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Enhancing Th2 immune responses against amyloid protein by a DNA prime-adenovirus boost regimen for Alzheimer's disease

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Abstract

Accumulation of aggregated amyloid β -protein ($A\beta$) in the brain is thought to be the initiating event leading to neurodegeneration and dementia in Alzheimer's disease (AD). Therefore, therapeutic strategies that clear accumulated $A\beta$ and/or prevent $A\beta$ production and its aggregation are predicted to be effective against AD. Immunization of AD mouse models with synthetic $A\beta$ prevented or reduced $A\beta$ load in the brain and ameliorated their memory and learning deficits. The clinical trials of $A\beta$ immunization elicited immune responses in only 20% of AD patients and caused T-lymphocyte meningoencephalitis in 6% of AD patients. In attempting to develop safer vaccines, we previously demonstrated that an adenovirus vector, AdPEDI-($A\beta$ 1-6)₁₁, which encodes 11 tandem repeats of $A\beta$ 1-6 can induce anti-inflammatory Th2 immune responses in mice. Here, we investigated whether a DNA prime-adenovirus boost regimen could elicit a more robust Th2 response using AdPEDI-($A\beta$ 1-6)₁₁ and a DNA plasmid encoding the same antigen. All mice ($n = 7$) subjected to the DNA prime-adenovirus boost regimen were positive for anti- $A\beta$ antibody, while, out of 7 mice immunized with only AdPEDI-($A\beta$ 1-6)₁₁, four mice developed anti- $A\beta$ antibody. Anti- $A\beta$ titers were indiscernible in mice ($n = 7$) vaccinated with only DNA plasmid. The mean anti- $A\beta$ titer induced by the DNA prime-adenovirus boost regimen was approximately 7-fold greater than that by AdPEDI-($A\beta$ 1-6)₁₁ alone. Furthermore, anti- $A\beta$ antibodies induced by the DNA prime-adenovirus boost regimen were predominantly of the IgG1 isotype. These results indicate that the DNA prime-adenovirus boost regimen can enhance Th2-biased responses with AdPEDI-($A\beta$ 1-6)₁₁ in mice and suggest that heterologous prime-boost strategies may make AD immunotherapy more effective in reducing accumulated $A\beta$.

Keywords

DNA vaccine; prime-boost strategies; adenovirus; Alzheimer's disease; IL-4; IL-10

1. Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disorder characterized by the progressive loss of memory and cognitive functions. Cardinal pathological changes found in the brains of patients with AD are loss of neurons and synaptic connections, neurofibrillary tangles and deposits of aggregated amyloid β -protein ($A\beta$) in neuritic plaques and cerebral vessels. The precise molecular mechanisms leading to such pathological and functional alterations are unknown and, to date, no satisfactory treatments are available for AD. The

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predominant hypothesis to explain the etiology of AD is the amyloid cascade hypothesis which states that accumulation of aggregated A β is the initiating event leading to neurodegeneration and dementia [1]. Therefore, therapeutic strategies that clear accumulated A β and/or prevent A β formation and its aggregation are predicted to be effective against AD [2].

Immunotherapy may be one of the most promising approaches to prevent and remove accumulation of aggregated A β [3]. This notion is supported by a number of animal experiments demonstrating the efficacy of immunotherapy. Parenteral immunization of AD mouse models with synthetic A β prevented or reduced A β deposits and improved their memory and learning deficits [4-6]. These impressive results prompted initiation of phase I/II clinical trials to evaluate the safety and tolerability of aggregated A β (AN1792) vaccination. The phase IIa clinical trial, however, was halted due to aseptic T-lymphocyte meningoencephalitis found in 6% of AD patients [7-9]. The initial report of the AN1792 clinical trial with a limited number of AD subjects by Hock et al. [10] indicated that responders showed significantly slower rates of decline in cognitive functions and activities of daily living. Recently, Gilman et al. [11] reported that the antibody responders had better scores only in a Neuropsychological Test Battery, but did not show improvements in other cognitive and functional tests compared with placebo groups. However, it was concluded that A β immunotherapy might be useful in AD. Neuropathologic analyses of three AD patients subjected to the clinical trial verified a profound reduction in amyloid plaques and astrogliosis, while neurofibrillary tangles and amyloid deposits in blood vessels remained [8,9,12]. These findings closely resemble the changes seen after immunotherapy in AD mouse models, indicating that immune therapy is effective in removing A β plaques in humans. Because peripheral administration of antibodies against A β also induced clearance of preexisting amyloid plaques in an AD mouse model [13] and because meningoencephalitis associated with the AN1792 trial is thought to be T-cell-mediated autoimmune responses, immunization modalities that elicit predominantly T helper (Th) type 2 (B cell) immune responses are considered to be safer for AD prevention and treatment [14-18].

