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DNA epitope vaccine containing complement component C3d enhances anti-amyloid- β antibody production and polarizes the immune response towards a Th2 phenotype

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Abstract

We have engineered a DNA epitope vaccine that expresses 3 self-B cell epitopes of A β ₄₂ (3A β ₁₋₁₁), a non-self T helper (Th) cell epitope (PADRE), and 3 copies of C3d (3C3d), a component of complement as a molecular adjuvant, designed to safely reduce CNS A β . Immunization of mice with 3A β ₁₋₁₁-PADRE epitope vaccine alone generated only moderate levels of anti-A β antibodies and a pro-inflammatory T helper (Th1 phenotype) cellular immune response. However, the addition of 3C3d to the vaccine construct significantly augmented the anti-A β humoral immune response and, importantly, shifted the cellular immune response towards the potentially safer anti-inflammatory Th2 phenotype.

Keywords

Alzheimer's disease; Amyloid-beta; DNA vaccine; Immunotherapy; Molecular adjuvant; AN-1792

1. Introduction

One of the major health concerns for elderly people is Alzheimer's disease (AD), which is the most common form of dementia with progressive loss of memory and general cognitive decline. The first immunotherapy clinical trial on Alzheimer's patients, AN-1792, was halted when a

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subset of those immunized with the vaccine containing the amyloid-beta peptide (A β ₄₂) developed adverse events (aseptic meningoencephalitis) in the central nervous system (CNS) (Orgogozo et al., 2003; Schenk, 2002; Steinberg, 2002). While the actual cause of the adverse events is unknown, speculation has centered on autoreactive T cells specific for the T cell epitope in A β , the conventional adjuvant (QS21), and the reformulation of the vaccine with polysorbate 80 during the phase IIa portion of the trial (Ferrer et al., 2004; Nicoll et al., 2003; Schenk, 2002). Subsequent analysis of postmortem brain tissue from patients that received the AN-1792 vaccine showed an overall reduction of A β burden in the CNS (Boche et al., 2007; Ferrer et al., 2004; Holmes et al., 2008; Masliah et al., 2005; Nicoll et al., 2006; Nicoll et al., 2003; Nitsch and Hock, 2007; Patton et al., 2006), and some suggestion of diminished progressive cognitive decline associated with the disease (Gilman et al., 2005; Hock et al., 2003), although this observation was not universal (Holmes et al., 2008). However, there was also evidence of increased CNS A β by ELISA (Patton et al., 2006), and increased incidence of cerebral vascular A β deposition (Holmes et al., 2008; Masliah et al., 2005; Nicoll et al., 2006; Patton et al., 2006). In the elderly AD patients, there were a low percentage of responders and the generally low titers in response to a self-A β antigen in the AN-1792 vaccine, even in the presence of a very potent adjuvant (Gilman et al., 2005; Patton et al., 2006). These results emphasize the difficulty facing active immunization approaches in elderly AD patients because the elderly generally develop functional deficits in their immune system or immunosenescence (Grubeck-Loebenstein and Wick, 2002). Accordingly, to avoid the problems associated with active immunization of elderly AD patients, recent clinical trials based on passive vaccination (AAB-001) were initiated. In these studies, different concentrations of humanized monoclonal anti-A β antibody are passively transferred to AD patients. However, passive immunotherapy requires the repeated administration of high doses of expensive humanized monoclonal anti-A β antibody. More importantly, this strategy is not likely to be useful for protective vaccination due to the substantial cost, invasive nature of the treatment, and the recurrent clinical visits necessary for effective delivery of the immunotherapy. Thus, there is growing consensus among some researchers based on both analysis of pre-clinical studies (Mamikonyan et al., 2007; Nickolic et al., 2007; Petrushina et al., 2007), as well as from the AN-1792 trial (Holmes et al., 2008; Patton et al., 2006; St George-Hyslop and Morris, 2008) that early preventive immunization prior to substantial neuropathology, neuronal loss, and cognitive deficits have become firmly established may be more effective and safer for future patients receiving immunotherapy. Especially if patients can be identified in a pre-clinical stage by validation of AD biomarkers (de Jong et al., 2006; Fagan et al., 2007a,b; Klunk et al., 2004).

