincides with the ability of antibodies to bind native plaques in brain tissue [11, 53] and trigger ex vivo phagocytosis [54]. Antibodies generated after gene gun immunization with $pA\beta_{42}$-IL-4 also recognize human $A\beta$ plaques in cortical tissues from a severe AD case (Fig. 6).

Successful DNA immunization with IL-4 as a molecular adjuvant to enhance Th2 type of immune responses to different immunogens has been previously reported [20, 21, 35–39]. However, to our knowledge no one examined whether anti-IL-4 antibody production is generated when IL-4 is used as a fusion protein with the immunogen. In this report we have demonstrated that mice immunized with $pA\beta_{42}$-IL-4 induced low levels of antibodies to self-IL-4 cytokine (Fig. 3). Such a response was not unexpected since gene therapy is used to induce immunological memory against self pro-inflammatory chemokines [55]. IL-4 cytokine is an anti-inflammatory cytokine with pleiotropic effects on immune cells of multiple lineages. Thus, production of antibodies to IL-4, unnaturally expressed after DNA vaccination, may be an important regulatory mechanism that protects an organism from overexpression of this cytokine.

The antibody isotype has been used as an indirect measure of the contribution of Th1 and Th2 cytokines to the humoral response [56]. We recently demonstrated that B6SJLF1 mice produce IgG2a[b] anti-$A\beta_{42}$ antibodies instead of IgG2a [57]. Accordingly, we analyzed the IgG1 and IgG2a[b] profiles of the humoral immune response after DNA immunization. Injections with $pA\beta_{42}$-IL-4 induced predominantly IgG1 and to a lesser extent IgG2b anti-$A\beta_{42}$ antibodies. Anti-$A\beta_{42}$ antibodies of IgG2a[b] isotype were negligible (Fig. 4). Thus, the IgG1/IgG2a ratio measured following immunization with our chimeric $A\beta_{42}$-IL-4 minigene implies that a highly polarized Th2-mediated immune response was generated. More importantly, IgG1 and IgG2b isotypes of anti-$A\beta_{42}$ antibodies may correlate with their therapeutic potential [11, 13, 53, 58]. The antibodies generated after $A\beta_{42}$-IL-4 minigene immunization are predominantly of the IgG1 isotype and bound to the first 15 amino acids of $A\beta_{42}$ and to $A\beta$ plaques in the AD brain tissue. Therefore, DNA immunization with minigene encoding $A\beta$ antigen and IL-4 molecular adjuvant induces therapeutically potent antibodies to $A\beta_{42}$.

Because T lymphocyte-mediated meningoencephalitis was observed in some patients after immunization with $A\beta_{42}$ [59], we analyzed lymphocyte infiltration into brains of immunized wild-type mice. Immunohistochemical analysis of brain sections did not reveal lymphocyte-mediated neuroinflammation or microglia activation (Fig. 7). Because antibodies raised against human $A\beta_{42}$ also recognize rodent $A\beta_{42}$, and therefore rodent APP, we speculate that pathological hallmarks in this case may be similar to the ones appearing after vaccination with self-antigen. Future studies with APP/Tg mice immunized with different $pA\beta$-IL-4 constructs are underway and these experiments will better define the therapeutic effects, such as reducing the deposition of $A\beta$ plaques, clearance of plaques from the brain, and protection of mice from developing functional memory deficits, following DNA vaccination.

4 Materials and methods

4.1 Mice

Eight- or ten-week-old female B6SJLF1 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the animal facility at the Institute for Brain Aging and Dementia, University of California, Irvine (UCI), CA. 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 the Institute for Brain Aging and Dementia at UCI.

4.2 DNA constructs

Three different plasmids encoding secreted forms of $A\beta$ peptide were constructed: $pA\beta_{42}$, encoding $A\beta_{42}$; $pA\beta_{28}$-IL-4 and $pA\beta_{42}$-IL-4, encoding chimeric proteins, where mouse IL-4 is fused with human $A\beta_{28}$ or $A\beta_{42}$, respectively (Fig. 1a, b). The $pA\beta_{42}$ was constructed by cloning $A\beta_{42}$ PCR fragment into pVAC (Invivogen, San Diego, CA) expression vector in frame with IL-2 signal sequence using BamHI and NheI restriction sites. $pA\beta_{42}$-IL-4 and $pA\beta_{28}$-IL-4 were constructed by fusion of $A\beta_{42}$ or $A\beta_{28}$ with IL-4 gene via overlapping PCR techniques using appropriate primers and then cloning into pVAC expression vector using the same restriction sites (IL-2 signal sequence was replaced by IL-4 signal sequence). The correct sequences of the generated plasmids were confirmed by nucleotide sequence analysis. The structure of plasmid psHA-mC3d$_3$ encoding HA of influenza and three copies of C3d component of complement was described earlier [28]. All plasmids have been purified by Endofree plasmid maxi kit (Qiagen, Valencia, CA). Purity of the DNA was confirmed by UV spectrophotometry (260 nm/280 nm absorbance ratio >1.7) and gel electrophoresis.