In order to induce Th2-polarized immune responses, some groups used B cell epitopes of A β [19] such as A β 1-15 [20-22], Th2-type adjuvants such as interleukin-4 [23,24], Alum [20, 25], mannan [26] monophosphoryl lipid A, cholera toxin B subunit and *E. coli* enterotoxin [27] and mucosal vaccination [28]. In attempting to induce such safer vaccines, we chose A β 1-6 as an antigen because A β 1-15 has been identified as a B cell epitope and A β 6-28 contains a T cell epitope [19]. We constructed cDNA encoding eleven tandem repeats of A β 1-6, (A β 1-6)₁₁, to overcome the hurdle of A β 's low immunogenicity (self-peptide) because tandem repeats of a small self-peptide are reported to permeate self tolerance [29]. Furthermore, we added the receptor-binding domain (Ia) of Pseudomonas exotoxin A (PEDI) as an adjuvant to (A β 1-6)₁₁ in order to facilitate receptor mediated endocytosis by antigen-presenting cells [29]. Thus, we produced an adenovirus vector, AdPEDI-(A β 1-6)₁₁, as a vaccine for delivery of a fusion protein of PEDI and (A β 1-6)₁₁, and showed that nasal vaccination with AdPEDI-(A β 1-6)₁₁ induced Th2-polarized responses in several mouse strains [30] and reduced cerebral A β load in an AD mouse model [31]. Qu et al. [32] and Okura et al. [33] also showed that plasmid DNA encoding A β elicited B cell immune responses without a significant T-cell-mediated immune response to A β in mice. Thus, DNA-vectored vaccines can be safer modalities for AD.

Another obstacle for A β -based immunotherapy is the difficulty to induce an appropriate anti-A β titer in AD patients. In the phase II clinical trial, 19.7% of AD patients developed a positive A β titer [11]. Heterologous prime-boost strategies are powerful vaccination regimens to induce very strong immune responses. The strategies involve the administration of two different vaccines, each expressing the same antigen, given several weeks apart. Most often, immune responses are primed by DNA vaccines and boosted by viral vaccines carrying the same antigens. The efficacy of this approach was first reported by Schneider et al. [34]. They

demonstrated that vaccination of the murine model of malaria with a DNA vaccine (priming) followed by a recombinant modified vaccinia virus as a booster, both of which encoded a malaria antigen, induced higher immune responses than homologous prime-boost regimens and unprecedented levels of protection against challenge. In addition, a number of investigators have shown that prime-boost strategies elicit greater levels of immunity to a variety of tumors and pathogens than homologous prime-boost strategies or a single vaccination of the same vector [35,36]. Therefore, we have investigated whether a DNA prime-adenovirus boost regimen can enhance vaccine efficacy for induction of humoral immune responses against A β in mice using AdPEDI-(A β 1-6)₁₁ and plasmid DNA encoding the same antigen.

2. Materials and methods

2.1. Plasmid and Adenovirus vectors

Construction and preparation of a plasmid, pCA-PEDI-(A β 1-6)₁₁, and an adenovirus vector, AdPEDI-(A β 1-6)₁₁, were described previously [30]. In brief, cDNA for a fusion protein of the receptor-binding domain (Ia) of Pseudomonas exotoxin A (PEDI) and eleven-tandem repeats of A β 1-6 was placed under the control of the cytomegalovirus enhancer/ β -actin (CA) promoter in pCA-PEDI-(A β 1-6)₁₁. The DNA fragment containing the CA promoter, cDNA for PEDI-(A β 1-6)₁₁ and β -globin poly A signal was isolated from pCA-PEDI-(A β 1-6)₁₁ by Sal I and Hind III digestion and cloned into the Xho I (compatible with Sal I) and Hind III site of pShuttle plasmid (AdEasy® Basic Kit, American Type Culture Collection, Manassas, VA) to generate pShut-CA-PEDI-(A β 1-6)₁₁. Other than CA-PEDI-(A β 1-6)₁₁, pShut-CA-PEDI-(A β 1-6)₁₁ contained adenovirus serotype 5 (Ad5) right and left arm homology sequences, left and right Ad5 inverted terminal repeat (ITR), encapsidation signal, pBR322 origin of replication and the kanamycin resistance gene. A recombinant adenovirus plasmid was generated by homologous recombination between pShut-CA-PEDI-(A β 1-6)₁₁ and pAdEasy-1 (AdEasy® Basic Kit) in *E. coli* BJ5183 cells and, then, used to produce AdPEDI-(A β 1-6)₁₁ in HEK293 cells as described previously [37,38].

