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## A peptide prime-DNA boost immunization protocol provides significant benefits as a new generation A $\beta$ 42 DNA vaccine for Alzheimer Disease

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### Abstract

Immunotherapy has the potential to provide a possible treatment therapy to prevent or delay Alzheimer Disease. In a clinical trial (AN1792) in which patients received this immunotherapy and received active A $\beta$ 1–42 peptide immunizations, treatment was stopped when 6% of patients showed signs of meningoencephalitis. Follow up on these patients led to the conclusion that the antibody response was beneficial in removing A $\beta$ 1–42 from brain but an accompanying inflammatory Th1 T cell response was harmful. As a safe alternative treatment targeting the same self protein, A $\beta$ 1–42, in brain, we and others are working on a DNA A $\beta$ 1–42 immunization protocol as the immune response to DNA immunizations differs in many aspects from immunizations with peptide antigens. Because the immune response to DNA vaccination has different kinetics and has a significantly lower antibody production, we evaluated two different prime boost regimens, A $\beta$ 1–42 DNA prime/ A $\beta$ 1–42 peptide boost and A $\beta$ 1–42 peptide prime/ A $\beta$ 1–42 DNA boost for their effectiveness in antibody production and possible side effects due to inflammatory T cell responses. While both boost regimes significantly enhanced the specific antibody production with comparable antibody concentrations, the absence of the A $\beta$ 1–42 T cell response (no proliferation and no cytokine production) is consistent with our previous findings using this DNA A $\beta$ 1–42 trimer immunization and greatly enhances the safety aspect for possible clinical use.

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## Keywords

Alzheimer Disease; Amyloid beta 1–42; immunotherapy; DNA vaccination; antibody production; regulatory immune response; peptide prime/DNA boost

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## 1. Introduction

Alzheimer Disease (AD) has been strongly associated with the accumulation, aggregation and deposition of amyloid beta (A $\beta$ ) peptides in brain and it was postulated 20 years ago that A $\beta$  deposition is an initial event in the pathogenesis of AD (Hardy, 1992, Selkoe et al. 1992, Hardy and Selkoe, 2002). A major component of senile plaques is aggregated extracellular amyloid (A $\beta$ 1–42 fibrils) and is thus, a major target for the development of an AD therapy. Mice transgenic for human FAD genes causing overproduction of human A $\beta$  peptides and plaque development in mouse brain were developed as mouse models of AD (Games et al. 1995, Dodart et al. 2002) and in these mice immunization with A $\beta$ 1–42 peptide lead to anti-A $\beta$ 1–42 antibody responses and reduction of A $\beta$ 1–42 from the brain, as well as a reduction in total number of plaques (Schenk et al. 1999). In addition, increased learning and memory have been described for A $\beta$ 42 immunized mice compared to the respective control animals (Janus et al. 2000, Morgan et al. 2000) and these findings lead to a first clinical trial, in which AD patients were vaccinated with A $\beta$ 1–42 peptide. This trial was stopped when 6% of immunized patients developed meningo-encephalitis (Fox et al. 2005, Gilman et al. 2005). A follow-up showed that A $\beta$ 1–42 peptide vaccination did indeed lead to a reduction in plaque load in patients who had been treated with A $\beta$ 1–42 peptide compared to the placebo control patients providing proof that it is possible to remove brain amyloid by immunotherapy (Holmes et al, 2008).

In an attempt to develop a safe treatment for Alzheimer Disease we and others are working on a DNA A $\beta$ 1–42 immunization protocol (Qu et al, 2004, 2006, 2007, 2010, Kim et al. 2007, Movsesyan et al. 2008, DaSilva 2009, Davtyan et al. 2010). The immune response to DNA immunizations in general is different from protein or peptide based immunizations and we have shown that in our model, a gene gun delivered DNA A $\beta$ 1–42 trimer immunization, a predominant Th2 antibody response and the absence of antigen specific T cell responses were the main characteristics. Both of these are indicative of a non-inflammatory immune response (Lambracht-Washington et al. 2009, Qu et al. 2010, Lambracht-Washington et al. 2011).