Based on the hypothesis that early treatment is better, we previously proposed an active vaccination strategy based on an epitope vaccine composed of the immunodominant self-B cell epitope of A β ₄₂ and a non-self T helper (Th) cell epitope. We demonstrated the feasibility of this strategy in wild-type (Agadjanyan et al., 2005) mice and then showed the efficacy and safety of epitope peptide vaccine in two different strains of APP/Tg mice (Mamikonyan et al., 2007; Petrushina et al., 2007). However, there are several problems associated with development of an epitope peptide vaccine for human clinical trials. First, there are technical limitations that are difficult to overcome due to the problems encountered in synthesizing large quantities of highly purified peptide epitope vaccines, which are also potent antigens. Secondly, there is the requirement for a potent conventional adjuvant and the only currently approved adjuvant for use in humans is Alum that does not induce robust anti-A β immune responses in APP/Tg mice (Ghochikyan et al., 2006). Our stratagem to overcome these problems was to design a DNA vaccine construct that includes the epitope vaccine concept linked to a potent molecular adjuvant, macrophage-derived chemokine (MDC/CCL21), to induce a robust anti-A β antibody response (Movsesyan et al., 2008). In this report, we have investigated another potential molecular adjuvant, complement (C') component C3d, which has previously been shown to enhance antibody responses to several antigens when three copies of C3d (3C3d)

were combined with the antigen as a fusion protein (Dempsey et al., 1996; Green et al., 2003; Ross et al., 2000). Our DNA epitope vaccine was re-engineered to include 3C3d as the molecular adjuvant and we found that the 3A β_{1-11} -PADRE-3C3d construct dramatically enhanced the anti-A β antibody response in mice compared to the immune response generated with the 3A β_{1-11} -PADRE DNA construct.

2. Materials and methods

2.1. Mice

Eight to ten week-old female C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in a temperature-and light-cycle controlled animal facility at the Institute for Brain Aging and Dementia, UCI. Animal use protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of UCI and were in accordance with guidelines of the National Institutes of Health.

2.2. Generation of plasmids

Two constructs (3A β_{1-11} -PADRE and 3A β_{1-11} -PADRE-3C3d) were generated based on the pSecTag2A mammalian expression vector (Invitrogen, CA). 3A β_{1-11} -PADRE (SfiI/BamHI) was ligated with 3C3d (BglII/NotI) and cloned into the vector using SfiI/NotI restriction sites. GS linker was used between each A β repeat as well as at juncture of C3d and PADRE. Linkers composed of GS(G₄S)₂GS were used between each C3d repeat. Plasmids were verified by appropriate restriction enzyme digestion and gel electrophoresis. Correct sequences of the generated plasmids were confirmed by nucleotide sequence analysis.

2.3. Transfection of cells and detection of plasmid expression by Western blot

CHO cells (8×10^5) were transiently transfected with 4 μ g plasmid DNA by Lipofectamine 2000 Reagent (Invitrogen, CA) according to the manufacturer's recommendation. After 48 h of growth (37 °C, 10% CO₂) in Dulbecco's Modified Eagles Medium supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine (Invitrogen, CA), proteins of interest was recovered from supernatants by immunoprecipitation (IP) with anti-A β antibody and analyzed using 10% SDS-PAGE followed by Western Blot analysis (WB). Supernatants from CHO cells transfected with vector as well as supernatants from non-transfected CHO cells were used as a negative control. Both IP and WB were performed using 6E10 anti-A β_{1-17} monoclonal antibody (Signet, MA). Proteins were visualized with enhanced chemiluminescence detection using Luminol reagent as recommended by manufacturer (Santa Cruz Biotechnology, CA).

2.4. Immunization

Plasmids encoding 3A β_{1-11} -PADRE-3C3d, 3A β_{1-11} -PADRE or the empty vector (pSecTag2A) were purified by Qiagen plasmid maxi kit (Qiagen, CA). The purity and concentration of DNA were measured by DNA gel electrophoresis and optical density reading at 260/280 nm. Three groups of C57BL/6 mice ($n=5$) were immunized biweekly with 3A β_{1-11} -PADRE-3C3d, 3A β_{1-11} -PADRE, or vector (9 μ g/mouse) total of 5 times. Immunizations were performed by gene gun bombardment on shaved abdominal skin as recommended by manufacturer (Bio-Rad, CA) and as previously described (Ghochikyan et al., 2003; Ross et al., 2000). Two independent experiments were performed with $n=5$ per group.