2.2. Western blot analysis

Expression levels of PEDI-(A β 1-6)₁₁ in HEK 293 cells after transfection with pCA-PEDI-(A β 1-6)₁₁ or pShut-CA-PEDI-(A β 1-6)₁₁ were determined by western blot analysis as previously described [39]. In brief, HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in 6 well plates and transfected with 2 μ g of pCA-PEDI-(A β 1-6)₁₁ or pShut-CA-PEDI-(A β 1-6)₁₁ using metafectene (Biontex, Munich, Germany) according to the manufacturer's protocol. Seventy-two hours after transfection, the cells were harvested and the proteins in the cells were separated by 4-15% SDS-PAGE. After electrotransfer to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA), expressed PEDI-(A β 1-6)₁₁ was visualized using the enhanced chemiluminescence system (Amersham, Arlington Heights, IL) and a rabbit polyclonal anti-A β serum (Zymed Lab, South San Francisco, CA). The membranes were reprobbed with monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (cell lysates) (Chemicon International, Temecula, CA). The relative concentrations of PEDI-(A β 1-6)₁₁ were determined by densitometric scanning of the membranes using HP Scanjet 4470c and a HP Precisionscan Pro 3.1 (Hewlett-Packard Development Company, Houston, TX) and normalized by the GAPDH signals. Three independent transfection experiments for each expression vector were performed.

2.3. Experimental animals

Groups of seven 2-month-old C57BL/6J female mice (Jackson Laboratory, Bar Harbor, ME) were immunized nasally with two doses of pCA-PEDI-(A β 1-6)₁₁ or pShut-CA-PEDI-(A β 1-6)₁₁ and the mice were boosted with two doses of AdPEDI-(A β 1-6)₁₁. In addition, seven

naïve mice were vaccinated nasally with two doses of AdPEDI-(A β 1-6)₁₁, pCA-PEDI-(A β 1-6)₁₁ or pShut-CA-PEDI-(A β 1-6)₁₁. As a control group, seven naïve mice were treated with two doses of PBS.

In order to test amyloid plaque immunoreactivity of sera obtained from mice subjected to the treatment regimens, a transgenic mouse model of Alzheimer's disease, Mo/Hu APPswe PS1dE9 [40], was used to obtain the brain sections for immunohistochemistry.

All animal protocols used for this study were prospectively reviewed and approved by the Institutional Animal Care and Use Committee of the University of Illinois College of Medicine at Peoria.

2.4. Immunization protocol

Intranasal inoculation was carried out by pipetting 100 μ g DNA plasmid in 20 μ l PBS into one of the nostrils of an anesthetized mouse at days 0 and 7 (Fig. 2). Intranasal inoculation of adenovirus vector AdPEDI-(A β 1-6)₁₁ (1×10^8 PFU, Plaque Forming Unit in 20 μ l PBS) was performed at days 14 and 21 (Fig. 2). Blood was collected through the tail at days 0, 14, 28, and 42.