In regard to immunizations with whole organisms, proteins and peptides, DNA vaccines in general are considered as third generation vaccines consisting of genetically engineered plasmids designed to produce one or two specific proteins against which the immune response is elicited. To overcome a significantly lower antibody production following DNA immunizations, so called prime-boost regimens have been shown to be highly effective. (Kim et al. 2007, Subramanian & Divya Shree, 2008, Davtyan et al. 2010). This greater level of immunity is based on a heterologous prime boost protocol in which the antigen is applied via different routes and immunization sites. The first immunization initiates the priming of the immune response and subsequent immunizations trigger further expansion of antigen specific cells and selection of cells with high antigen avidity to boost the specific

responses (Woodland, 2004). We show here the results from two different boost regimens: The peptide boost of a DNA primed immune response and the DNA boost of a peptide primed immune response. Both appear to be effective to increase the antibody responses but they show marked differences in respect to the cellular immune response.

## 2. Material and Methods

### 2.1 Mice

All experiments were performed in 4 to 6 month old B6SJLF1/J female mice (n=24) purchased from The Jackson Laboratory (Bar Harbor, Maine). Animal use for this study was approved by the UT Southwestern Medical Center Animal Research Committee. In the first set of experiments we immunized four mice 3x with the DNA A $\beta$ 42 trimer followed by 3 immunizations with A $\beta$ 42 peptide (DNA prime/peptide boost) and another group of four mice received 3 A $\beta$ 42 peptide immunizations first followed by 3 immunizations with DNA A $\beta$ 42 trimer (peptide prime/DNA boost). The experiments were repeated with 16 mice in 4 groups: 6x DNA A $\beta$ 42 trimer, 3x DNA A $\beta$ 42 trimer/ 3x A $\beta$ 42 peptide, 6x A $\beta$ 42 peptide, 3x A $\beta$ 42 peptide/3x DNA A $\beta$ 42 trimer. All mice received a total of six immunizations.

### 2.2 Immunization with A $\beta$ 1–42

DNA vaccinations with a double plasmid DNA system (activator plasmid encoding Gal4 transcription factor and responder plasmid encoding three copies of human A $\beta$ 1–42) were performed using a Helios gene gun (Bio-Rad, Hercules, CA) with a helium pressure of 300 psi into the mouse ears without the use of adjuvant. Human A $\beta$ 1–42 peptide was mixed with QuilA as adjuvant which is very similar to Qs21 which had been used in the clinical trial and was given by i.p. injections. The immunizations procedures have been described in detail previously (Qu et al. 2007, Lambracht-Washington et al. 2009, Qu et al. 2010, Lambracht-Washington et al. 2011).

### 2.3 Plasma and splenocyte collections

The mice were killed ten days following the final immunization. Blood was collected by cardiac puncture; spleens were aseptically removed and processed for tissue culture as previously described (Lambracht-Washington et al. 2009).

### 2.4 Analysis of cell proliferation by CFSE dilution

This method has been described in detail previously (Lambracht-Washington et al. 2011). Fluorescence of the cells was measured using an Accuri C6 Flow Cytometer (Ann Arbor, MI) and analyzed with CFlow Plus and FCS Express Version 3 software programs.

### 2.5 ELISA and ELISPOT assays

ELISA assays for antibody levels in mouse plasma and cytokine concentrations from cell culture supernatants and ELISPOT assays to determine the numbers of cytokine producing cells were done as described previously (Lambracht-Washington et al. 2009).

## 2.6 Statistics

For statistics of proliferation, ELISA and ELISPOT data points, which were all performed in triplicate samples we used GraphPad Prism version 5.02 for Windows (San Diego, CA, [www.graphpad.com](http://www.graphpad.com)). Mean values and standard deviations were calculated using an unpaired t test with two tailed p values and column statistics. *P*-values of  $\leq 0.05$  were considered significant.

