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DNA prime–protein boost increased the titer, avidity and persistence of anti-Aβ antibodies in wild-type mice

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Abstract

Recently, we reported that a DNA vaccine, composed of three copies of a self B cell epitope of amyloid-β ($A\beta_{42}$) and the foreign T-cell epitope, Pan DR epitope (PADRE), generated strong anti-Aβ immune responses in wild-type and amyloid precursor protein transgenic animals. Although DNA vaccines have several advantages over peptide–protein vaccines, they induce lower immune responses in large animals and humans compared with those in mice. The focus of this study was to further enhance anti-$A\beta_{11}$ immune responses by developing an improved DNA vaccination protocol of the prime–boost regimen, in which the priming step would use DNA and the boosting step would use recombinant protein. Accordingly, we generated DNA and recombinant protein-based epitope vaccines and showed that priming with DNA followed by boosting with a homologous recombinant protein vaccine significantly increases the anti-Aβ antibody responses and do not change the immunoglobulin G1 (IgG1) profile of humoral immune responses. Furthermore, the antibodies generated by this prime–boost regimen were long-lasting and possessed a higher avidity for binding with an $A\beta_{42}$ peptide. Thus, we showed that a heterologous prime–boost regimen could be an effective protocol for developing a potent Alzheimer's disease (AD) vaccine.

Keywords

DNA vaccine; β-amyloid; prime–boost regimen

Introduction

Alzheimer's disease (AD) is the leading cause of dementia in older people, both in the United States and worldwide. More than 5 million Americans currently suffer from AD, and the projections are that the number will balloon to 10–15 million over the next few decades. Some

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Conflict of interest
The authors declare no conflict of interest.

data suggest that this devastating disease will affect over 106 million people worldwide by the middle of this century.[1,2] The clinical criteria for the diagnosis of AD include the insidious onset and progressive impairment of memory and other cognitive functions; however, a definitive diagnosis of AD can currently be made only during an autopsy, by examining the brain tissue for amyloid plaques and neurofibrillary tangles. The extracellular amyloid plaques and intracellular neurofibrillary tangles represent examples of proteopathies, which results from the aberrant accumulation of misfolded or aggregated proteins that are believed to interfere with normal functions, thereby directly or indirectly contributing to disease pathogenesis.3 Currently, therapeutic strategies for circumventing AD have focused on the reduction and blocking of the production or assembly of aberrant forms of amyloid-β ($A\beta_{42}$) and the promotion of clearance of these misfolded peptides.[4] It seems that one promising approach in halting disease progression is currently associated with an Aβ immunotherapy strategy.[5,6]

The first AD clinical trial, AN-1792, used fibrillar $A\beta_{42}$ formulated in a strong Th1-type adjuvant QS21 to vaccinate patients with mild-to-moderate AD.[4] However, the trial had to be halted because of the harmful meningoencephalitis that occurred in the brains of AD patients.[7–10] Despite this outcome, there were some important findings from this clinical trial that will help in advancing our understanding of this type of vaccine. The results showed that vaccination reduced amyloid plaques in the brains of the AD patients but without a significant improvement in cognitive functions and with an increased severity of cerebral amyloid angiopathy and meningoencephalitis.[7–13] In addition, only 20% of vaccinated subjects responded to the vaccine, generating relatively low titers of anti-Aβ anti-bodies. [9,10,12] The suggestion that arises, based on these data and data from other clinical and pre-clinical studies using AD mouse models,[11,14–16] is that the development of a safe and effective Aβ-based immunotherapy for AD may require the generation of high titers of anti-Aβ antibodies while avoiding autoreactive proinflammatory Th1 responses. The latter are believed to be responsible for the adverse events (meningoencephalitis) that occurred during the course of the AN-1792 trial.[7–10]

In fact, it has never been shown that the AN-1792 vaccine induced T-cell responses specific to Aβ, although one study showed that cognitively normal elderly and AD patients have an increase in autoreactive T cells specific to the Aβ peptide.[17,18] Interestingly, although this group showed increased levels of anti-Aβ T cells in the elderly and AD patients, recent data with peptide microarrays showed a decrease in natural anti-oligomeric antibodies in these populations.[19] If data reported by Britschgi *et al.*[19] are confirmed by other scientists, it will be necessary to analyze in detail the mechanism of the immunity to self Aβ antigen in the elderly and AD patients.

In any case, the clinical trial data indicate that a safe AD vaccine should be able to produce therapeutically relevant titers of anti-Aβ antibodies and avoid the activation of autoreactive T cells. One way to achieve this aim is to replace the self Aβ T helper (Th)-cell epitope with a foreign Th-cell epitope while keeping the self-B-cell epitope intact. Fortunately, studies on mapping B- and T-cell epitopes in $A\beta_{42}$ peptide showed that these two epitopes are distinct.[20–32] We previously generated AD epitope vaccines based on DNA (DepVac) and on the multiple antigenic peptide, and showed that both vaccines are able to generate strong anti-Aβ antibody responses and reduce AD pathology, inhibiting cognitive decline in amyloid precursor protein transgenic animals, without generating autoreactive Th cells.[14,15,33,34] These studies suggest that although a peptide epitope vaccine is capable of inducing strong anti-Aβ antibody responses, some problems associated with this type of vaccine still exist.[35] One of these problems is that a peptide epitope vaccine is immunogenic when it is synthesized as a multiple antigenic peptide, but this is not suitable for clinical trials because it cannot be easily scaled up. At the same time, the generation of an AD vaccine solely based on a recombinant protein, is not trivial because of the high cost of its purification. Another issue

associated with any protein-based vaccine strategy is that a potent conventional adjuvant is necessarily required and adds an element of toxicity and cost.

