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DNA Immunization Against Amyloid beta 42 has High Potential as Safe Therapy for Alzheimer's Disease as it Diminishes Antigen-Specific Th1 and Th17 Cell Proliferation

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Abstract

The pathogenesis of Alzheimer's disease (AD) has been strongly associated with the accumulation of amyloid beta ($A\beta$) peptides in brain, and immunotherapy targeting $A\beta$ provides potential for AD prevention. A clinical trial in which AD patients were immunized with $A\beta_{42}$ peptide was stopped when 6% of participants showed meningoencephalitis, apparently due to an inflammatory Th1 immune response. Previously, we and other have shown that $A\beta_{42}$ DNA vaccination via gene

gun generates a Th2 cellular immune response, which was shown by analyses of the respective antibody isotype profiles. We also determined that in vitro T cell proliferation in response to A β 42 peptide re-stimulation was absent in DNA A β 42 trimer-immunized mice when compared to A β 42 peptide-immunized mice. To further characterize this observation prospectively and longitudinally, we analyzed the immune response in wild-type mice after vaccination with A β 42 trimer DNA and A β 42 peptide with Quil A adjuvant. Wild-type mice were immunized with short-term (1–3 $\times$ vaccinations) or long-term (6 $\times$ vaccinations) immunization strategies. Antibody titers and isotype profiles of the A β 42 specific antibodies, as well as cytokine profiles and cell proliferation studies from this longitudinal study were determined. Sufficient antibody titers to effectively reduce A β 42, but an absent T cell proliferative response and no IFN γ or IL-17 secretion after A β 42 DNA trimer immunization minimizes the risk of inflammatory activities of the immune system towards the self antigen A β 42 in brain. Therefore, A β 42 DNA trimer immunization has a high probability to be effective and safe to treat patients with early AD.

Keywords

Amyloid beta; Alzheimer's disease; Immunotherapy; Gene gun; Immune response; Inflammation; Th17

Introduction

Amyloid beta peptide (A β 1–42), a small end product from processing of the amyloid precursor protein (APP), is the dominant component in senile plaques found in the brains from Alzheimer's patients. Accumulation of A β 1–42 peptides in plaques has been strongly associated with the progression of Alzheimer's disease and dementia (Hardy 1996; Hardy and Selkoe 2002; Bertram and Tanzi 2004; Rosenberg 2005). It has been shown that soluble A β oligomers and dimers are particularly toxic; impair synaptic plasticity and decrease dendritic spine density in the hippocampus. These toxic A β species are likely derived from the plaque cores present at high numbers in AD brain which may release locally active A β molecules (Selkoe 2008; Shankar et al. 2008). Research using transgenic mice overexpressing human A β transgene had shown that immunization with A β 42 peptides can lead to a reduction of plaque numbers in the brain as well as an improvement in learning behavior and memory in these mice (Schenk et al. 1999, Janus et al. 2000, Morgan et al. 2000). A subsequent clinical study in which early Alzheimer patients received A β 42 peptide immunizations was stopped due to autoimmune reactions causing meningoencephalitis in 6% of the participants (Fox et al. 2005, Gilman et al. 2005). The adverse effect had been associated with an inflammatory T cell response to the auto antigen A β 42 in brain (Monson et al. 2006) and it has been shown that activated T cells easily cross the blood brain barrier (Qing et al. 2000). To avoid this particular complication, research for future clinical trials using A β 42 immunization in Alzheimer's patients has been focused on alternative strategies. One such strategy is the immunization with A β 42 encoding DNA via gene gun which is well established, and it has been shown that the intradermal ballistic injection of DNA results in a strongly Th2-biased immune response. This has been shown in several studies including ours by analyses of the respective antibody isotype profiles (DaSilva et al. 2009; Davtyan et al. 2009; Ghochikyan et al. 2003; Kim et al. 2007a, b, Movsesyan et al. 2008a, b; Qu et al. 2004, 2006, 2007, 2010). However, we also found that specific in vitro T cell proliferation to A β 42 peptide was missing in DNA A β 42 trimer-immunized mice when compared to A β 42 peptide-immunized mice which had been analyzed in parallel (Lambracht-Washington et al. 2009). Our prior results clearly demonstrated that DNA A β 42 vaccination led to the production of A β 42-specific IgG1 antibodies demonstrating the function of Th2 T cell responses, yet paradoxically, T cell proliferative responses were not detected. These results led us to hypothesize that DNA

A β 42 vaccination leads to a transient self-limiting T cell response. We examined this hypothesis by comparing the T cell immune response following vaccination with A β 42 trimer DNA delivered by gene gun at several different time points and compared it to the responses in mice which had been immunized by i.p. injections of A β 42 peptide.