## 3. Results

### 3.1 Th1 and Th2 T cell subsets provide help for increased antibody production

The antibody response after DNA A $\beta$ 1–42 immunization delivered via gene gun bombardment into the skin is a characteristic Th2 response with antibodies of the IgG1 and IgG2b isotypes only. In the prime boost experiments performed here we found that boosting with A $\beta$ 1–42 peptide as well as DNA A $\beta$ 1–42 trimer, lead to remarkable increases in specific antibody production (Figure 1). The antibody levels found were similar to levels found in peptide only immunized mice,  $341.3 \pm 9.25$   $\mu\text{g/ml}$  in the peptide boosted mice and  $217.9 \pm 16.5$   $\mu\text{g/ml}$  in the DNA boosted mice, but the signature Th2 antibody response characteristic for the DNA regimen disappeared following the boost immunizations in both groups.

After 3 immunizations with DNA A $\beta$ 1–42 trimer or A $\beta$ 1–42 peptide, respectively, we found good antibody production in both groups:  $191.6$   $\mu\text{g/ml} \pm 14.52$  in the peptide immunized mice ( $n=8$ ) and  $92.05$   $\mu\text{g/ml} \pm 65.05$  in the DNA immunized mice ( $n=8$ ),  $P = 0.0009$  for the comparison of the two groups. The antibody isotypes after 3 immunizations showed a mixed antibody response in the peptide immunized mice with IgG1/IgG2a ratios of 1.0005 (Table 1) and a predominant IgG1 antibody response in the DNA immunized mice (IgG1/IgG2a ratio of 7.677, Table 1) consistent with our published results (Lambracht-Washington et al. 2009). Following the switch in the immunization procedure (peptide boost and DNA boost) the isotype profile changed completely. Now three of the groups, peptide only, peptide primed/DNA boost, DNA primed/peptide boost, showed a mixed anti-A $\beta$ 42 antibody isotype profile. Only the mice which had received 4 DNA A $\beta$ 42 peptide immunizations ( $n=4$ ) kept the predominant IgG1 antibody profile. Due to the mixed background in the mice we were using (B6SJLF1) and the known allelic differences for the IgG2a locus in the B6 and SJL mouse strains, we included the IgG2c (IgG2a<sup>b</sup>) isotype in our final analyses of the Th1/Th2 antibody ratios (Table 1, IgG1/IgG2a-IgG2c). We had seen in our isotype analyses a substantial amount of the IgG2c antibody from B6SJLF1 mouse plasma (data not shown).

### 3.2 Epitope analyses from plasma of prime-boost mice revealed a previously undetected B cell epitope difference and showed the influence of T cells during the priming immunizations

The antibody epitope for A $\beta$ 1–42 specific antibodies lays with in the N-terminal peptide sequence, A $\beta$ 1–15. To search for different antibody binding features in the mouse groups we tested antibody binding to a number of overlapping peptides within the known B cell epitope, A $\beta$ 1–17, A $\beta$ 2–17, A $\beta$ 3–17, A $\beta$ 4–17, A $\beta$ 5–17, and A $\beta$ 6–17. Furthermore, we dissected the antibody binding in regard to IgG isotypes, IgG1 and IgG2a/IgG2c. While

IgG1 and IgG2a/c antibodies from A $\beta$ 1–42 peptide immunized mice detected A $\beta$ 1–17, A $\beta$ 2–17, and A $\beta$ 3–17, some of the IgG1 antibodies from DNA A $\beta$ 1–42 trimer immunized mice detected also A $\beta$ 4–17 and A $\beta$ 5–17. Due to the high antibody levels this new epitope becomes very obvious in the prime-boost mouse groups: In the DNA prime/peptide boost mice IgG1 antibodies from five of eight plasma samples reacted with A $\beta$ 4–17 and A $\beta$ 5–17 and for the IgG2a/c isotypes four of eight plasma samples detected A $\beta$ 4–17 and A $\beta$ 5–17 (Figure 2). In the peptide prime/DNA boost mice only one from seven plasma samples recognized A $\beta$ 4–17 and A $\beta$ 5–17. Thus, it appears as if the DNA priming guided the antibody response towards this new B cell epitope. Antibodies reacting with A $\beta$ 4–17 can be found in 62.5% of the plasma samples from DNA primed mice and only 14.2% of the plasma samples from peptide primed mice.