DNA-based vaccination provides a unique alternative method of immunization, and is extra valuable because in addition to protecting against AD it shows properties that may be advantageous in developing a range of vaccines against a variety of pathogens and for human diseases, such as cancer, autoimmune disorders, and others.[36–40] In addition, a DNA vaccine is safe in humans,[41–43] and its large-scale production is easy and inexpensive. Although based on our previous work and studies conducted by other groups we have observed that a DNA-based vaccine has many advantages, we also saw that this type of vaccine still needs to be improved. We launched our study hoping to analyze ways to improve the potency of this type of vaccine and to study the possibility of reducing its setbacks. One of these setbacks is that although a DNA vaccine is effective in mice, it induces only very low immune responses in large animals and humans.[35,44–46] Therefore, translation of DepVac to the clinic would likely require a further enhancement of its immunogenicity, specifically an improvement in the DNA delivery system and the immunization protocol. The DNA prime–protein boost regimen is known to be one of such effective protocols,[47–52] and researching on how it can be applied to an AD vaccine was the main aim of this study.

Accordingly, in this study, we generated a recombinant protein vaccine (PepVac) composed of three copies of a self B-cell epitope of $A\beta_{42}$ ($3A\beta_{11}$) and the foreign T-cell epitope, Pan DR epitope (PADRE), fused with a macrophage-derived chemokine (MDC). We showed that priming with DepVac followed by boosting with PepVac significantly enhances the production of long-lasting high-avidity immunoglobulin G1 (IgG1) antibodies specific to Aβ.

Results

Heterologous prime– boost regimen induces high titers of long-lasting anti-Aβ antibodies

The chimeric gene encoding MDC-$3A\beta_{11}$-PADRE was subcloned into the *Escherichia coli* expression vector pET11d (Figure 1a) as described in the Materials and methods section and the recombinant homologous protein was purified from *E coli*. The purity and integrity of the protein was analyzed in a 10% Bis-Tris polyacrylamide gel electrophoresis. The protein band of ~17.5 kDa was visualized by staining with Coomassie dye (Figure 1b). The identity of this band was confirmed by western blot analysis using anti-Aβ 20.1 antibody (Figure 1c).

The generation of the DepVac vaccine was described earlier.[15] C57Bl6 mice were immunized to evaluate the effect of boosting with PepVac on the anti-Aβ antibody responses in DepVac-primed mice. More specifically, three immunization regimens were used: a DNA prime–DNA boost, a DNA prime–protein boost and a protein prime–protein boost (Figure 2a). It is to be noted that recombinant proteins purified from *E. coli* cells are usually contaminated with bacterial lipopolysacharide (endotoxin) that can act as an adjuvant. Taking advantage of this, we immunized mice with a recombinant protein containing 60 ng of endotoxin in 1 mg of protein.

As we had expected based on our previous studies, DepVac immunization induced the production of relatively high levels of antibodies (62.2 μg ml^{-1}) after three immunizations. Importantly, the levels of anti-Aβ antibodies were significantly higher (367.9 μg ml^{-1}, $P<0.01$) in animals primed with DNA and boosted with recombinant protein. This response was even higher when compared with the antibody response generated after three protein immunizations with PepVac (249.95 μg ml^{-1}) (Figure 2b), although this difference was not significant.

Previously, we had reported[15,33,53] that antibodies induced by an epitope vaccine are therapeutic and bind to different forms of amyloid, including plaques, in the brains from AD

cases. As we had expected, antibodies generated by the DNA prime–protein boost also bound to β-amyloid plaques in human brain tissue (Figure 2c) and this binding was almost completely abrogated by $A\beta_{42}$ peptide (Figure 2d). Thus, the DNA prime–protein boost regimen significantly enhanced the production of therapeutically potent anti-Aβ antibodies. It is to be noted that this increase is not associated with the presence of endotoxin in PepVac, as DepVac mixed with the same concentration of endotoxin did not show the same effect (data not shown).

Next, we assumed that the immunization regimens may influence not only the magnitude, but also the longevity of the induced immune responses. To assess the duration of the antibody response in mice immunized by different regimens, the antibody concentrations in the sera of mice were monitored for 52 weeks after vaccination. It is to be noted that as the strength and longevity of antibody responses in mice that were immunized and boosted with PepVac was not significantly different from that detected in mice primed with DepVac and boosted with PepVac, we only compared the longevity of the antibody generated after the DNA prime–DNA boost and DNA prime–protein boost in our further analysis (Figure 3a).

Pharmacokinetic analysis was performed to compare the continuous decline in antibody titers in both groups of mice and to evaluate the potency of two immunization protocols (Figure 3b). The area under the curve determined that significantly lower levels of antibodies were produced by the DNA prime–DNA boost as compared with the DNA prime–protein boost regimen. The maximum value observed after a DNA prime–protein boost was significantly higher, and the peak ratios were less than 0.2. In addition, the rate of decay (λz) for antibody concentrations was faster in the DNA prime–DNA boost regimen; the half-life $(T\frac{1}{2})$ of anti-Aβ antibodies induced by DNA immunization was 15.4 weeks in comparison with 25.8 weeks for antibodies induced after the DNA prime–protein boost. These results clearly showed that the DNA prime–protein boost regimen is a more powerful protocol for generating high and persistent titers of anti-Aβ antibodies than DNA prime–DNA boost immunizations.

Priming with DNA and boosting with PepVac enhances immunoglobulin G1 (IgG1) antibody responses specific to Aβ

In our previous studies, we showed that antibodies produced after DepVac immunization are predominantly of the IgG1 isotype,[15] and this is an indirect measure of the relative contributions of the Th2-type immune responses.[54–56] The same results were obtained in this study when mice were primed and boosted with DepVac (Figure 4a). Keeping in mind that recombinant protein contains *E. coli* endotoxin that is supposed to act as a Th1-type adjuvant,[57,58] it was interesting to analyze the $IgG1/IgG2a^b$ profile of the immune responses after protein immunizations and after the DNA prime–protein boost regimen. As we had expected, the protein prime–protein boost regimen induced mostly the Th1 type of humoral immune responses, as $IgG1/IgG2a^b$ ratio was equal to 1. Interestingly, when mice were primed with DNA, a further boost with protein did not change the isotype pattern of the antibodies (Figure 4a). Boosting with protein enhanced the production of IgG1, but only slightly increased the levels of $IgG2a^b$ isotypes without changing the $IgG1/IgG2a^b$ ratio (Figure 4b).