Materials and Methods

Mice

All experiments were performed in 4- to 8-month-old B6SJLF1/J mice purchased from The Jackson Laboratory (Bar Harbor, Maine). Animal use for this study was approved by the UT Southwestern Medical Center Animal Research Committee.

Immunization with A β 1-42

DNA vaccinations with plasmid DNA encoding the human A β 42-trimer and human A β 42 peptide were performed as described previously (Qu et al. 2007, 2010; Lambracht-Washington et al. 2009). The cloning of this particular construct and comparison with other DNA A β 42 constructs has been presented in detail in Qu et al. 2010. Due to an endosomal targeting sequence following the sequence coding for A β 42 peptide (3 $\times$), it is expected that the antigen will be processed via the MHC class II pathway. In the first set of experiments (Group A), twelve mice received gene immunizations into the skin of the lower back (six, three, two, and one immunization time point(s)). For this procedure, a small section (1–2 cm²) of the lower back was shaved with precaution not to damage the skin. The skin was swabbed with ethanol to remove surface oils and enhance penetration. The DNA-coated gold particles were injected into the skin via gene gun and with a helium gas pressure of 300 psi. Each vaccination consisted of two injections into the lower back (2 μ g DNA total per immunization). The immune response in this group was compared to one B6SJLF1 mouse which had received one A β 42 peptide injection. The experiment was repeated with twelve mice which received four, three, and one immunization (three mice each which received gene gun injections into both ears (4 μ g DNA/immunization) and compared to a group of three mice which had received three A β 42 peptide/Quil A immunizations (Group B). In the last set of experiments, we compared the immune response in mice which had received six DNA immunizations into the skin of both ears with mice which had received six peptide immunizations, mice which had received three DNA immunizations with mice which had received three peptide immunizations, and mice which had received one DNA immunization with mice which had received one peptide immunizations, respectively ($n = 18$, 3 mice per group, Group C). All peptide immunizations were via the intraperitoneal route (100 μ g A β 42 peptide/ 20 μ g Quil A).

Preparation of Leukocytes

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).

Analysis of Cell Proliferation by CFSE Dilution

Cells for labeling were re-suspended in PBS and immediately mixed with an equal volume of CFSE (carboxyfluorescein diacetate succinimidyl ester) diluted in PBS. The cells were incubated for 5 min at room temperature, labeling was stopped by adding complete RPMI medium and removal of the solution from the cells by centrifugation. Cells were plated in round-bottom 96-well cell culture plates at 1×10^6 cells per well and re-stimulated with A β 42 peptide or Con A for 6 days. After harvesting cells were re-suspended in FACS buffer (PBS/1% BSA/0.1% NaN₃) and directly stained with APC conjugated antibodies, CD4 and

CD8 (BD Biosciences, San Jose, CA). 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.

ELISPOT and ELISA assays

ELISPOT assays for murine IL-2, IL-5, IL-17, and IFN γ (eBioscience, San Diego, CA) were performed in triplicates according to standard procedures. Spots were counted with an automated ELISPOT plate reader (Bioreader 5000, Biosys, Karben/Germany). ELISA assays for cytokine concentrations and antibody levels in mouse plasma were done as described previously (Lambracht-Washington et al. 2009).

Statistics

For statistics (unpaired t test with two tailed p values, column statistics) we used GraphPad Prism version 5.02 for Windows (San Diego, CA, www.graphpad.com). *P* values of 0.05 were considered significant.