2.5. ELISA for determination of serum titers

After incubating blood at 4°C for 3 h, the blood was centrifuged for 10 min at 10,000g. The serum was collected and frozen at -80°C. Anti-A β immunoglobulin (IgM, IgG, IgG1, IgG2a and IgG2b) titers were determined by enzyme-linked immunosorbent assay (ELISA). Microtiter wells were coated with A β 1-42 (5 μ g/ml) in 50 mM carbonate buffer pH 9.6 overnight at 4°C and rinsed three times with washing buffer [phosphate-buffered saline (PBS) containing 0.05% Tween-20]. Microtiter wells were treated with blocking buffer (5.0% goat serum, 1% BSA and 0.05% Tween-20 in PBS) for 2 h at room temperature. The serum samples were diluted with PBS and added to the microtiter wells. After incubation for 2 h at room temperature, the plates were washed five times with the washing buffer and incubated for 1 h with an appropriate horseradish peroxidase (HRP)-conjugated detection antibody. The detection antibodies (Zymed, South San Francisco, CA) were diluted in the blocking buffer at 1:2,000 for anti-mouse IgG and IgM and 1:1,000 for anti-mouse IgG1, IgG2a, and IgG2b. After washing, plates were incubated with 3,3',5,5'-tetramethylbenzidine (TMB) (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) for 15 min and the reaction was stopped with the addition of 1N H₂SO₄. Optic densities at 450 nm were determined using a Microplate Reader (Labsystems, Finland). Anti-A β antibody titers in the mouse sera were determined using serial dilutions of 6E10 (monoclonal anti-A β antibody) as the standard. Therefore, the concentrations (μ g/ml) of the serum titers presented here reflect the concentrations of 6E10 antibody, which produce the same ELISA readings, and may not accurately represent the absolute amounts. The linear regression was found between 0.1 and 24 ng/ml of IgG. The minimum detectable anti-A β titer was 0.1 ng/ml which was determined by adding two standard deviations to the mean absorbance obtained when the zero standard was assayed 32 times. Comparison of treatment groups was performed by ANOVA and two-tailed Student's t-test. $P < 0.05$ was considered statistically significant.

2.6. Cytokine-specific ELISA

Interferon- γ (IFN- γ), interleukin (IL)-2, IL-4, and IL-10 in mouse sera were determined by the BD OptEIA ELISA system (BD Biosciences - Pharmingen, San Jose, CA) according to the manufacturer's protocol using TMB as a chromogen. Optic densities at 450 nm were determined using a Microplate Reader (Model 680XR, BioRad) within the linear regression. Each sample was measured in triplicates. The minimum detectable concentrations were 3.1 pg/ml for IL-2, 31.3 pg/ml for IFN- γ , 7.8 pg/ml for IL-4, and 31.3 pg/ml for IL-10.

2.7. Immunoreactivity of antisera to amyloid plaques in the brain

A 7-month-old-transgenic mouse (Mo/Hu APPswe PS1dE9) was deeply anesthetized and perfused transcardially with 4% paraformaldehyde. The brain was removed and postfixed in the perfusate for 16 h. The brain was then stored overnight in 30% sucrose in 0.1 M PBS. Coronal sections (5 μ m thick) were cut by a cryostat and prepared for immunofluorescent labeling of amyloid plaques using different dilutions of the sera from vaccinated mice. After quenching autofluorescence by 10 mg/ml sodium borohydride (NaBH₄) and/or 0.05% Sudan Black B, the sections were washed with 0.5% Triton-X in PBS and blocked with 10% normal goat serum in PBS for 60 min at room temperature. After washing with PBS, the sections were incubated with diluted sera in PBS containing 10% normal goat serum for 16 h at 4°C and, then, with goat anti-mouse IgG1 antibody conjugated with Alexa Fluor 488 (Molecular Probe, Eugene, OR) for 1 h at room temperature. The sections were washed with PBS, mounted in 0.1% *p*-phenylene diamine in 10% PBS and 90% glycerol and observed under Olympus IX71 automated fluorescence microscope. The sera from PBS-treated mice were used as controls. The pictures were taken through an Olympus DP70 digital camera system.

3. Results

3.1. Confirmation of antigen expression from plasmid DNAs

To determine expression levels of PEDI-(A β 1-6)₁₁ in cultured cells, HEK293 cells were transiently transfected with pCA-PEDI-(A β 1-6)₁₁ or pShut-CA-PEDI-(A β 1-6)₁₁. The levels of PEDI-(A β 1-6)₁₁ were determined by western blot analysis using a polyclonal antibody against A β and normalized by GAPDH expression (Fig. 1 A and B). HEK293 cells transfected with pCA-PEDI-(A β 1-6)₁₁ expressed significantly more PEDI-(A β 1-6)₁₁ than cells transfected with pShut-CA-PEDI-(A β 1-6)₁₁ ($P < 0.05$, Student's *t*-test). Thus, higher antigen expression was achieved by pCA-PEDI-(A β 1-6)₁₁ in cultured cells.