4.3 Transfection of cells and expression of $A\beta$ by $psA\beta_{42}$, $pA\beta_{28}$-IL-4 and $pA\beta_{42}$-IL-4

CHO cells (0.8×10^6) were transiently transfected with 2 μg of the appropriate plasmid ($pA\beta_{42}$, $pA\beta_{42}$-IL-4, or $pA\beta_{28}$-IL-4) by

Lipofectamine Plus Reagent (Invitrogen, Carlsbad, CA) and expressions of these plasmids were analyzed in the supernatants or lysates of cells. Cells transfected with vector were used as a negative control. All proteins were detected by combination of immunoprecipitation (IP) and WB using 6E10 anti-Aβ antibodies (Signet, MA). Tricine-SDS-polyacrylamide gel (16%, for Aβ_{42}) or 15% Tris-SDS-polyacrylamide gel (for fusion proteins) were used for electrophoresis. The antibody-antigen complexes were detected using horseradish peroxidase (HRP)-conjugated anti-mouse antibodies and Luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA).

4.4 Flow cytometry

The flow cytometry analysis was used for detection of Aβ_{42} peptide or Aβ_{42}-IL-4 and Aβ_{28}-IL-4 fusion proteins in transfected CHO cells. Transiently transfected CHO cells were treated by inhibitor of intracellular transport brefeldin A (Sigma, St. Louis, MO) at a final concentration of 5 μg/ml for 16 h. Aβ_{42} expression was analyzed using monoclonal antibodies 6E10 (0.5 μg per 2.5×10^5 cells) following staining with FITC-conjugated goat anti-mouse polyclonal antibodies (BD PharMingen, San Diego, CA). Double staining with both 6E10 and PE-conjugated anti-mouse IL-4 (0.2 μg per 2.5×10^5 cells; BD PharMingen) antibodies were used for analyses of Aβ_{42}-IL-4 and Aβ_{28}-IL-4 expression. Flow cytometry analyses were performed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA), and data were analyzed by CellQuest software (Becton Dickinson).

4.5 Immunization

Immunizations of mice with experimental plasmids (two independent experiments with eight animals for each plasmid) were performed on shaved abdominal skin using the Helios gene gun (Bio-Rad, Hercules, CA) as we described [28]. Briefly, mice were bombarded two times with doses containing 1 μg of DNA per 0.5 mg of ~1-μm gold beads (DeGussa-Huls Corp., Ridefield Park, NJ) at a helium pressure setting of 400 psi. Mice were immunized and boosted by the same method biweekly and sera were collected 7–8 days after the final boost. As a control we used animals (eight in each group) immunized by gene gun bombardment with psHA-mC3d$_3$ (positive), or with vector (negative) [28].

4.6 ELISA

Total anti-Aβ_{42} and anti-HA antibodies have been detected as described previously [7, 19, 28, 57]. Briefly, wells of 96-well plates (Immulon II; Dynatech) were coated with 2.5 μM of human or mouse Aβ_{42} in bicarbonate coating buffer (pH 9.7) and incubated 3 h at 37°C or overnight at 4°C. They were then washed and blocked with 3% non-fat dry milk in Tween-20 Tris buffer solution (TTBS) for 1–2 h at 37°C. After

washing of the wells primary sera from experimental and control mice were added in duplicate at the indicated dilutions. After incubation (O/N at 4°C) and washing, HRP-conjugated anti-mouse IgG was added as recommended by manufacturer (Jackson Laboratories). Plates were incubated for 1 h at 37°C, washed, and freshly prepared OPD substrate solution (o-phenylendiamine in 0.05 M phosphate-citrate buffer, pH 5.0; Sigma) was added to develop reaction. All plates were analyzed spectrophotometrically at 405 nm. Anti-HA antibodies have been detected by ELISA exactly as we described earlier [28]. Anti-IL-4 antibodies were detected as described above except that plates were coated with 200 pg/ml recombinant mouse IL-4 (BD PharMingen).