Heterologous prime–boost regimen significantly increased the binding avidity of anti-Aβ antibody

Besides the quantitative characterization of antibody responses generated by our immunization protocols, we also analyzed the avidity for binding of these antibodies to the antigen by using sodium thiocyanate (NaCSN) displacement enzyme-linked immunosorbent assay (ELISA).[47] The effective concentration of NaSCN required for the release of 50% of antiserum from the ELISA plate (half-maximal effective dose) was calculated. Using this technique we showed that a DNA prime–DNA boost regimen generated antibodies that can be released from an

antigen at a concentration of NaSCN equal to 2.4 M (Figure 5a). In contrast, the antibodies that were induced after priming with DNA and boosting with protein required much higher concentration of NaSCN (5.1 M). It is to be noted that this concentration was significantly higher than the chemical concentration used to release antibodies generated by the protein prime–protein boost regimen (4.05 M). On the basis of these data, we concluded that the avidity of antibodies generated after a DNA prime–protein boost regimen is significantly higher than those induced after a DNA prime–DNA boost regimen.

Heterologous prime–boost regimen significantly enhanced anti-PADRE-specific T-cell responses

Keeping in mind that T-cell help is an essential factor in robust B-cell activation, and that a protein boost of DNA-primed mice enhanced the titers of anti-$A\beta_{11}$ antibodies, we went on to examine whether the protein boost may also amplify the PADRE-specific T-cell proliferation that is induced after DNA priming.

It is to be noted that we had previously reported that splenocytes isolated from animals vaccinated with a DNA- or protein-based epitope vaccine proliferated after *in vitro* re-stimulation with PADRE but not with $A\beta_{40}$.[14,15,33,34] Similarly, priming and boosting mice with a DNA epitope vaccine generated moderate levels of anti-PADRE (Figure 5b), but not anti-Aβ T-cell responses (data not shown). Importantly, the proliferation of splenocytes isolated from mice immunized by a DNA prime–protein boost regimen was significantly higher than in mice immunized with DepVac only, and the former was as effective as the protein prime–protein boost regimen (Figure 5b). Thus, the anti-PADRE T-cell proliferation was significantly lower in the group primed and boosted with DepVac compared with the other two groups.

PepVac boosted antibody production regardless of the concentrations of antibodies generated after DNA priming

Next, we assumed that the concentration of anti-Aβ antibodies at the time of the PepVac boost may influence the enhancement of humoral responses. To address this question, mice were immunized with DepVac four times with biweekly intervals, and the antibody responses were tested at nearly half a year after vaccination (Figure 6a). As we had expected based on the data presented above (Figure 3a), the mice continued to produce significant amounts of antibodies, although the variability of antibody titers between individual mice was high (Figure 6b). Thus, we decided to group these mice based on the concentrations of generated anti-Aβ antibodies: low ≤ 5 μg ml^{-1}, moderate 5–50 μg ml^{-1} and high ≥ 50 μg ml^{-1} concentrations. Half of these animals were boosted with PepVac and the other half were injected with DepVac as a control (Figure 6a). No enhancement of antibody responses was observed after a boost with DepVac in the groups possessing moderate and high concentrations of antibodies. When the group of mice with low concentration of antibodies was boosted with DepVac, the antibody response increased; however, this enhancement was not significant. In contrast, a boost with PepVac significantly enhanced the antibody responses in all of the concentration groups (Figure 6c).

Discussion

The AN-1792 clinical trial data showed significant individual variability in anti-Aβ antibody responses in vaccinated AD patients.[11,59] Importantly, although there was no significant improvement of cognitive functions in vaccine-responding subjects, the trial suggested a connection between the titers of antibodies and the efficacy of the AD vaccine.[10,11,16,60] Vaccinated AD patients who generated Aβ-plaque-recognizing antibodies showed a dose-dependent relationship between the increase in serum antibodies and the slowing of cognitive decline associated with the disease.[16] An improvement in the memory domain of the

Neuropsychological Test Battery and the decreased cerebrospinal fluid tau levels[11] were observed in a small subset of antibody responders compared with the placebo group. Follow-up studies of patients enrolled in the phase I trial showed the clearance of amyloid plaques in the brains of AD patients, but did not prevent progressive neurodegeneration. [10] At the same time, data from patients enrolled into phase IIa trial were more encouraging, showing that after almost 5 years responders to AN-1792 vaccine not only retained detectable levels of anti-Aβ antibody, but also showed some functional and dependence benefits compared with placebo-treated patients.[60]

Previously, we had reported that a DNA vaccine induced strong anti-PADRE T-cell responses as well as therapeutically potent titers of anti-Aβ antibodies without activating autoreactive T cells in wild-type and amyloid precursor protein transgenic mice.[15,34,61] However, despite many advantages of DNA-based vaccines and the encouraging results in animal models, clinical trials with DNA vaccines have documented suboptimal potency of immune responses. One reason for these results is the low transfection efficacy of naked DNA.[44,45] It is likely that a selection of an optimal DNA delivery system, such as a gene gun or electroporation, along with utilization of molecular adjuvants, could significantly enhance the efficiency of DNA vaccines.[62–67] Additional augmentation of immune responses may be achieved by developing more effective immunization protocols. One such approach is a heterologous immunization, in which priming is performed with DNA followed by boosting with recombinant proteins or recombinant viruses.[47–52,68–70] Furthermore, this approach enhances not only the magnitude of antibody responses primed by DNA immunization, but also the overall quality of these responses (avidity or neutralizing activities).[71] Although currently the mechanism for efficacy of heterologous immunization is not fully understood, it has been hypothesized that the use of two versions of the same immunogen may activate different subsets of immune cells. For example, DNA immunization is more effective in inducing $CD4^+$ T-cell responses and priming antigen-specific B cells, whereas protein immunization is more effective in stimulating the proliferation of memory B cells into antibody-secreting plasma cells.[71] On the basis of these data, we endeavored to test a DNA prime–protein boost regimen to enhance the efficacy of our AD epitope vaccine. To our knowledge, only one group has studied the DNA prime–peptide boost regimen using a whole-length $Aβ_{42}$ peptide, but could not enhance the immune response without adding an adjuvant (oligo-deoxynucleotides, CpG motif).[72] In contrast, in this study we show that a DNA prime–protein boost strategy induces a significantly higher anti-PADRE T-cell proliferation (Figure 5b) and a higher production of anti-Aβ antibodies compared with a DNA prime–DNA boost regimen (Figure 2b).