3.2. Immune response to immunization

Two groups of seven mice were nasally immunized twice at days 0 and 7 with either pCA-PEDI-(A β 1-6)₁₁ or pShut-CA-PEDI-(A β 1-6)₁₁ plasmid DNA. None of the mice in these groups had detectable serum titers by A β -specific ELISA at days 28 and 42. No further experiments were performed on these groups. Out of seven mice subjected to nasal vaccination of AdPEDI-(A β 1-6)₁₁ at days 14 and 21, four had measurable serum IgG titers by A β -specific ELISA; on average, 0.86 and 2.79 μ g/ml at day 28 and 42, respectively (Fig. 2). Two groups of seven mice were nasally vaccinated with either pCA-PEDI-(A β 1-6)₁₁ or pShut-CA-PEDI-(A β 1-6)₁₁ twice at days 0 and 7 and then boosted with AdPEDI-(A β 1-6)₁₁ at days 14 and 21. All mice in the latter two groups had readily detectable serum titers by A β -specific ELISA at days 28 and 42. The mean serum titers of mice subjected to pShut-CA-PEDI-(A β 1-6)₁₁ prime-adenovirus boost vaccination were 2.20 and 10.2 μ g/ml at day 28 and 42, respectively. The mean serum titer induced by pShut-CA-PEDI-(A β 1-6)₁₁ prime-adenovirus boost vaccination was significantly higher than that by AdPEDI-(A β 1-6)₁₁ only at day 42 ($P < 0.05$) (Fig. 2). The mean serum titers of mice subjected to pCA-PEDI-(A β 1-6)₁₁ prime-adenovirus boost vaccination were 5.78 and 19.5 μ g/ml at days 28 and 42, respectively and significantly greater compared with mice subjected to pShut-CA-PEDI-(A β 1-6)₁₁ prime-adenovirus boost vaccination ($P < 0.05$) (Fig. 2). Thus, the heterologous prime-boost regimens elicited anti-A β antibody in all mice and greater serum titers compared to the homologous prime-boost regimens.

3.3. IgG isotype profile

Immunoglobulin isotype-specific anti-A β titers were quantified by ELISA. In mice, the production of IgG1 is primarily induced by Th2-type cytokines, while IgG2a is produced

through Th1-type cytokines. Nasal vaccination with only AdPEDI-(A β 1-6)₁₁ elicited a predominant IgG1 response in C57BL/6 mice (Fig. 3A). The IgG1/IgG2a ratio at day 42 was 6.2 for only adenovirus. The induced anti-A β antibodies by the DNA prime-adenovirus boost regimens were predominantly of the IgG1 isotype, also (Fig. 3B and C). A β specific IgM titers were on average lower than other isotype-titers for DNA prime-adenovirus boost regimens. The IgG1/IgG2a ratios at days 28 and 42 were approximately 2.2 and 3.3 for the pShut-CA-PEDI-(A β 1-6)₁₁ prime-adenovirus boost regimen and 3.2 and 3.0 for the pCA-PEDI-(A β 1-6)₁₁ prime-adenovirus boost regimen, respectively. These results suggest that the DNA prime-adenovirus regimens induce Th2-biased immune responses.

3.4. Serum cytokine responses

IL-4 and IL-10 are specifically produced by Th2 cells, while Th1 cells are characterized by secretion of IFN- γ and IL-2. Serum levels of cytokines at days 0 and 42 were quantified by ELISA in mice treated with the pCA-PEDI-(A β 1-6)₁₁ prime-adenovirus boost regimen (Fig. 4) and with PBS (data not shown). Serum levels of IL-10 (1.7 ng/ml) at day 42 in mice subjected to the pCA-PEDI-(A β 1-6)₁₁ prime-adenovirus boost regimen were significantly higher than those in the naïve (preimmune) and PBS-treated mice (< 0.25 ng/ml, $P < 0.002$). The levels of IL-4 at day 42 in the mice treated with the pCA-PEDI-(A β 1-6)₁₁ prime-adenovirus boost regimen were greater than those (less than detectable) in the naïve and PBS-treated mice ($P < 0.003$). There were no differences in levels of serum IFN- γ and IL-2 between the mice treated with the pCA-PEDI-(A β 1-6)₁₁ prime-adenovirus boost regimen and the naïve or PBS-treated mice ($P > 0.05$). Thus, the pCA-PEDI-(A β 1-6)₁₁ prime-adenovirus boost regimen upregulated mainly IL-10 in C57BL/6 mice.