Results

In order to identify the nature of the immune response elicited following human DNA A β 42 trimer immunization, T cell proliferative responses were determined following one, three, and six immunizations and comparisons were made with control mice that had been vaccinated with human A β 42 peptide. In a wild-type mouse the immunization with human A β 1-42 results in a response against a foreign antigen but our ongoing experiments with human APP transgenic mice show similar results (unpublished). For analysis of the T cell proliferation a CFSE dilution assay (illustrated in Fig. 1) was utilized. This technique allows for the identification of proliferating cell populations by the dilution of CFSE fluorescence in combination with cell surface markers which made it possible to distinguish within the proliferating cell populations whether the cells belong to the CD4 or the CD8 lineages using multicolor flow cytometry.

T Cell Proliferation and Humoral Immune Response

For the CD4 + T cell response a weak proliferative response was detected from the peptide-immunized ($22.37 \pm 9.59\%$; *P* value 0.0975) and DNA A β 42-immunized mice ($12.07 \pm 4.1\%$ *P* value 0.9265) following a single immunization (Fig. 2a). Although T cell proliferation was detected above the level of an unimmunized mouse, CD4 proliferation from both groups was not significantly increased above the negative control where no antigen was added (medium only). Following three and six immunizations, CD4 + T cell proliferation was strongly increased in the peptide-immunized mice ($44.47 \pm 2.237\%$ and 44.8 ± 6.092 , respectively) (Fig. 2b, c). By contrast, the DNA A β 42 trimer-immunized mice showed weak CD4 T cell proliferative responses following three and six immunizations (13.97 ± 5.843 and $10.53 \pm 3.323\%$, respectively). Significant CD8 T cell responses were only detected in the peptide-immunized group after three and six immunizations.

In the analyses of the humoral immune response, only low levels of an A β 42 specific antibody response, IgG and IgM, was detected after one immunization with A β 42 peptide and DNA A β 42 trimer (Fig. 2d). We found a strong IgM response and a low IgG response which is typical for this early immunization time point as the initial antibody response upon immunization is always IgM which converts to IgG antibodies after germinal center reactions, T helper cell help, and production of cytokines had occurred. After three immunizations, the IgG levels were increased and most importantly all of the IgG antibodies found at this time point in the DNA A β 42 trimer-immunized mice had already switched to the IgG1 isotype indicating that Th2 cell help had occurred (Fig. 2e) and antibody levels

increased further after six immunizations with IgG1 as the dominant IgG isotype (Fig. 2f). In the A β 42 peptide-immunized mice a steady increase of IgG antibody levels was observed for three and six immunization time points; the antibody isotype profile showed similar levels of IgG1 and IgG2a antibodies indicative for a mixed Th1/Th2 immune response. Additional antibody epitope studies showing that antibodies elicited by DNA A β 1-42 trimer as well as A β 1-42 peptide immunization detected the main B cell epitope, A β 1-15, and will thus react with A β 1-42 and A β 1-40, have been presented before (Lambracht-Washington et al. 2009; Qu et al. 2010). We were surprised that we could not find increasing numbers of proliferating T cells in the DNA A β 42 trimer-immunized mice as the antibody isotype switching clearly indicated that a Th2 T cell response was operative.

Epitope Studies, Precursor Frequencies, and Cytokine Secretion

The lack of strong T cell proliferation in DNA A β 42-immunized mice led us to examine T cell responses to the T cell epitope A β 10-26 for this particular mouse strain as this might provide a stronger signal to the respective T cell receptor. Splenocytes from mice that had been immunized three times with A β 42 peptide or DNA A β 42 were cultured in the presence of media alone, A β 42 (10 μ g/ml) or the A β 10-26 peptide (10 μ g/ml) and proliferation was detected by CFSE dilution. As shown in Fig. 3a, an equivalent number of CD4 + T cells from peptide-immunized mice proliferated in response to full-length A β 1-42 ($44.77\% \pm 1.756$) and the A β 10-26 peptide ($44.1\% \pm 2.762$). CD4 + T cells from DNA A β 42-immunized mice failed to proliferate to A β 42 ($14.4\% \pm 5.973$) yet they strongly proliferated to A β 10-26 ($45.07\% \pm 6.787$). It is interesting to note that the CD4 proliferative responses to A β 10-26 were nearly identical between the A β 42 peptide and DNA A β 42 trimer-immunized mice. CD8 T cell responses from A β 42 peptide-immunized mice showed a significant increase in proliferation after re-stimulation with A β 10-26 ($23.7\% \pm 5.635$) as compared to A β 1-42 ($12.93\% \pm 1.501$). For both peptides, A β 42-specific CD8 T cell proliferation is highly significant (*P* values 0.0022 and 0.0002) when compared to the medium control. The effects of stimulation with A β 10-26 were not seen in CD8 T cells from DNA A β 42 trimer-immunized mice where only a modest increase in proliferation was seen following culture with A β 10-26 ($7.867\% \pm 2.631$) and full-length A β 1-42 ($2.5\% \pm 1.559$) (*P* values of 0.0783 and 0.7120).