The post-vaccination production of anti-Aβ antibody over time may be considered a pharmacological response of the immune system to the vaccine. Antibody production is a dose-dependent response to vaccination that is followed by gradual degradation. This degradation of antibodies can be characterized by half-lives. Considering the fact that the production and elimination of antigen-specific antibodies is dose and time dependent, we have decided to perform pharmacokinetic analysis to compare the potency of two immunization protocols: DNA prime–DNA boost and DNA prime–protein boost. The calculation of pharmacokinetic parameters allowed us to show that DNA prime–protein boost vaccination regimen not only induces higher level of antibodies, but also slows the rate of decay of antibody concentrations, and increases the half-life of antibodies when compared with antibodies induced by the DNA prime–DNA boost regimen.

Antibody isotyping has been used as an indirect measure of the contribution of Th1 (IgG2a) and Th2 (IgG1) cytokines during the stimulation of the humoral response.54 DNA immunization mostly induced IgG1 isotypes of anti-Aβ antibodies (as expected based on our previous data15), in which immunization with PepVac with a small quantity of endotoxin (lipopolysacharide) induced both IgG1 and $IgG2a^b$ antibodies. Importantly, the boost with

PepVac did not change the isotype pattern of anti-Aβ antibody responses that were initially generated by priming with DepVac.

Another important characteristic of antibodies is their avidity for binding to antigens. Higher avidity leads to the formation of stronger antigen–antibody complexes, suggesting a more efficient immune elimination of antigens. In this study, we compared the avidity of antibodies elicited by vaccination using three immunization regimens: DNA prime–DNA boost, DNA prime/protein boost or protein prime–protein boost. Our results show that a DNA prime–protein boost regimen induces antibodies with significantly higher avidity for binding to Aβ as compared with the other regimens (Figure 5a). These data are in agreement with previously published experiments generated with Env antigen from T-cell-line-adapted virus.[47]

Data accumulated over several years suggest that AD vaccination will be effective when started before the accumulation of toxic forms of β-amyloid (prophylactic vaccination) or at least at the early stages of AD.[5,6,73,74] We assumed that when a DNA epitope vaccine is a safe modality in humans, it could be effectively used for priming and one or two booster injections with a protein may be used to rapidly enhance the production of therapeutic antibodies later, during AD progression. In this study we showed that priming mice with a DNA vaccine and boosting them with a protein vaccine after a prolonged resting period significantly increased the production of antibodies, regardless of the initially generated antibody concentrations. In contrast, when after a resting period, mice were boosted with a DNA vaccine, the antibody responses increased only in mice that had a low concentration of anti-Aβ antibodies at the time of boosting (Figure 6c).

We believe that further translational studies in amyloid precursor protein transgenic mice as well as in rabbits and monkeys with a regimen consisting of priming with a DNA vaccine and boosting with a protein vaccine (formulated in a conventional adjuvant) may allow us to move this strategy into the clinic. This vaccination regimen could be cost effective and might be advantageous for humans, allowing the generation of high titers of high-avidity antibodies over a long period of time.

Materials and methods

Mice

Female, 5- to 6-week-old C57Bl/6 mice were purchased from The Jackson Laboratory (Sacramento, MN, USA). All animals were housed in a temperature- and light cycle-controlled animal facility at the Institute for Memory Impairments and Neurological Disorders, University of California Irvine. Animal use protocols were approved by the institutional animal care and use committee of the university and were in accordance with the guidelines of the National Institutes of Health.

DNA constructs

The construction strategy of DepVac was previously described.[15] To prepare the recombinant MDC-3Aβ_{11}-PADRE protein, we subcloned the 3Aβ_{11}-PADRE gene from a mammalian expression vector into an *E. coli* expression plasmid pET11d/MDC-myc/6xHis (gift from Dr Biragyn, National Institute on Aging) in frame with mature MDC gene at the N-terminal and myc/6xHis-Tag at the C-terminal using the PCR technique. A nucleotide sequence analysis confirmed that the generated plasmid contained the correct sequence.

Purification of recombinant protein

The recombinant protein was purified from *E. coli* BL21 (DE3) cells transformed with pET11d/MDC-3Aβ_{11}-PADRE plasmid. Cells were grown up to the optical density 0.7–0.8 at 600 nm

in the Luria Bertani medium containing 100 μg ml^{-1} of ampicillin. Gene expression was induced by adding isopropyl-β-D-1-thiogalactopyranoside at a final concentration of 1 mM and incubating for 4 h at 28 °C. Cells harvested by centrifugation were re-suspended in the lysis buffer, and the integrity of those cells was disrupted using sonication. The MDC-3Aβ_{11}-PADRE protein appeared as inclusion bodies and was separated from cell debris using the BugBuster protein extraction reagent as recommended by the manufacturer (Novagen, New Canaan, CT, USA). The final protein pellet was dissolved in 8 M of urea buffer and purified using Ni-agarose column (Qiagen, Valencia, CA, USA). Positive fractions were combined and the protein was refolded by dilution in refolding solution (0.1 M Tris HCl, pH 8.0/0.5 M L-arginine/4 mM oxidized glutathione/2 mM EDTA, pH 8.0) followed by dialysis against 20 mM Tris-HCl, pH 7.5. Protein was concentrated using centricon filters (Millipore, Billerica, MA, USA). The final concentrate of recombinant protein was analyzed in 10% Bis-Tris gel electrophoresis (NuPAGE Novex Gel, Invitrogen, Carlsbad, CA, USA) (Figure 1).

The level of endotoxin was measured using E-TOXATE kits as recommended by the manufacturer (Sigma, St Louis, MO, USA).

Immunizations

The C57Bl/6 mice were primed twice with a 2-week interval and boosted once after another 2-week interval. The groups were DepVac only, PepVac only and DepVac followed by PepVac boosting (Figure 2a).