2.5. Detection of anti-A β antibody concentration and isotypes

Eight days after each boost, blood was collected and the serum was analyzed for A β -specific antibodies by ELISA. The assay was performed as described earlier (Cribbs et al., 2003; Ghochikyan et al., 2003). The reaction was developed by adding 3,3',5,5'-tetramethylbenzidine

(TMB) (Pierce, IL) substrate solution and stopped with 2 M H₂SO₄. Plates were read in a spectrophotometer at 450 nm (Biotek, Synergy HT, VT) and antibody concentrations were calculated using a calibration curve generated with known concentrations of 6E10 monoclonal antibody (Signet, MA). The isotypes of anti-A β antibodies were detected using HRP-conjugated anti-IgG1, IgG2a^b, IgG2b and IgM as secondary antibodies (Zymed, CA) as previously described (Agadjanyan et al., 2005; Ghochikyan et al., 2003).

2.6. Detection of T cell response

For analysis of PADRE-specific CD4⁺ T cell proliferation we used the succinimidyl ester of carboxyfluorescein diacetate (CFSE) Flow cytometry assay. Briefly, splenocyte cultures from experimental and control groups were stained with 1 μ M CFSE (Molecular Probes, CA) as recommended by manufacturer. After washing, cells were incubated for 3 days in HL-1 media alone or with PADRE peptide (5 μ M). After incubation, cultures were stained with PE-labeled rat anti-mouse CD4 monoclonal antibodies (BD Pharmingen, CA). Since dead cells might fluoresce nonspecifically, these cells were excluded from the assay by staining with a nucleic acid dye (7-amino actinomycin D, 7-AAD, BD Pharmingen, CA) and proliferation of viable cells was analyzed by FACScan flow cytometer (BD Biosciences, CA) according to the manufacturer's instructions. CD4⁺ T cell population was analyzed using CellQuest software (BD Biosciences, CA). After overlaying of histograms generated with *in vitro* re-stimulated or non-stimulated splenocyte cultures, we calculated the delta (Δ = percent of proliferating CD4⁺ T cells in re-stimulated culture minus that in non-stimulated culture).

2.7. Detection of different cytokines concentrations in splenocyte culture media

Four different cytokines (IL-4, IL-5, IFN- γ and TNF) were simultaneously detected in supernatants from PADRE-re-stimulated or non-re-stimulated splenocyte cultures by Flow cytometry using the Mouse Th1/Th2 Cytokine CBA kit as recommended by manufacturer (BD Biosciences, CA). Concentrations of cytokines were calculated using the calibration curves generated with known concentrations of standards for each cytokine. Ratios of Th2/Th1 cytokines have been calculated by dividing the concentration value of each Th2 cytokine on that of Th1 cytokines.

2.8. Detection of cytokines production by splenocytes

ELISPOT assay was used to determine the number of antigen-specific cells producing cytokines (IFN- γ and IL-4) in PADRE re-stimulated splenocyte cultures from experimental and control mice. Assay was performed according to the manufacturer's recommendation (BD Pharmingen, CA) and as we previously described (Cribbs et al., 2003; Ghochikyan et al., 2006). Spots were counted using a dissecting microscope (Olympus, Japan) by two independent observers. Difference in number of spots per 10⁶ splenocytes in re-stimulated minus non-re-stimulated samples was calculated.

2.9. Testing the functionality of generated anti-A β antibodies

Sera from vaccinated and control mice (at 1 μ g/ml concentration) were added to 50 μ m-thick brain sections of formalin-fixed cortical tissue from patients with severe AD and Tg2576, as well as 3 $\times$ Tg-AD mice. Sections were pretreated with 90% formic acid, and exogenous peroxidase was quenched. We used the same dilutions of the pre-immune sera, and 6E10 monoclonal antibody (Signet, MA) for the negative and positive controls, respectively. For two additional controls, we stained the brain sections from a non-AD patient, as well as that of a non-transgenic mouse (BALB/c). Finally, to test the specificity of binding, immune sera were pre-absorbed for 1 h at 37 °C with A β ₄₂ peptide at 1 μ M final concentration, and adjacent sections of all brains were immunostained in the same experiment. Binding of sera to brain sections was detected by Vectastain Elite ABC Mouse anti-IgG/biotin-avidin/HRP system

with DAB (both from Vector Laboratories, CA) according to the manufacturer's recommendations. A digital camera (Olympus, Japan) was used to capture images of the plaques at 10× original magnification.