T cell precursor frequencies reflect how many antigen-specific cells existed in a given cell population as it calculates back from all divided cells which frequency of antigen-specific cells has been present in the original cell population. Precursor frequencies can be obtained from the proliferation analyzes as well as from the ELISPOT experiments. High frequencies of antigen-specific T cells in both assays harbor potential danger in form of an autoimmune response against A β 42 as cerebral self antigen. We showed here the analysis of the precursor frequencies from the CFSE proliferation which had been calculated with the FCS Express 3 Proliferation software. In comparison to precursor frequencies of the A β 42 peptide-immunized mice much lower numbers were obtained for CD4 T cell precursors from the DNA A β 42 trimer-immunized mice against the full-length A β 42 peptide but not the epitope peptide A β 10-26 (Fig. 3b).

Cytokines have an important function as mediators for the immune response and can be differentiated into pro-inflammatory and anti-inflammatory activators. Thus, we determined the effects of DNA and peptide immunization on T effector cell differentiation by analysis of cytokine expression (IFN γ , IL-5, IL-17, IL-2) by ELISPOT and used again both peptides, full-length A β 42 and A β 10-26. In Fig. 3c–f, the results from a representative ELISPOT experiment were shown. Numbers of cytokine secreting cells from spleens of DNA A β 42 trimer- and A β 42 peptide-immunized mice were compared, and we found that consistent with the T cell proliferation results, the mice immunized with A β 1-42 peptide developed

much higher numbers of cytokine secreting cells in response to A β 1-42 peptide re-stimulation than DNA A β 42 trimer-immunized mice. For IFN γ , a mean of 21 ($\pm$ 7) IFN γ secreting cells per 10⁶ cells was found in mice which had received one A β 1-42 peptide immunization. This number increased to 102 ($\pm$ 7.7) IFN γ secreting cells per 10⁶ splenocytes after three peptide immunizations and to 194 ($\pm$ 38.1) cells per 10⁶ splenocytes after six peptide immunizations. Higher numbers of IFN γ secreting cells were found after re-stimulation with the T cell epitope A β 10-26 for the one time and three times immunized mice (76 $\pm$ 12 and 102 $\pm$ 7 cells, respectively) and lower cell numbers were found in the six times immunization time point re-stimulated with A β 10-26 (118 $\pm$ 26 cells). Low numbers of IFN γ secreting cells were measured for all three time immunization points of the DNA A β 42 trimer-immunized mice with mean numbers of 24, 23 and 16 IFN γ secreting cells, respectively, and higher numbers of antigen specific cells in the wells which were re-stimulated with A β 10-26 in the mice which had received one and three DNA immunizations (mean numbers of 59 and 57 cells, respectively) but not for the six immunization time point. The IL-2 ELISPOT analysis as a general T cell growth factor showed consistent results with more spots in the A β 42 peptide-immunized mice and less spots in the DNA A β 42 trimer-immunized mice. Also for the anti-inflammatory cytokine IL-5 higher numbers of cytokine secreting cells were measured in the six times A β 42 peptide-immunized mice with 90 $\pm$ 22 IL-5 secreting cells in 10⁶ splenocytes compared to 3 $\pm$ 2.6 IL-5 secreting cells in the six times DNA A β 42 trimer-immunized mice. A very interesting new finding is the high number of IL-17 secreting cells (137.3 $\pm$ 14 cells per 10⁶ splenocytes) in the six times A β 42 peptide-immunized mice which had been re-stimulated with the full-length A β 1-42 peptide, but not in the A β 10-26 re-stimulated cells from these mice nor any other mouse splenocytes sample (A β 42 peptide or DNA A β 42 trimer-immunized mice) (Fig. 3f).