In another set of experiments, mice ($n = 82$) were immunized four times biweekly with DepVac (Figure 6a). After a 26-week resting period, these mice were grouped on the basis of the concentrations of the generated anti-Aβ antibodies. Each group was divided in half and boosted either with DepVac or PepVac.

The DNA vaccine was administered using gene gun bombardment (Bio-Rad, Hercules, CA, USA), as previously described.[15,40,61] Each bullet carried 0.5 mg gold (Bio-Rad) coated with 3 μg of DNA. Each mouse received three shots (total 9 μg DNA) on shaved abdominal skin using a gene gun with helium gas at 400ψ.

Mice from the protein vaccine group were injected with 50 μg of PepVac subcutaneously, as previously described.[34,75]

Detection of anti-Aβ antibody concentration and binding avidity by enzyme-linked immunosorbent assay (ELISA)

On day 12 after each immunization, blood was collected for analysis of anti-Aβ antibodies using ELISA as described previously.[14,33,76] In brief, wells of 96-well plates (Immulon II; Dynatech Laboratories, Chantilly, VA, USA) were coated with 2.5 μM soluble Aβ_{42} (pH 9.7, o/n and 4 °C). Wells were then washed and blocked, and sera from experimental mice were added to the wells at different dilutions. After incubation and washing, horseradish peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used as secondary antibody. Plates were incubated and washed, and the reaction was developed by adding 3,3′,5,5′ tetramethylbenzidine (Pierce, IL, USA) substrate solution and stopped with 2N H_2SO_4. The optical density was read at 450 nm (Biotek, Synergy HT, VT, USA), and antibody concentrations were calculated using a calibration curve generated with 6E10 monoclonal antibody (Signet, Dedham, MA, USA). Horseradish peroxidase-conjugated anti-IgG1, IgG2a[b], IgG2b (Zymed, South San Francisco, CA, USA) were used for characterizing the isotype profiles of antibodies. Sodium thiocyanate displacement ELISAs were performed using the method described by Richmond *et al*.[47] Concentrations of antibodies from all mice were equalized in the sera. Bound antibodies were detected as described above,

with an exception: after incubation with primary sera, plates were washed thrice times with Tris-buffered saline containing 0.5% Tween-20, once with Tris-buffered saline buffer containing 0, 1, 3, 5, 10 M NaCSN for 15 min, and then six more times with Tris-buffered saline containing 0.5% Tween-20. The results are expressed as a percentage of antibody concentration in the absence of NaSCN.

Detection of Aβ plaques in human brain tissues

Sera from immunized mice were screened for the ability to bind to human Aβ plaques using 50-μm brain sections of formalin-fixed cortical tissue from a severe AD case as described.[34,40] A digital camera (Olympus, Tokyo, Japan) was used to capture images of the plaques at a ×10 magnification. The binding of anti-$A\beta_{11}$ sera to the β-amyloid plaques was blocked by 2.5 μM of $A\beta_{42}$ peptide as described.[34]

T-cell proliferation

To detect T-cell proliferation, we used a [^{3}H]thymidine incorporation assay as described earlier.[77] In brief, splenocytes from experimental and control mice (5×10^5 in 100μ) were re-stimulated *in vitro* with 5 μM of PADRE. Cells were first incubated for 72 h, then 1 μCi of [^{3}H]thymidine (Amersham Biosciences, South Plainfield, NJ, USA) was added to each well for 16–18 h. Cells were harvested using the Tomtec Mach III harvester, and [^{3}H]thymidine uptake (c.p.m.) was counted on a MicroBeta 1450 Trilux scintillation counter (Wallac Oy, Turku, Finland). The stimulation index was calculated as previously described.[77]

Statistical analysis

Statistical parameters (mean, s.d., significant difference and so on) were calculated using Prism 3.03 software (GraphPad Software, Inc., San Diego, CA, USA). Statistically significant differences were examined using a *t*-test, or analysis of variance and Tukey's multiple comparisons post-test (a *P*-value of <0.05 was considered significant).

Data of antibody concentration in the sera of immunized mice ($n = 8$) were used in pharmacokinetic analyses, in which all concentrations of antibodies at time 0 were set to zero. All pharmacokinetic calculations were performed using WinNonlin 5.2 (Pharsight Corporation, St Louis, MA, USA). The area under the concentration-time curve (AUC_{0-t}) was calculated by the linear trapezoidal method. Peak concentration (C_{max}) was the highest concentration observed. The time to reach peak concentration (T_{max}) was estimated as the sampling time at which C_{max} occurred. The apparent decay rate constant (λ_z) was estimated from the terminal $\log_e$-linear, concentration-time points. The decay half-life ($T_{1/2}$) was calculated as $\log_e(2)/\lambda_z$. The area to infinity ($AUC_{0-\infty}$) was calculated by extrapolating the AUC_{0-t} value by adding of the quantity C_t/λ_z, in which C_t is the value for the last measured concentration.

Statistical analyses of the pharmacokinetic parameters were performed using the general linear model procedure of the SAS statistical program (PC version 9.1.3) (SAS Isntitute Inc., Cary, NC, USA). Confidence intervals (95%) for DNA prime–DNA boost to DNA prime– protein boost area and C_{max} ratios of means were calculated using the *t*-test approach (2, 1-sided) at $\alpha=0.05$ overall, $\alpha=0.025$ each side, both before and after $\log_e$ (ln) transformation of the values.

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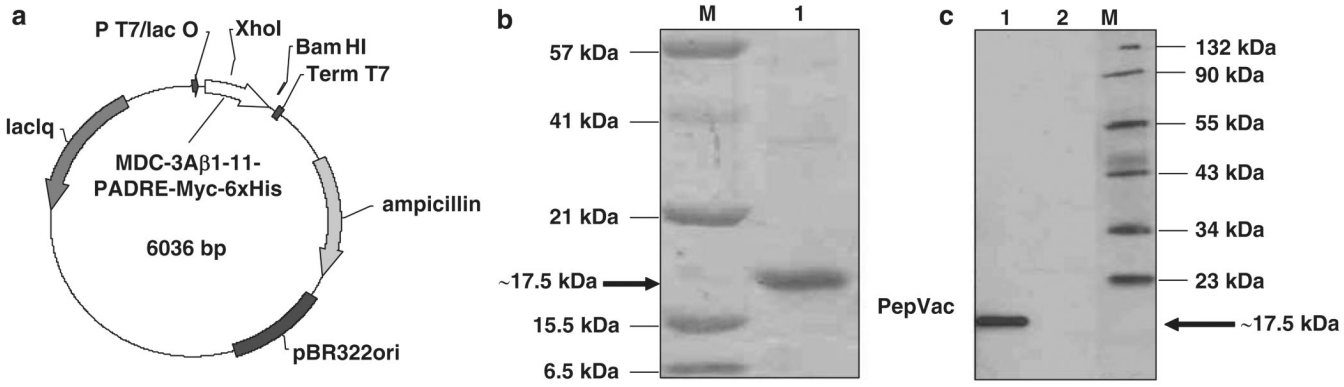


Figure 1.