3.5. Induced anti-A β antibodies are immunoreactive to A β deposits in the brain

Frozen brain sections from a 7-month old Mo/Hu APPswe PS1dE9 mouse were immunostained with sera (day 42) from the mice treated with the pCA-PEDI-(A β 1-6)₁₁ prime-AdPEDI-(A β 1-6)₁₁ boost regimen and with only AdPEDI-(A β 1-6)₁₁. Both sera reacted with amyloid plaques in the brain (Fig. 5A and B), whereas sera from mice administered with PBS failed to label amyloid plaques (Fig. 5C). The sera from the DNA prime-adenovirus boost regimen seemed to visualize more amyloid plaques than those from only adenovirus treatment. The results indicate the specificity of anti-sera against amyloid deposits in the brain and suggest that the heterologous prime-boost regimen may be superior to the homologous counterpart.

4. Discussion

The clinical trials of peptide A β vaccine (AN1792) brought to light the problems associated with the modality; A β immunization caused aseptic T cell-mediated meningoencephalitis in 6% of AD patients and induced anti-A β antibody in only 20% of AD patients [11]. Because passive immunization with anti-A β antibodies was effective in clearing A β deposits in AD mouse models, vaccination modalities which induce humoral (Th2-type) immune responses are currently exploited in order to avoid the adverse effect [14-18]. As we demonstrated previously, nasal administration of AdPEDI-(A β 1-6)₁₁ can elicit predominantly humoral immune responses in several strains of mice [30,31]. In this study, we show that the DNA prime-adenovirus boost regimen delivering A β 1-6 elicited anti-A β antibodies composed predominantly of IgG1, suggesting induction of Th2-biased responses. This heterologous prime-boost regimen increased the number of mice positive for anti-A β antibody, the value of the antibody titer and the affinity of the antibody to amyloid plaques. Thus, the DNA prime-adenovirus boost regimen may be more efficacious than its homologous counterparts in reducing accumulated A β in AD patients as well as AD mouse models.

The vast majority of the research demonstrated that heterologous prime-boost regimens elicit robust cellular immune responses [35,36,41]. A number of DNA prime-adenovirus boost regimens were intended to induce cellular immune responses through intramuscular injection [42-49]. Some research groups, however, reported that DNA prime-virus boost regimens stimulated strong humoral immune responses. Chikhlikar et al. [50] demonstrated that immunization of BALB/c mice with the DNA prime-recombinant adeno-associated virus boost regimen induced a markedly increased antibody response, mainly of the IgG1 isotype resulting from the activation of the Th2 subset of CD4⁺ T cells. They used DNA encoding a chimeric protein of HIV-1 Gag and lysosomal associated membrane protein-1. When BALB/c mice were primed with both plasmid DNA encoding HIV-1 Gag and CpG followed by adenovirus boost, robust humoral and cellular immune responses were elicited [51]. A DNA prime-adenovirus boost regimen against domain four of *Bacillus anthracis* protective antigen induced both humoral and cellular immune responses in A/J mice [52]. Rollier et al. [53] reported that the DNA prime-adenovirus boost regimen against nonstructural protein 3 of hepatitis C virus induced B and T cell responses in rhesus macaques and that adjuvantation with Th1 cytokines (IL-2- and IL-12) reduced Th2 responses. Here, we demonstrated that DNA prime-adenovirus boost regimens enhanced Th2 responses. Thus, differences in antigens, adjuvant/cytokines, genetic backgrounds of animals and administration routes may influence the type of immune responses (Th1 versus Th2, CD4 versus CD8) induced by heterologous prime-boost regimens.