In addition, we never found A $\beta$ 4–17 antibody binding in other A $\beta$ 1–42 peptide immunized mice but in some of the DNA A $\beta$ 1–42 trimer immunized mice from mouse strains B6SJLF1/J and B6C3F1/J carrying the mixed *H2* haplotypes *b/s* and *b/k* (data not shown).

### 3.3 DNA boosted T cells down-regulate T cell proliferation in peptide primed mice

T cell proliferation was analyzed with a CFSE dilution assay and staining for CD4 and CD8 T cells as described previously (Lambracht-Washington et al. 2011). In the first set of experiments we found no T cell proliferation in the mice which had received the peptide prime/DNA boost regimen (CD4 T cell proliferation index of 1, n=3), while for the mice which had received the DNA prime/peptide boost regimen elevated CD4 T cell proliferation was observed (CD4 T cell proliferation index of  $1.66 \pm 0.14$ , and A $\beta$ 42 specific CD4 T cell precursor frequencies of  $7.41\% \pm 1.6$ , n=4) (Figure 3). Thus, even though both groups had received three DNA and three peptide immunizations, the latter immunization, DNA or peptide, dominated in the T cell response, which meant that DNA boosted T cells prevented the T cell proliferation in the peptide primed mice, while the peptide boosted T cells proliferated despite the presumed presence of DNA primed A $\beta$ 1–42 specific T cells. In these experiments we had also included wells in which we used plate-bound CD28 antibody to provide a secondary signal in addition to TCR signaling to improve T cell proliferation. No differences to the above described results were found (data not shown).

These results were confirmed in the second set of experiments in which we tested in parallel mice which had received six DNA A $\beta$ 1–42 trimer immunizations and six A $\beta$ 1–42 peptide immunizations respectively.

### 3.4 Cytokine secretion in the prime-boost models

Splenocytes from six times A $\beta$ 1–42 peptide immunized mice secreted IFN $\gamma$  (shown by ELISPOT, Figure 4), IL-17, and IL-5, whereas the splenocytes from six times DNA A $\beta$ 1–42 trimer immunized mice secreted none of these three cytokines. The ELISPOT analysis showed that indeed the DNA boost regimen apparently suppressed the T cell response in peptide primed mice. While the peptide prime/DNA boost mice showed only a slight increase in IFN $\gamma$  secreting cells compared to the respective medium controls and the levels found in the DNA only immunized mice ( $26.3 \pm 6$  spots per  $10^6$  cells after A $\beta$ 1–42 re-stimulation and  $56 \pm 14.7$  spots after A $\beta$ 10–26 re-stimulation), the DNA prime/peptide boost

mice showed  $105 \pm 27.7$  IFN $\gamma$  ELISPOTs after A $\beta$ 1–42 re-stimulation and  $85.6 \pm 0.5$  IFN $\gamma$  ELISPOTs after re-stimulation with A $\beta$ 10–26 peptide. This difference in IFN $\gamma$  producing cells in these two groups of mice, peptide prime/DNA boost and DNA prime/peptide boost, is highly significant ( $P = 0.0067$ ). Furthermore, while the peptide prime/DNA boost mice showed no IL-17 secreting cells in the ELISPOT analysis, the DNA prime/peptide boost mice showed an increase in IL-17 spot count ( $36.33 \pm 18.18$ ) compared to the spot count in the peptide prime/DNA boost mouse group. However, due to the high standard deviation this difference is not significant ( $P = 0.06$ ). For the Th2 cytokine IL-5 the DNA prime/peptide boost mouse group has the highest number of cytokine secreting cells with  $37.67 \pm 17.01$  spots per  $10^6$  cells, but again due to the high standard deviation the difference to the peptide prime/DNA boost group as well as the peptide only immunized mouse group were not significant ( $P$  values of 0.037 and 0.252, respectively).