Preparation of recombinant protein, PepVac. (**a**) Strategy for cloning the gene encoding MDC-3Aβ_{11}-PADRE-myc/6xHis (PepVac) into the *E. coli* expression vector pET-11d. (**b**) The purity and integrity of PepVac isolated from *E. coli* was analyzed in a 10% Bis-Tris polyacrylamide gel electrophoresis (PAGE). The protein band of ~17.5 kDa was visualized using Coomassie dye (M, molecular weight marker; lane 1, PepVac). (**c**) The specificity of the band was confirmed using western blot (WB). It was performed using 20.1 anti-Aβ antibody followed by horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G (IgG) (M, molecular weight marker; lane 1, PepVac; lane 2, irrelevant recombinant protein).

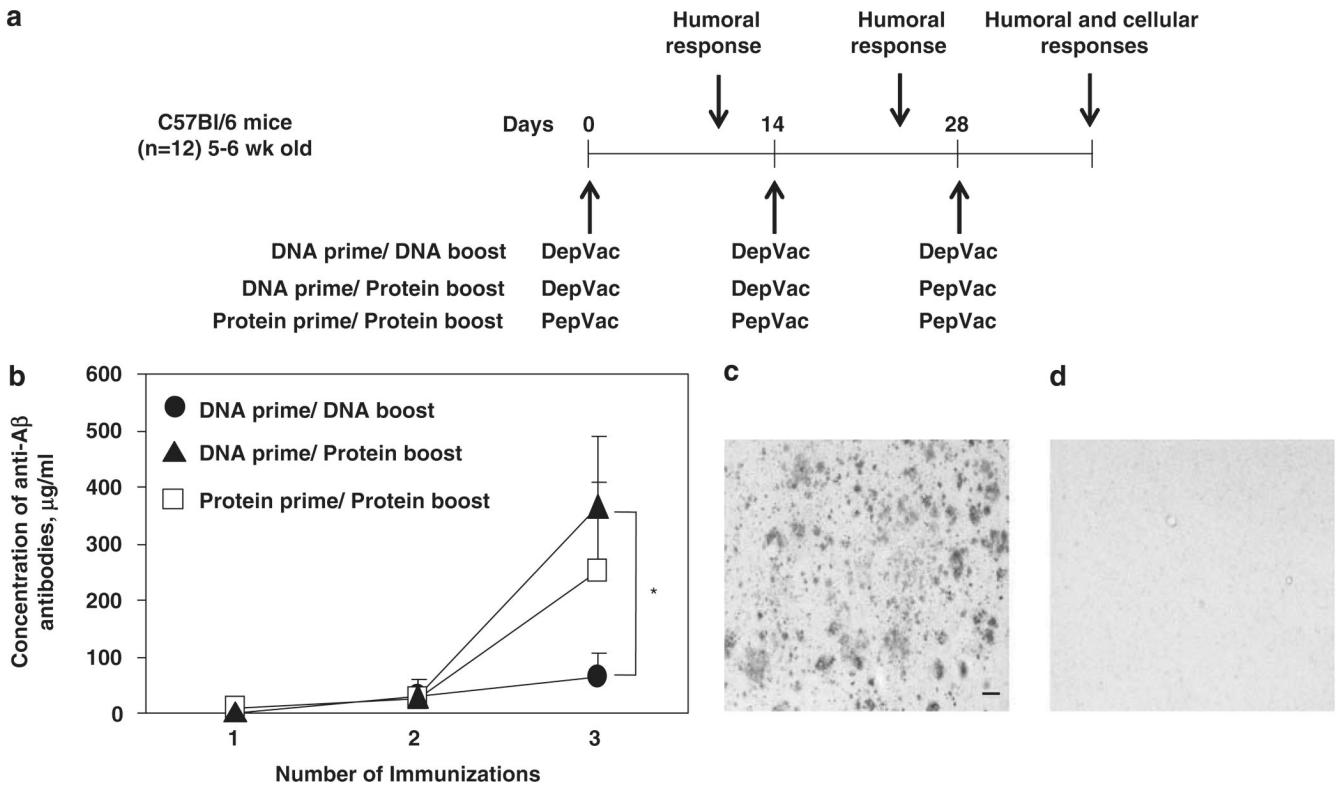
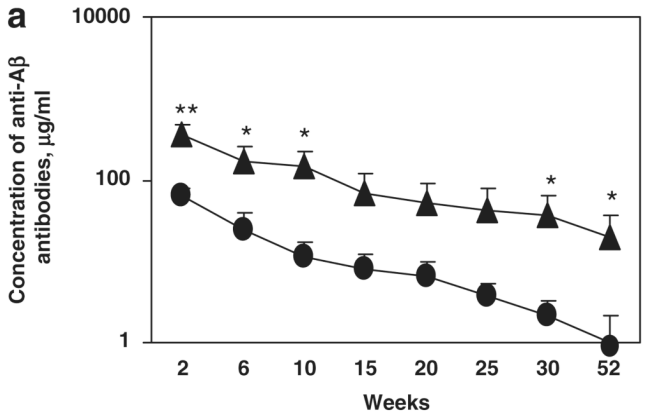


Figure 2.