The inverted terminal repeat (ITR) sequences of the adeno-associated virus in plasmid DNA vaccine encoding HIV-1 Gag or Env under the control of CMV enhancer/promoter augment humoral and cellular immune responses [50,54]. This booster effect of the ITR sequences is thought to be associated with upregulation of transgene expression in mice because an addition of the ITR sequences to plasmid DNA increases transgene expression in cultured cells. The ITR sequences of adenovirus serotype 5 is located approximately 200 bp upstream of CMV enhancer in pShut-CA-PEDI-(Aβ1-6)₁₁ while pCA-PEDI-(Aβ1-6)₁₁ lacks the ITR sequences. The adenovirus ITR functions as an enhancer in certain cells [55]. Therefore, we made a comparison between pShut-CA-PEDI-(Aβ1-6)₁₁ and pCA-PEDI-(Aβ1-6)₁₁ in cultured cells and in DNA prime-adenovirus boost vaccination. The expression level of PEDI-(Aβ1-6)₁₁ is greater in cultured cells transfected with pCA-PEDI-(Aβ1-6)₁₁ compared with pShut-CA-PEDI-(Aβ1-6)₁₁. Consistent with the cell culture experiments, a greater anti-Aβ titer in mice was found when pCA-PEDI-(Aβ1-6)₁₁ was used in the DNA prime-adenovirus boost regimen. The adenovirus ITR in pShut-CA-PEDI-(Aβ1-6)₁₁ may not function as an enhancer under our experimental conditions.

Bacterial plasmids that contain stimulatory CpG motifs can function as adjuvant, presumably through toll-like receptor (TLR) 9 activation (reviewed by Klinman [56] and Krieg [57]). The optimal sequence motif in mice consists of two 5' purines + CpG + two 3' pyrimidines. We found 5 and 25 CpG motifs in pCA-PEDI-(Aβ1-6)₁₁ and pShut-CA-PEDI-(Aβ1-6)₁₁, respectively. Type 5 adenoviral DNA, however, is known to have unusually high contents of neutralizing CpG motifs that block immune activation by stimulatory CpG motifs [58]. pShut-CA-PEDI-(Aβ1-6)₁₁ included genomic DNA from serotype 5 adenovirus and had more neutralizing than stimulatory CpG motifs. Such inflammatory responses initiated by CpG-motifs and TLR9 interactions could be the reason why the pCA-PEDI-(Aβ1-6)₁₁ prime-adenovirus boost regimen induced higher anti-Aβ titers than the pShut-CA-PEDI-(Aβ1-6)₁₁ prime-adenovirus boost regimen. Alternatively, because immune responses induced by CpG-motif-containing plasmid DNA vaccines remain unchanged between TLR9-sufficient and TLR9- or Myd88-deficient mice [59], activation of innate immune responses by CpG-motifs may have little to no effects on vaccine immunogenicity.

Nasal immunization of mice with only pShut-CA-PEDI-(Aβ1-6)₁₁ or pCA-PEDI-(Aβ1-6)₁₁ failed to induce serum anti-Aβ antibody assessed by ELISA. Nonetheless, the DNA prime-

adenovirus boost regimen induced higher levels of anti-A β antibody titers in more animals than adenovirus alone. The precise mechanisms by which heterologous prime-boost regimens induce more robust immune responses than homologous ones are not fully understood. One postulated mechanism is that immune responses to a target antigen are synergistically amplified through stimulating multiple immune pathways with the antigen delivered by different vectors [35]. Another possible mechanism for enhanced antigen immunogenicity is the avoidance of anti-vector immunity with the heterologous booster immunizations. Because administration of plasmid DNA encoding A β by gene gun or muscular injection can elicit discernible humoral immune responses in AD mouse models [32,33], DNA priming through epidermal or muscular route followed by adenovirus boosting may enhance immune responses far greater than nasal DNA priming. We are currently testing heterologous administration routes for DNA prime-adenovirus regimens in order to optimize Th2 responses.

Amyloid plaques and neurofibrillary tangles (NFTs) are two hallmarks of Alzheimer's disease. The major constituent of NFT is hyperphosphorylated tau protein. The molecular mechanism which links both pathologic lesions is unknown. Because NFTs correlate better with AD symptom progression than amyloid plaques [60], such tau pathology can be another therapeutic target for AD. Neuropathological examination of AD patients subjected to A β vaccination revealed clearance of amyloid plaques but persistence of NFTs and neuropil threads, suggesting that A β immunotherapy is not effective in reducing tau pathology [9,12,61]. Recently, Oddo et al. [62,63] demonstrated that A β immunotherapy ameliorated cognitive deficits by reducing soluble A β and tau without significant reduction in insoluble A β (plaques) and tau (tangles) in aged triple-transgenic mice expressing mutant forms of human APP, PS1 and tau. They concluded that A β immunotherapy represented an effective strategy for ameliorating cognitive decline even in aged brains marked by resistant amyloid plaques and NFTs. It, however, remains to be determined if the DNA prime-adenovirus boost regimen presented here is effective in improving behavioral deficits and reducing A β and tau in AD mouse models.