2.10. Statistical analysis

GraphPad Prism software was used for statistical analysis of mean, standard deviation, and significant differences. Significant differences between experimental and control groups were analyzed by t test or one-way ANOVA, using Tukey's post-test. Differences were considered statistically significant when P-value was less than 0.05.

3. Results

3.1. Generation of DNA epitope vaccines

To test the potency of the C3d component of *C'* as a molecular adjuvant, we generated a DNA epitope vaccine composed of 3A β ₁₋₁₁-PADRE antigen attached to 3 tandem repeats of C3d (Fig. 1). *In vitro* expression of plasmids encoding 3C3d-fused and non-fused epitope vaccines were analyzed in conditioned media of CHO cells transiently transfected with 3A β ₁₋₁₁-PADRE-3C3d, 3A β ₁₋₁₁-PADRE or pSecTag2A vector by IP and WB using 6E10 monoclonal anti-A β antibody. The analysis revealed a protein of expected size, ~111 kDa, in case of 3A β ₁₋₁₁-PADRE-3C3d and ~8.3 kDa in case of 3A β ₁₋₁₁-PADRE (Fig. 1). A 43 kDa protein detected in CHO cells transfected with 3A β ₁₋₁₁-PADRE-3C3d likely represents a truncated form of the antigen. Importantly, there was no protein of a similar size detected in supernatants of the vector-transfected or non-transfected CHO cells (Fig. 1). Importantly, both plasmids produced approximately the same amount of protein when expressed in CHO cells.

3.2. 3C3d significantly enhances the A β -specific humoral immune response induced by DNA epitope vaccine

Two groups of C57BL/6 mice (*n*=5) were immunized and boosted biweekly by gene gun bombardment with 9 μ g/mouse of 3A β ₁₋₁₁-PADRE-3C3d or 3A β ₁₋₁₁-PADRE expressing DNA alone. Mice in the control group were vaccinated with empty vector. All groups received a total of 5 immunizations. Sera collected from vaccinated mice showed a steady increase in total anti-A β antibody level with each boost for both vaccines, reaching an average of 65 μ g/ml following the final immunization for 3A β ₁₋₁₁-PADRE-3C3d and 34.8 μ g/ml for 3A β ₁₋₁₁-PADRE (Fig. 2A). Although the 3A β ₁₋₁₁-PADRE expressing DNA vaccine induced high levels of anti-A β antibodies, the fusion of this antigen with 3C3d enhanced the titers of these antibodies (*P*<0.01 or *P*<0.001). Interestingly, this molecular adjuvant directed the humoral immunity towards the generation of stronger Th2 type responses as shown by the detection of higher IgG1 than IgG2a^b subclasses. The ratio of IgG1 to IgG2a^b was significantly higher (*P*<0.05) in mice immunized with 3A β ₁₋₁₁-PADRE-3C3d versus 3A β ₁₋₁₁-PADRE (Fig. 2B). Thus, C3d not only increased the anti-A β antibody titer, but also polarized the humoral immune response towards Th2 phenotype, which may help to reduce the incidence of pro-inflammatory-mediated adverse events in elderly AD patients.

3.3. Fusion of the DNA epitope vaccine to 3C3d enhances T cell proliferation and promotes a Th-2 cell phenotype

Several reports indicate that C3d can modulate the immune response, not only on B cells, but also on T cells, and the nature of the antigen fused to 3C3d is crucial for this modulation (Kaya et al., 2001). To determine the effect of 3C3d molecule on T cell responses induced by the DNA epitope vaccine, experimental and control mice were sacrificed on the 10th day after the last boost, and *in vitro* proliferation of PADRE-specific CD4⁺ T cells were analyzed by FACS. Although both vaccines induced considerable antigen-specific CD4⁺ T cell proliferation,

3A β_{1-11} -PADRE fused with 3C3d induced a significantly greater ($P<0.01$) response (5.3%) versus 3A β_{1-11} -PADRE (2.3%) (Fig. 3).