#### 4. Discussion

We present here data for two different immune boost regimens to enhance the antibody production in a wild-type mouse model for DNA A $\beta$ 1–42 immunization as possible therapy for Alzheimer Disease. The immune response following DNA immunizations in general is much lower compared to peptide immunizations and to enhance the antigen specific response boost immunizations are performed to enhance this response. Most commonly used is a protein/peptide or viral boost immunization to enhance the antibody response in DNA immunizations (Kim et al. 2007, Subramanian & Divya Shree, 2008, Davtyan et al. 2010). Our results show that it is also very efficient to augment a peptide immunization with a DNA boost. To our knowledge this is also the first time, DNA immunizations were used to boost an existing immune response. The benefits from this vaccination approach (peptide prime/DNA boost) may lay in the down regulation of a T cell response which harbors complications of an inflammatory immune response. Therefore, the protocol described here with a peptide prime/DNA boost approach provides the basis for future vaccination protocols.

Mice were primed for three immunization time points to establish a well directed immune response which was tested with the antibody isotype analysis after the initial immunizations. In line with our previous data, we found a mixed immune response (IgG1/IgG2a antibodies) in the peptide primed mice and a predominant Th2 immune response with almost exclusively IgG1 antibodies in the DNA primed mice as shown with the high IgG1/IgG1a ratio (Table 1).

This changed immediately with the boost immunizations: The DNA prime/peptide boost mice switched from a predominant IgG1 response (IgG1/IgG2a ratio of 7.7) to the mixed isotype profile with an IgG1/IgG2a ratio of 4.4 after four immunizations and ratios of 1.1 and 1.3 after five and six immunizations which is similar to the peptide immunizations with ratios of 1.0 and 1.1 indicative of equal IgG1 and IgG2a A $\beta$ 1–42 antibody isotype levels.

Antibodies from the DNA primed group maintained the small overlapping epitope specificity and bound strongly to A $\beta$ 4–17 and A $\beta$ 5–17, whereas this epitope is undetected in the peptide primed/DNA boost group or the A $\beta$ 1–42 peptide only group. Thus, a large

portion of the high level antibodies and the respective antibody secreting B cells is derived directly from the three DNA or peptide priming vaccinations and enhanced with the latter peptide boost immunizations. After six immunizations both groups which had received the boost immunization, peptide or DNA respectively, had similar high antibody levels with a mixed antibody isotype pattern. As both showed similar levels of IgG1 and IgG2a antibodies indicative for the type of ongoing immune response, Th1 or Th2, we expected to find a similar cellular immune response as well. While we do find strong T cell reactivity in the DNA boost/peptide primed mice, we do not find T cell responses as tested by proliferation assays or the analysis of cytokine secretion in the peptide primed/DNA boost mice. T cells from DNA prime/peptide boost mice showed good proliferation and secretion of IFN $\gamma$  and IL-17 while T cells from peptide prime/DNA boost mice did not proliferate and made no IFN $\gamma$  or IL-17. The DNA boost dominated in the T cell immune response as it down regulated antigen specific T cells consistent with what we have shown before (Lambracht-Washington et al. 2011) and a peptide prime/DNA boost immunization regimen is thus beneficial as it minimizes the risk of an inflammatory immune response.

A number of positive functions of A $\beta$  immunotherapy were directly associated with the anti-A $\beta$ 42 antibodies. Several studies have shown that the removal of the toxic A $\beta$ 42 depositions in brain with antibody therapy preserves synaptic structures and neuron morphology (Buttini et al. 2005, Serrano-Pozo et al. 2010) and again the positive effects were attributed to the antibodies only. Recently it has been shown that antibodies binding to the N-terminal portion of A $\beta$ 1–42, which is the known B cell epitope, bind and neutralize soluble A $\beta$  species and prevent synaptotoxicity (Zago et al. 2012). In regard to the mixed antibody response (IgG1 and IgG2a) in our peptide prime/DNA boost model, the beneficial antibodies used in the Zago study were all of the IgG2a (Th1) isotype. Thus, the antibody itself even with the IgG2a (Th1) isotype does not appear to be harmful per se. Furthermore, as stated in this paper the mouse antibody 3D6, which has the IgG2a isotype is the parent of the humanized anti-A $\beta$ 42 antibody, bapineuzumab, which is FDA approved and in phase III clinical trials (Zago et al. 2012). Earlier it has been shown that antibodies of the IgG2a isotype are more potent in reducing amyloid burden from APP transgenic mouse brains compared to antibodies of the IgG1 or IgG2b isotypes (Bard et al. 2003).