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Fig 1. Expression of PEDI-(Aβ1-6)₁₁ in HEK293 cells

HEK293 cells were transfected with either pShut-CA-PEDI-(Aβ1-6)₁₁ or pCA-PEDI-(Aβ1-6)₁₁. Seventy-two hours after transfection, expressed PEDI-(Aβ1-6)₁₁ was detected by western blot analysis using an anti-Aβ serum. The same western membranes were stripped and reprobed with anti-GAPDH antibody for normalization. Three independent transfections were performed and a representative western blot is shown (**A**). Lane 1, untransfected; lane 2; transfected with pShut-CA-PEDI-(Aβ1-6)₁₁; lane 3, transfected with pCA-PEDI-(Aβ1-6)₁₁. The relative density of the western signals [PEDI-(Aβ1-6)₁₁] normalized by the GAPDH signals are plotted in % as bar graphs in (**B**). The error bar is M.S.E. * $P < 0.05$ for pCA-PEDI-(Aβ1-6)₁₁ compared with pShut-CA-PEDI-(Aβ1-6)₁₁.

Fig 2. Induction of anti-A β IgG after prime-boost immunization regimens

For heterologous prime-boost regimens, mice were nasally administered with either pShut-CA-PEDI-(A β 1-6)₁₁ ($\blacktriangle$) or pCA-PEDI-(A β 1-6)₁₁ ($\blacksquare$) at days 0 and 7 (depicted by thin arrows) followed by AdPEDI-(A β 1-6)₁₁ boosting at days 14 and 21 (depict by bold arrows). For adenovirus prime-adenovirus vaccination ($\blacklozenge$), nasal administration of AdPEDI-(A β 1-6)₁₁ was performed twice at days 14 and 21. The results from DNA prime-DNA boost regimens are not shown because this treatment did not induce anti-A β antibody in mice. Anti-A β IgG on average by ELISA is shown. The error bars are standard error of the mean (SEM). Each anti-A β titer from each mouse at day 42 is also shown on the right of the day 42 average titers.

Fig 3. Isotypes of anti-Aβ antibodies

Isotype-specific titers for anti-Aβ antibodies at indicated dates were determined by ELISA using anti-mouse IgM, IgG1, IgG2a, and IgG2b antibodies. **A.** Titers in mice subjected to only adenovirus vector, AdPEDI-(Aβ1-6)₁₁. **B.** Titers in mice subjected to pCA-PEDI-(Aβ1-6)₁₁ priming followed by adenovirus boosting. **C.** Titers in mice subjected to pShut-CA-PEDI-(Aβ1-6)₁₁ priming followed by adenovirus boosting. The error bars are SEM.

Fig 4. Levels of Th1 (IFN- γ and IL-2) and Th2 (IL-4 and IL-10) cytokines in mice treated with a pCA-PEDI-(A β 1-6)₁₁ prime-AdPEDI-(A β 1-6)₁₁ boost regimen

The mean serum cytokine levels (day 0 for preimmune and day 28 for after vaccination) determined by ELISA in mice subjected to a pCA-PEDI-(A β 1-6)₁₁ prime-adenovirus boost regimen are shown. The error bars are SEM.

Fig 5. Sera from mice subjected to a homologous or heterologous prime-boost regimen react with amyloid plaques in the brain

Brain sections from a 7-month old Mo/Hu APPswe PS1dE9 mouse were incubated with sera from mice treated with a pCA-PEDI-(A β 1-6)₁₁ prime-adenovirus boost regimen (**A**), only AdPEDI-(A β 1-6)₁₁ (**B**) or PBS (**C**), followed by visualization with Alexa Fluor 488 - conjugated anti-mouse IgG1 antibody. Representative pictures are shown. Scale bar 250 μ m.