To determine the phenotype of Th response we first analyzed production of IL-4 and INF- γ cytokines in the cultures of re-stimulated immune and control splenocytes using an ELISPOT assay. We detected approximately equal numbers of INF- γ producing splenocytes isolated from mice immunized with both types of DNA epitope vaccines (Fig. 4). However, the DNA epitope vaccine fused with 3C3d generated significantly higher numbers of immune cells producing IL4 cytokines. These data were confirmed by measuring the concentrations of four different cytokines in the supernatants of re-stimulated splenocytes (Fig. 5). Thus, both DNA epitope vaccines induced Th1 and Th2 cytokines (Fig. 4), however the 3C3d molecular adjuvant stimulated production of IL-4 and IL-5 to a higher degree than the 3A β_{1-11} -PADRE construct (Table 1). Therefore, fusion of 3C3d to the DNA epitope vaccine biased the cellular immunity towards an anti-inflammatory Th2-type response, even in a Th1-prone mouse strain (Gessner et al., 1993; Huang et al., 1997; Nogaki et al., 2000).

3.4. Anti-A β antibodies from immune mice are therapeutically functional

Brain sections from an AD case, as well as brain sections from transgenic mice, were used for testing the binding ability of anti-A β antibodies from sera of vaccinated animals. As shown in Fig. 6, sera of mice immunized with 3A β_{1-11} -PADRE-3C3d bound to A β plaques in AD brain sections (Fig. 6A), as well as to brain sections of 3 $\times$ Tg-AD and Tg2576 transgenic mice (Fig. 6B), demonstrating the potential therapeutic efficacy of these antibodies. The non-AD brain sections and non-transgenic BALB/c mouse brain sections were used as negative controls for non-specific binding. As a positive control for staining of A β plaques, 6E10 anti-A β monoclonal antibodies were used. Sera from vector-injected mice did not show any specific binding (data not shown). The specificity of antibodies was further confirmed by incubation of immune sera with A β_{42} peptide prior to staining of the sections, which completely eliminated recognition of plaques in AD and transgenic mice brain tissues. Sera from 3A β_{1-11} -PADRE immunized mice demonstrated similar patterns of A β plaque immunostaining (data not shown).

4. Discussion

Previously, we reported that a DNA vaccine expressing full-length A β_{42} peptide could induce a significant anti-A β antibody response in wild-type mice (Ghochikyan et al., 2003). However, we subsequently found that the same DNA vaccine induced only very low titers of anti-A β antibodies in Tg2576 mice (Cribbs and Agadjanyan, 2005). Several groups confirmed our results on the generation of low titers of antibodies after DNA vaccination (Okura et al., 2006; Qu et al., 2004; Schultz et al., 2004). Additionally, they suggested that these DNA vaccines did not generate autoreactive T cells. However, A β_{42} is a T-dependent antigen, since immunization in two types of nude mice with fibrillar A β_{42} peptide formulated in a strong conventional adjuvant did not induce any humoral responses (data not shown). Therefore, it is not surprising that A β_{42} based DNA vaccines did not induce detectable anti-self T helper responses since these vaccines did not generate high titers of anti-A β antibodies. Of note, pre-clinical studies on AD mouse models (Chen et al., 2007; Petrushina et al., 2007) and data from the AN-1792 trials (Holmes et al., 2008; Patton et al., 2006) demonstrated a correlation between high titers of anti-A β antibodies and a reduction of A β plaque pathology. These data implied that effective and safe DNA vaccination should be able to induce therapeutically potent titers of anti-A β antibodies in the absence of autoreactive T cells.

To generate a vaccine that circumvents the activation of autoimmune T cells, but is still capable of inducing high titers of therapeutically potent anti-A β antibodies, we recently developed and tested a DNA epitope vaccine composed of a strong foreign T helper epitope fused with a B cell epitope of A β_{42} (Movsesyan et al., 2008). To enhance immune responses, we included in

this DNA vaccine a molecular adjuvant, macrophage-derived chemokine (MDC/CCL22). In the current study, we attempted to use another fusion protein construct to augment antibody responses to the A β -B cell epitope by using C3d as a molecular adjuvant.