4.7 Antibody isotyping

To determine the specific isotypes, sera from individual mice were diluted 1:250 and tested in duplicate as described above. To detect mouse IgG1, IgG2b, or IgM isotypes, we used anti-mouse Ig-subclass-specific HRP-conjugated secondary antibodies (Zymed, San Francisco, CA). To detect mouse IgG2c (IgG2a^b) isotype, we used biotin-conjugated mouse anti-mouse IgG2a^b (Igh-1b) monoclonal antibody (PharMingen, San Diego, CA), followed by incubation with HRP-conjugated streptavidin (Vector Laboratories, Burlingame, CA). For detection of IgE and IgA isotypes we used appropriate isotype-specific anti-mouse antibodies (Zymed).

4.8 Mapping of Aβ_{42} antigenic determinants by competition ELISA

Previously, we have detected antigenic determinants in Aβ_{42} peptide using a competition ELISA assay where the small overlapping peptides mixed with antisera compete with binding to the Aβ_{42}-coated plates [19]. Briefly, plates were coated with Aβ_{42}, washed, and blocked, as described above. Sera from immunized individual mice (diluted 1:250) were mixed with 2.5 μM of Aβ_{42} peptide or small overlapping peptides spanning amino acids 1–15 (Aβ_{1-15}), 6–20 (Aβ_{6-20}), 11–25 (Aβ_{11-25}), 16–30 (Aβ_{16-30}), 21–35 (Aβ_{21-35}), and 16–42 (Aβ_{16-42}) of Aβ_{42} and incubated 1 h at 37°C. The peptide-treated sera were added in duplicate to Aβ_{42}-coated plates and antibody binding was analyzed by ELISA as described above. The percent of inhibition by small peptides and by control full-length peptide was calculated based on binding of sera without competing peptides to Aβ_{42} as 100%.

4.9 Detection of Aβ plaques in human brain tissues

Sera from immunized mice were screened for an ability to bind to Aβ plaques in the human brain. Briefly, pooled sera were added to the serial 50-μm brain sections of formalin-fixed frontal cortical tissue from a patient (an 80-year-old

man with neuropathological and behavioral patterns typical for severe AD). Sections were pretreated with 90% formic acid, and exogenous peroxidase was quenched. Three different titers of mice antisera were tested (1:500, 1:750, and 1:1,000 dilutions). As a negative control, we used the same dilutions of pre-immune sera. As a positive control, monoclonal anti-human Aβ antibody 6E10 (dilution 1:1,500) was used to immunostain plaques in AD brain sections. Binding of antisera to the plaques was blocked by soluble $A\beta_{42}$ peptide in a concentration of 2.5 μM. Binding of antibodies to the brain sections was determined via VECTASTAIN Elite ABC Mouse IgG /DAB substrate biotin-avidin system (both kits from Vector Laboratories), according to manufacturer's recommendations. Digital camera (Olympus, Japan) was used to view the plaques at 20× image magnification.

4.10 Detection of neuroinflammation in mouse brains

Mice were sacrificed and brains were removed, fixed in 4% paraformaldehyde for 24 h at 4°C, and subsequently cut in serial 50-μm-thick sections. Sections were pretreated with 0.3% H_2O_2 for 30 min to eliminate endogenous peroxidase activity. The following primary antibodies were used after blocking of sections: CD54 (ICAM) (3E2), CD45 (30-F11), CD11b (M1/70), CD4 (GK1.5), CD8a (53–6.7) (all from BD PharMingen). All antibodies were optimally diluted in 0.1 M Tris, 0.1% Triton X-100 and 2% BSA as recommended by the manufacturer. Bound antibodies were detected using biotinylated anti-hamster (Vector Laboratories) or preabsorbed biotinylated anti-rat (Jackson Immunoresearch) antibodies followed by incubation with avidin:biotin peroxidase complex (ABC), using the Vectastain Elite kit (Vector Laboratories). Staining reactions were performed with 3,3-diaminobenzidine (DAB; Sigma) according to manufacturers' protocols. For negative control, the primary antibody was omitted from the diluent.

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