The published data about the interrupted clinical trial with active A $\beta$ 1–42 immunization in AD patients (AN1792) has lead to the conclusion that the antibody response was beneficial but the accompanying inflammatory Th1 T cell response was harmful and was responsible for the meningoencephalitis in 6% of the patients (Gilman et al. 2005, Holmes et al, 2008). Thus, even though we have a mixed antibody response, we assume that the peptide prime/DNA boost regimen is safe and has the advantage that it increases strongly the antibody response and at the same time the DNA boost down regulates the antigen specific T cell immune response which minimizes the risk of an inflammatory autoimmune response.

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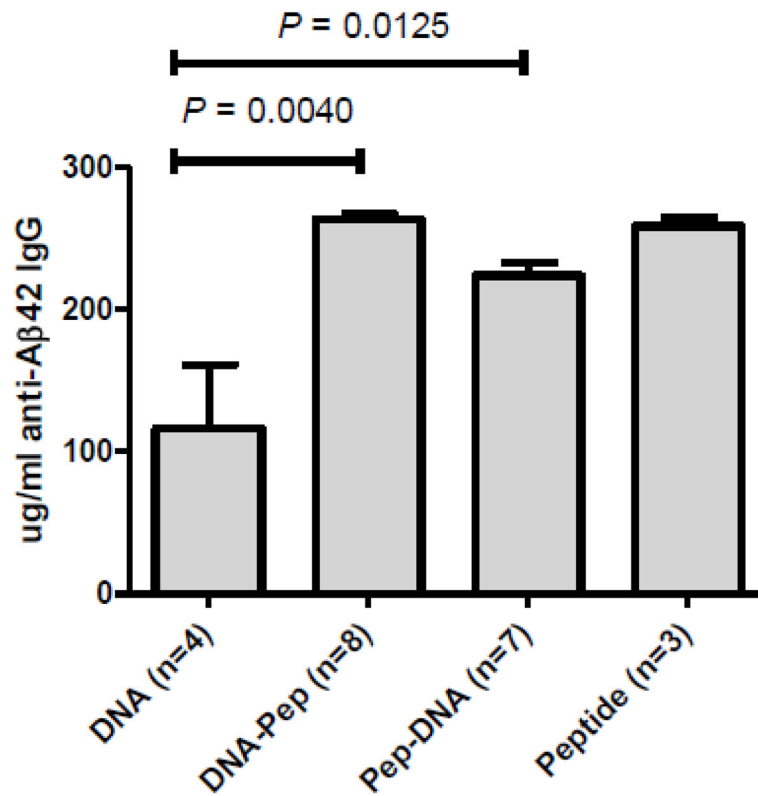
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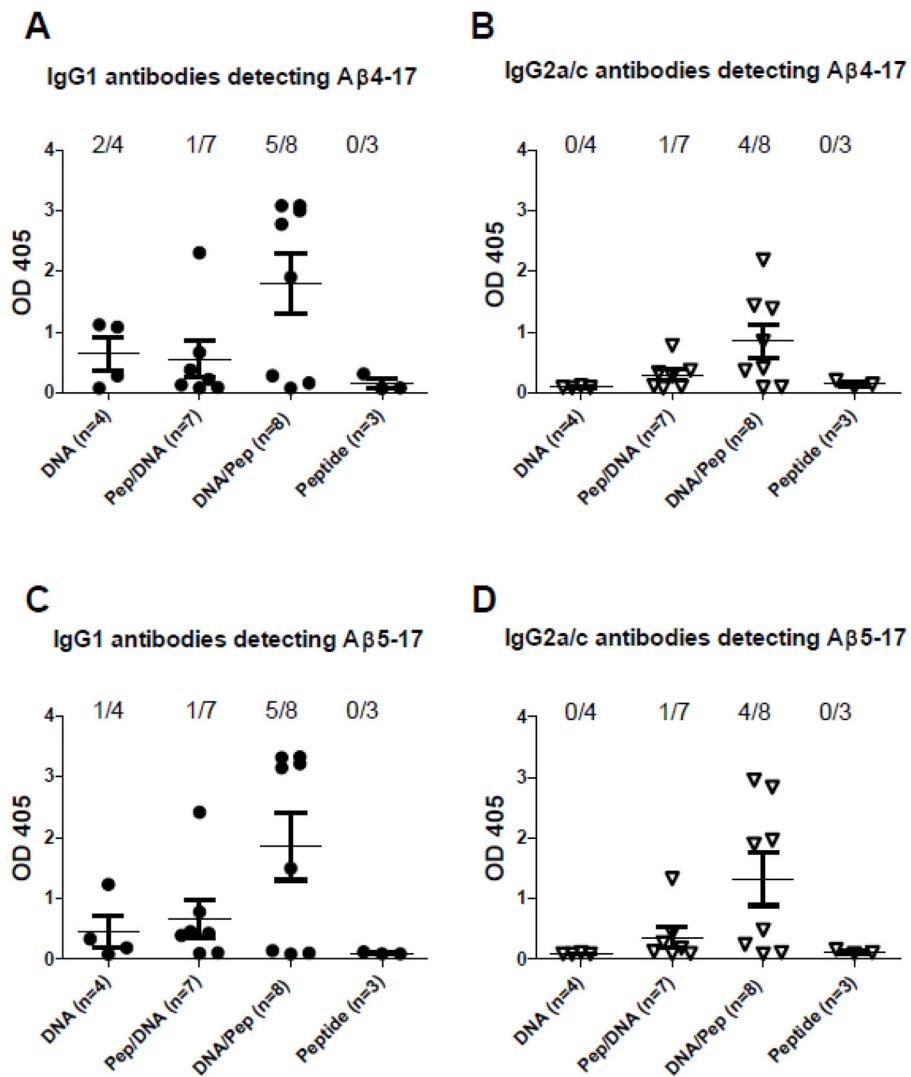