Immunization with DNA (DepVac) followed by boosting with protein (PepVac) is an effective vaccination regimen for generating therapeutically potent anti-Aβ antibodies. (**a**) Experimental protocol: 5- to 6-week-old C57Bl/6 mice were immunized thrice with a 2-week interval, blood was collected after each immunization and humoral immune responses were analyzed in sera. After the last immunization, mice were killed and cellular immune responses were analyzed in splenocytes. (**b**) Humoral immune responses: concentrations of anti-Aβ antibodies were measured in the sera of individual mice after immunizations with DNA prime–DNA boost (DepVac), protein prime–protein boost (PepVac) or DNA prime–protein boost (DepVac/PepVac). Error bars represent average±s.d. for $n = 4$ in each group (*$P<0.05$). (**c, d**) Specificity of antibodies generated after DNA prime–protein boost regimen (the original magnification is ×10 and the scale bar is 50 μm): immune sera generated after the DepVac prime–PepVac boost vaccination regimen (at dilution 1:1000) bound to the 50-μm brain sections of cortical tissues from a severe Alzheimer's disease (AD case) (**c**). Binding of sera to amyloid plaques was blocked by pre-absorption of the sera with 2.5 μM $A\beta_{42}$ peptide (**d**).



b

Parameter	Least-Squares Means*		Ratio**
	DNA/DNA	DNA/Protein	
AUC 0-t (μg-hr/ml)	469	3928	0.119
AUC 0-∞ (μg-hr/ml)	504	4609	0.109
C_{max} (μg-hr/ml)	65.7	360	0.182
λ_z,(1/hour)	0.0605	0.0286	2.119
T½ (hour)	15.4	25.8	0.595

* Least-squares geometric means for ln-transformed data.

** Ratio calculated as DNA prime/DNA boost least-squares mean divided by the DNA prime/Protein boost least-squares mean

Figure 3.

DNA prime–protein boost regimen induces long-lasting anti-Aβ antibodies. (**a**) The longevity of humoral immune responses: the concentrations of antibody were monitored for 52 weeks in mice primed with DepVac and boosted with either PepVac or DepVac. Error bars represent average ± s.d. for $n = 4$ in each group (*P<0.05; **P<0.01). (**b**) Pharmacokinetic analyses were performed as described in Materials and methods. AUC_{0-t}, area under the concentration-time curve; C_{max}, peak concentration; T_{max}, the time to reach peak concentration. λ_z, decay rate constant; ($T_{1/2}$), the decay half-life; $AUC_{0-\infty}$, the area to infinity.

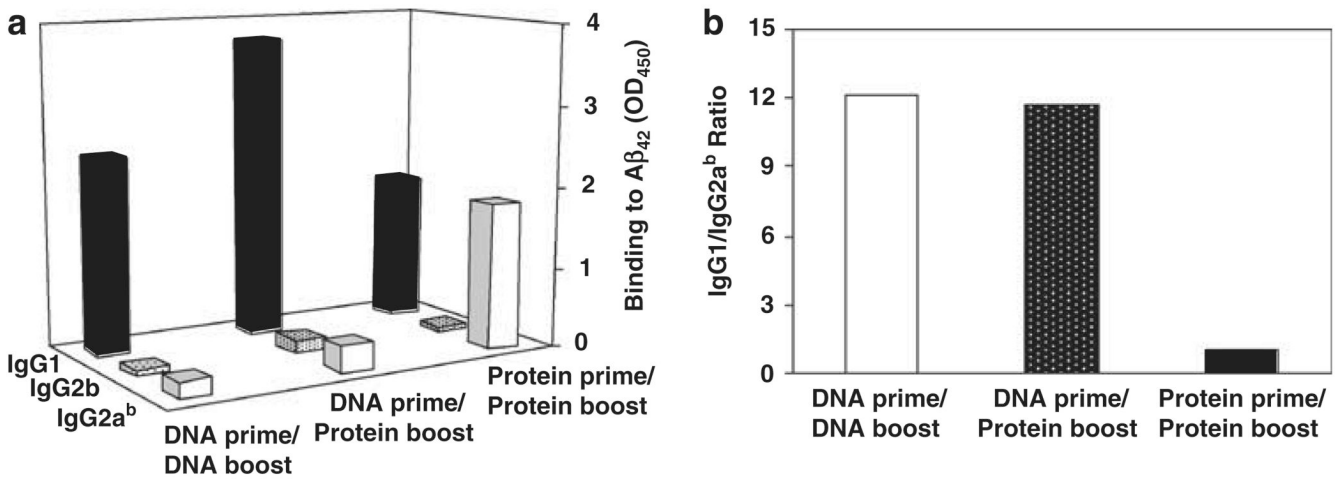


Figure 4.

The boost with PepVac did not change the isotype pattern of anti-Aβ antibody responses initially generated by priming with DepVac. (**a**) Isotyping of antibody responses in pooled sera of mice (dilution 1:500) immunized with DNA prime–DNA boost, DNA prime/protein boost or protein prime–protein boost regimens. (**b**) IgG1/IgG2a^b ratios were calculated based on data presented in (**a**). The experiment was repeated twice with the same results.

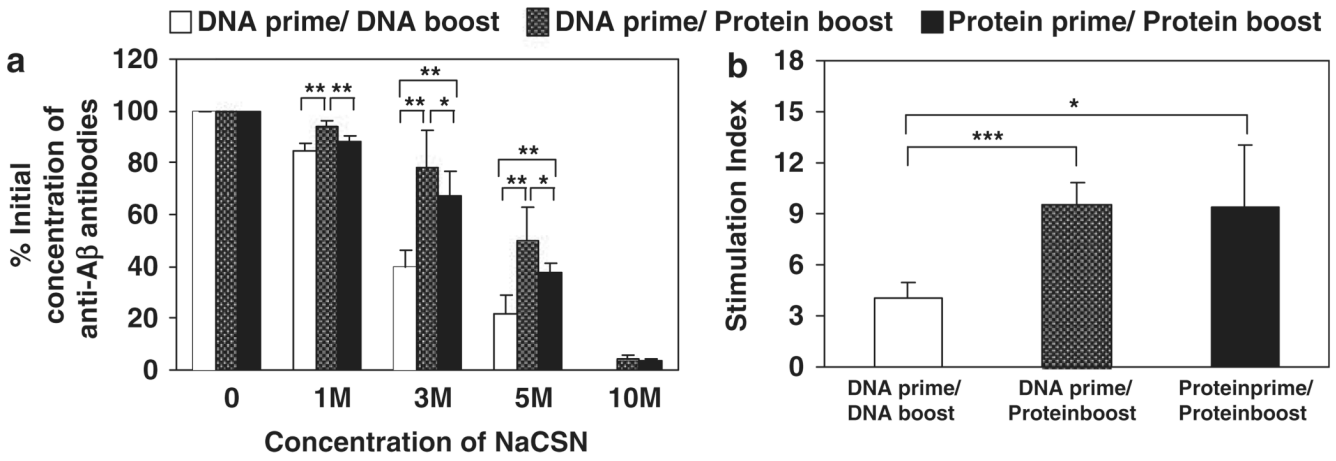


Figure 5.