A major function of complement, an important component of the innate immune response system, is the opsonization of antigen-immune complexes. This is partially mediated by the covalent attachment of cleavage fragments of C3 and C4 to the surface of pathogens, which links the innate and the adaptive immune responses by targeting immune complex to specific complement receptors type 1 (CD35) and type 2 (CD21) (Carroll, 1998; Fearon, 2000). The CD21 expressed on mature B cells as a non-covalent complex with CD19 that functions as a specialized membrane adaptor protein for antigen-specific B cell receptors (BCR) (Ahearn et al., 1996; Croix et al., 1996). Simultaneous engagement of BCR and CD21/CD19 by antigen-C3d complex dramatically reduced the BCR:antigen affinity threshold, which significantly prolongs BCR residency in lipid rafts, and induces much stronger B cell activation than antigen alone (Carter and Fearon, 1992; Cherukuri et al., 2001b; Mongini et al., 1997). Thus, immunization of mice with recombinant hen egg lysozyme containing 3 copies of C3d molecule dramatically enhanced (10,000-fold) antibody formation (Dempsey et al., 1996). More recently, we and others showed that the efficacy of DNA vaccines encoding antigens from the different pathogens (influenza, HIV, pseudorabies virus (PrV) gC) dramatically increased after fusion of these immunogens with 3C3d molecular adjuvant (Mitchell et al., 2003; Ross et al., 2000, 2001; Tong et al., 2006).

In this report, we propose to take advantage of the capacity of C3d to act as a molecular adjuvant that can amplify the humoral response to the self-antigen, A β . A plasmid, encoding the 3A β ₁₋₁₁-PADRE epitope vaccine was fused with 3C3d and its potential to induce anti-A β antibodies were compared with non-3C3d fused vaccine (Fig. 1). To exclude the differences in antibody responses caused by different levels of protein expression, we tested the expression of both plasmids in CHO cells and demonstrated that they provide comparable levels of protein expression and secretion (Fig. 1). Mice immunized with the DNA epitope vaccine fused with 3C3d generated significantly higher titers anti-A β antibodies than the epitope vaccine alone (Fig. 2). Because antibody isotyping can be used as an indirect measure of the contribution of Th1 (IgG2a) and Th2 (IgG1) cytokines to the immune response (Finkelman et al., 1990), we used this methodology to investigate the Th phenotype induced by the two different DNA vaccines. Fusion of 3A β ₁₋₁₁-PADRE with 3C3d molecular adjuvant dramatically changed the ratio of IgG1/IgG2a^b anti-A β antibodies, demonstrating that the 3C3d-modified epitope vaccine induced predominantly anti-inflammatory immune responses. The therapeutic potency of anti-A β antibodies generated in mice immunized with our DNA epitope vaccine was demonstrated by its binding to A β plaques in brain tissues from an AD case and two different mouse models of AD (Fig. 6).

Targeting of the antigen to CD21 by C3d tagging can increase antigen processing and presentation by all B cells regardless of their specificity (Cherukuri et al., 2001a; Thornton et al., 1994), as well as professional APC (follicular dendritic cells) (Fearon, 2000). Thus, we hypothesized that our epitope vaccine may increase activation of anti-A β B cells, but also increase presentation of immunogen and subsequently augment the stimulation of T cells. The higher percent of proliferating PADRE-specific CD4⁺T cells in splenocyte culture of mice immunized with p3A β ₁₋₁₁-PADRE-3C3d (Fig. 3) suggests that this enhancement may be associated with more effective presentation of antigen. In this report, we demonstrated that supplementation of our DNA epitope vaccine with 3C3d enhanced the humoral (Fig. 2) and cellular (Fig. 3) immunity, but also polarized the immune response towards an anti-inflammatory Th2 phenotype (Figs. 4, 5, Table 1). Currently, we are testing the therapeutic potency of the p3A β ₁₋₁₁-PADRE-3C3d vaccine construct in the Tg2576 mouse model of AD.

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Fig. 1.

Schematic representation and *in vitro* expression of plasmids encoding AD epitope vaccines. (A) 3Aβ₁₋₁₁-PADRE and 3Aβ₁₋₁₁-PADRE-3C3d genes were cloned into the pSecTag2A mammalian expression vector containing CMV promoter for initiation and BGH polyadenylation site for termination of transcription. Fusion to Ig κ chain signal sequence allows the expressed proteins to be secreted through the cell membrane. (B) Both 3Aβ₁₋₁₁-PADRE-3C3d and 3Aβ₁₋₁₁-PADRE proteins were expressed in CHO cells transfected with appropriate plasmids. WB of supernatants following IP with 6E10 antibodies revealed the expression of both 3Aβ₁₋₁₁-PADRE-3C3d (111 kDa — Lane 4) and 3Aβ₁₋₁₁-PADRE (8.3 kDa — Lane 3) proteins. No expression was observed in controls: supernatants of CHO cells transfected with vector (Lane 2) and non-transfected CHO cells (Lane 1).