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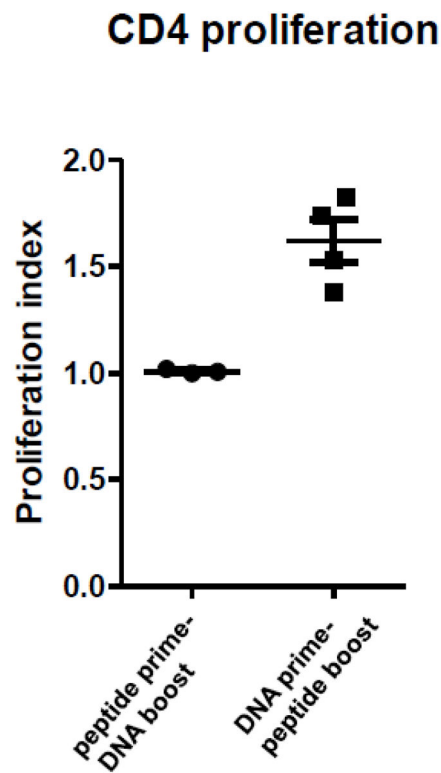


**Figure 1. Boost immunizations strongly increase the Aβ1–42 specific antibody response**  
 Bars show the amount of antigen specific IgG levels in mouse plasma combined from the individual median antibody levels for each mouse. The number of mouse samples analyzed is indicated as n. Both of the tested boost immunizations (DNA prime/Peptide boost and Peptide prime/DNA boost) significantly increased the levels of Aβ1–42 specific antibodies in mouse plasma compared to the DNA Aβ1–42 trimer