(**a**) DNA prime–protein boost regimen induced antibodies with higher avidity for binding with $A\beta_{42}$ peptide compared with antibodies generated by DNA prime–DNA boost or protein prime–protein boost regimens. Relative avidity for antibody–antigen binding was determined using sodium thiocyanate (NaSCN) displacement enzyme-linked immunosorbent assay (ELISA). The effective concentration of NaSCN required to release 50% of antiserum from the ELISA plate (half-maximal effective dose (ED_{50})) was 2.4 M for DNA prime–DNA boost, 4.05 M for protein prime–protein boost and 5.1 M for DNA prime–protein boost. Error bars indicate ±s.d. (**P*<0.05; ***P*<0.01). (**b**) T-cell proliferation in mice immunized by DNA prime–protein boost or protein prime–protein boost regimens is significantly higher than in mice immunized and boosted with a DNAvaccine. Splenocytes from individual mice (n = 12) from three groups were re-stimulated *in vitro* with the Pan DR epitope (PADRE) peptide and the average value of the stimulation index was calculated. Error bars indicate±s.d. (**P*<0.05; ****P*<0.001).

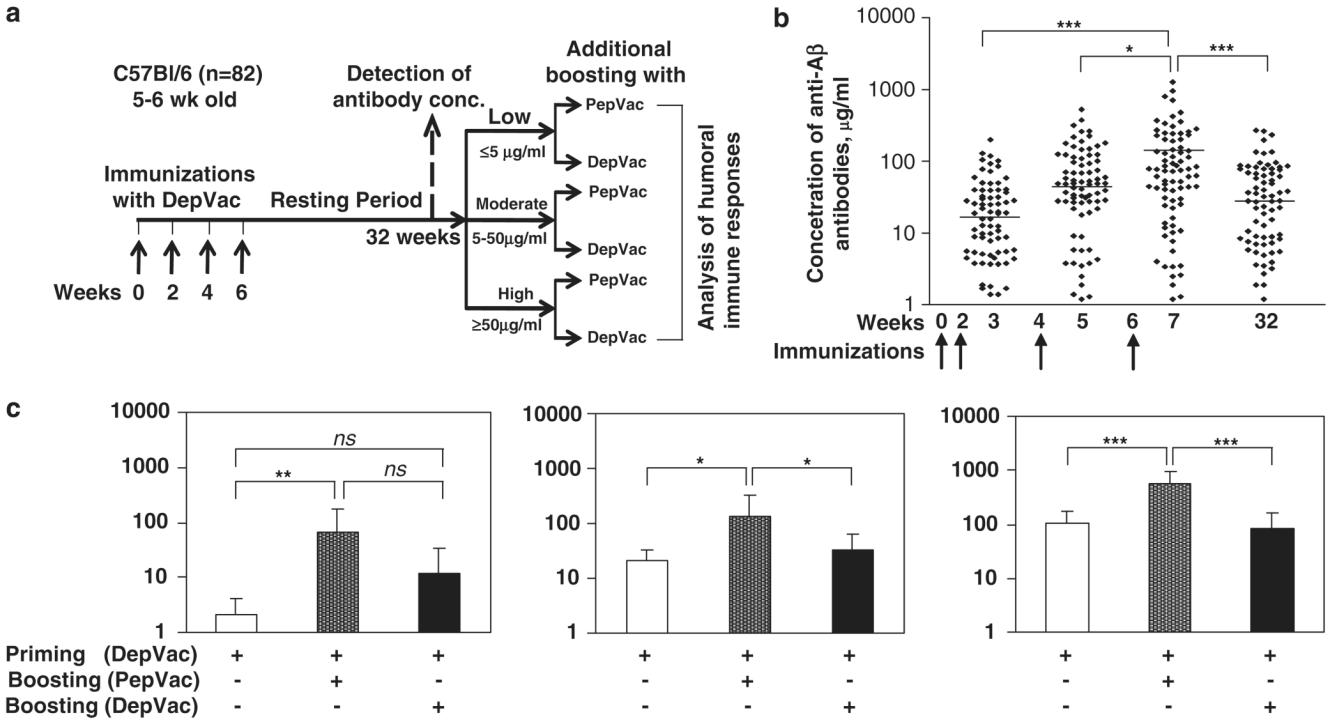


Figure 6.

(**a**) Schematic presentation of the immunization protocol. Mice were immunized with DepVac four times and then divided into groups of low, moderate and high responders and boosted either with DepVac or PepVac after a 26-week resting period (see details in Materials and methods). (**b**) Kinetics of the immune responses in mice immunized with DepVac. After 4 immunizations with DepVac, mice continued to produce significant concentrations of anti-Aβ antibodies during the 26-week resting period (results measured at week 32 from the experiment start date). Error bars indicate ±s.d. (n = 82 and *P<0.05; ***P<0.001). (**c**) The effect of a PepVac boost in mice is more profound than a DepVac boost. Boosting with PepVac at week 32 significantly increased the antibody responses in all concentration groups. In contrast, boosting with DepVac slightly increased the antibody production only in mice that had a low concentration of antibodies before the booster injection. Error bars represent the average±s.d. for mice with low (n = 12), moderate (n = 16) and high (n = 13) anti-Aβ antibody concentrations (*P<0.05; **P<0.01; ***P<0.001).