Fig. 2. 3C3d molecular adjuvant significantly enhances anti-A β antibody response induced by DNA epitope vaccine and dramatically increased IgG1 (Th2)/IgG2a^b (Th1) ratio. (A) Concentrations of anti-A β antibodies in the sera of mice immunized with 3C3d-fused epitope vaccine was significantly higher than that in mice immunized with epitope vaccine lacking 3C3d (** $P < 0.01$, *** $P < 0.001$). (B) Isotype analysis of anti-A β antibodies demonstrated that the immune response in mice immunized with 3C3d-modified epitope vaccine were significantly more Th2 polarized (ratio of IgG1/IgG2a^b) (* $P < 0.05$). Sera from mice immunized with empty vector, as well as a construct expressing 3C3d fused with irrelevant antigen did not contain any detectable

A β -specific antibodies (data not shown). Average data and SD from two experiments are shown ($n=5$ for each group in each experiment).

Fig. 3.

Fusion of epitope vaccine with 3C3d molecular adjuvant increases the activation of PADRE-specific Th cells. Proliferation of antigen-specific CD4⁺ T cells detected by CFSE FACS assay were significantly higher (** $P < 0.01$) in mice immunized with 3Aβ₁₋₁₁-PADRE-3C3d compared to mice immunized with 3Aβ₁₋₁₁-PADRE. Data shown in the figure was the Δ percent, which was calculated as a percent of proliferating CD4⁺ T cells in peptide re-stimulated cultures minus that in non-re-stimulated cultures. This experiment was performed twice ($n=5$ for each group in each experiment), and representative data from one experiment is shown.

Fig. 4.

Epitope vaccine fused with 3C3d induces predominantly IL-4-producing splenocytes. To determine phenotype of Th response we analyzed production of IL-4 and INF- γ cytokines in the culture of re-stimulated immune and control splenocytes using ELISPOT assay. Both 3C3d-fused and non-fused DNA epitope vaccines induced similar numbers of splenocytes producing INF- γ (Th1) cytokine. However, the addition of molecular 3C3d to the composition of DNA epitope vaccine significantly increased the numbers of IL-4-producing cells (** $P < 0.01$). The splenocytes isolated from mice injected with a vector control did not respond to *in vitro* re-stimulation with PADRE (see details in Materials and methods). These experiments were performed twice ($n=5$ per group) and average and SD of two experiments is shown.

Fig. 5.

Fusion of 3C3d to DNA epitope vaccine drives the cellular immunity towards the desired anti-inflammatory Th2 type response. Concentrations of four different cytokines in splenocyte cultures of immunized and non-immunized mice were detected using Mouse Th1/Th2 Cytokine CBA kit by flow cytometry. The DNA epitope vaccine fused or non-fused with 3C3d increased concentrations of both Th1 and Th2 cytokines. However, 3C3d stimulated production of IL-4 and IL-5 to a significantly higher degree (* $P < 0.05$), while differences in production of INF- γ and TNF were not significant. This experiment was performed twice ($n=5$ for each group in each experiment) with similar results.

Fig. 6.

Immune sera generated against the DNA vaccine fused with 3C3d are therapeutically functional based on binding to amyloid plaques. At a concentration of 1 µg/ml anti-Aβ antibodies bind to amyloid plaques on the brain section of cortical tissues from an AD case (A) and two mouse models of AD (B). These antibodies did not bind control brain sections from a non-AD case or wild-type mice (A, B). The binding of antisera collected from mice immunized with the plasmid encoding 3Aβ₁₋₁₁-PADRE-3C3d to the amyloid-β-plaques was specific, because binding was blocked by pre-absorption of the sera with 1 µM Aβ₄₂ peptide (A, B). The original magnification is 20× and the scale bar is 50 µm. All sections are from the cortex area, both human and mouse brain tissues.

Table 1

Detection of Th2/Th1 type cytokines ratios in mice immunized with DNA vaccine fused or non-fused with 3C3d molecular adjuvant

Vaccine	Th2/Th1 cytokine ratio			
	IL-4/IFN- γ	IL-5/IFN- γ	IL-4/TNF	IL-5/TNF
3A β_{1-11} -PADRE-3C3d	0.65	1.1	0.97	1.64
3A β_{1-11} -PADRE	0.21	0.63	0.41	1.23