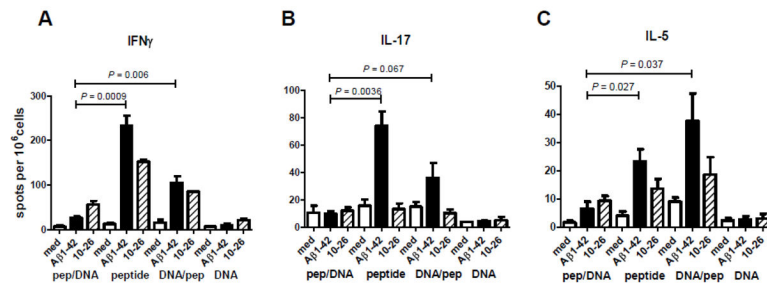
**Figure 2. Detection of the B cell epitope A $\beta$ 4-17 in DNA immunized mice**

Plasma samples from the 6x immunization time point of all the mice used in this study were analyzed by ELISA in parallel. DNA A $\beta$ 1-42 immunized mice had no anti-A $\beta$ 1-42 IgG2a antibodies and thus no anti-A $\beta$ 4-17 or anti-A $\beta$ 5-17 IgG2a antibodies were detected (B and D) while some (2/4 and 1/4) mouse samples showed specific IgG1 antibody binding to this epitope (A and C). Despite high antibody levels against A $\beta$ 1-42 in the A $\beta$ 42 peptide immunized mice which detected the B cell epitope A $\beta$ 1-17, no antibody binding was found for A $\beta$ 4-17 and A $\beta$ 5-17 (A, B, C, and D). IgG1 antibodies reacting with A $\beta$ 4-17 can be found in 62.5% (5/8) of the plasma samples from DNA primed mice and only 14.2% (1/7) of the plasma samples from peptide primed mice (A). IgG2a antibodies reacting with A $\beta$ 4-17 can be found in 50% (4/8) of the plasma samples from DNA primed mice and again 14.2% (1/7) of the plasma samples from peptide primed mice (B). Identical reactivities were found for the A $\beta$ 5-17 epitope (C and D)



**Figure 3. No CD4 T cell proliferation in mice which received the peptide prime/DNA boost A $\beta$ 42 immunizations**

Pooled splenocytes from mice which had received the respective 6 immunizations were re-stimulated *in-vitro* with A $\beta$ 1–42 peptide and CFSE dilution was analyzed at day 6. No proliferation was found in mice which had received the DNA boost (CD4 T cell proliferation index of 1), while the DNA boosted mice which had received 3 peptide boost immunizations showed elevated CD4 T cell proliferation (CD4 T cell proliferation index of 1.6).



**Figure 4. ELISPOT analysis of prime boost groups indicates down-regulation of cytokine secretion due to the DNA boost immunizations**

Pooled splenocytes from the four immunization groups were analyzed for IFN $\gamma$  (A), IL-17 (B) and IL-5 (C) secreting cells after A $\beta$ 42 peptide re-stimulation *in-vitro* in ELISPOT assays. The four immunization groups are indicated below the graphs (peptide prime/DNA boost, peptide, DNA prime/peptide boost, DNA). White bars show the respective number of cytokine secreting cells in the medium controls, black bars show cytokine secretion after re-stimulation with full-length A $\beta$ 1–42 peptide, and the striped bars show cytokine secreting cells after re-stimulation with the T cell epitope peptide, A $\beta$ 10–26. The differences for IFN $\gamma$  secreting cells in the two prime boost groups (peptide prime/DNA boost and DNA prime/peptide boost) is significant (P value 0.006) and it appears that the DNA boost regimen down regulates the inflammatory cytokine response in these mice. The comparison of IFN $\gamma$  and IL-17 secreting cells in the peptide prime/DNA boost group and the peptide group shows for both of these cytokines significantly less cytokine secreting cells in the peptide prime/DNA boost group (P values of 0.0009 and 0.0036 respectively) which is indicative for the down regulated immune response due to the DNA boost immunizations.

**Table 1**

Analysis of changes in the IgG1/IgG2a ratios due to different boost regimens

Immunization times	IgG1/IgG2a-2c ratio			
	DNA A $\beta$ 42 trimer	DNA prime/peptide boost	A $\beta$ 42 peptide	Peptide prime/DNA boost
3 ×	7.677		1.005	
4 ×	10.5	4.438	1.026	1.033
5 ×	9.717	1.198	1.113	1.023
6 ×	9.293	1.385	1.113	1.008