Discussion

Immunotherapy against the cerebral self antigen A β 42 has great potential to prevent or treat AD, the most common dementia found in the elderly. However, active immunization against A β 42 harbors also the potential of the development of an autoimmune response and can cause brain inflammation which depends largely on the type of immune response elicited. During the cellular immune response naive T cells differentiate into one of several antigen specific CD4 + T helper subtypes, Th1, Th2, and Th17 and/or antigen specific cytotoxic CD8 + T cells, which all have different effector functions and secrete different cytokines. Based on the following polarization of the immune response the outcome of an immunization can be inflammatory or anti-inflammatory. Genetic immunization in which the immunizing agent is DNA encoding A β 42 peptide differs in important points from peptide immunization and appears to be safe as preventive therapy for AD. Consistent with our previous findings we confirmed the absence of an A β 42 specific in vitro T cell proliferation after full-length A β 42 re-stimulation and the absence of effector cytokine production in the A β 42 DNA trimer-immunized mice at the six times immunization time points. While we did find similar levels of CD4 T cell proliferation in response to the T cell epitope peptide, A β 10-26, in A β 42 peptide and DNA A β 42 trimer-immunized mice, we did not find significant cytokine production in response to the epitope peptide or to the full-length A β 42 peptide in the DNA immunized mice. Thus, the A β 42 T cells generated in the DNA-immunized mice do not have the same effector functions as T cells generated in the peptide-immunized mice as we found both increasing proliferation and strong cytokine secretion in these mice.

Cytokines as important mediators of the immune system are not only produced by cells of the immune system. Thus, the presence of a particular cytokine is only a relative indicator for the polarization of an immune response into an inflammatory or a non-inflammatory pathway. The inflammatory cytokine IFN γ is initially secreted by natural killer cells and

macrophages, and in case of an ongoing specific immune response it is secreted by CD4 and CD8 T cells. Differences in the IFN γ levels in response to full-length A β 1-42 and the T cell epitope peptide A β 10-26 indicated that the cytokine was secreted by different cell types and a lower secretion of IFN γ after A β 10-26 re-stimulation was due to cytokine production from T cells and it was lower compared to the full-length peptide as this measures IFN γ also from other cells. The high number of IL-17 secreting cells in the ELISPOT from the six times A β 42 peptide-immunized mice is the first description of a Th17 immune response after A β 42 peptide immunization and is of particular interest as this group of CD4 T cells (Th17) has been described as important T cell subset in the pathogenesis of multiple sclerosis (MS) and its respective mouse model, experimental autoimmune encephalitis (EAE) (Gocke et al. 2007; Aranami and Yamamura 2008; O'Connor et al. 2008; Murphy et al. 2010). A direct role for Th17 cells as effector cells causing neuronal dysfunction and neuroinflammation has recently been described by in vivo imaging experiments in an EAE mouse model (Siffrin et al. 2010). It is possible that both A β 42 specific Th1 and A β 42 specific Th17 cells might have been involved in the occurrence of the meningoencephalitis in the AD patients from the clinical trial AN1792 in which A β 42 peptide immunization had been used (Fox et al. 2005, Gilman et al. 2005) and further characterization of the Th17 subset which was found in response to A β 42 peptide immunization might add important information to the analysis of neurodegenerative diseases as A β 1-42 is a brain self antigen as myelin oligodendrocyte glycoprotein (MOG) or myelin basic protein (MBP), which are the neuroantigens involved in MS. A recent report in which the authors showed that Th17 T cells were increased in AD patients (Saresella et al. 2011) favors the speculation that A β 42 specific Th17 T cells might be indicative for neuroinflammation and in this context the immunization with A β 42 peptide might further an underlying inflammatory immune response increasing Th17 development in AD patients.

In general, DNA vaccinations are potent inducers of CD8 T cell responses (Gurunathan et al. 2000) which are a necessary immune defense against viral infections as CD8 T cells can kill the infected cells. As a particularly interesting result, we did not find any CD8 T cell response in our DNA A β 42 trimer immunizations while we do find A β 42 specific CD8 T cells in the peptide-immunized mice. One of the possible explanations for the lack of a CD8 T cell response in the DNA A β 42 trimer-immunized mice compared to A β 42 peptide-immunized mice might be due to the different antigen concentrations as it has been reported that low levels of antigen exposure lead to clonal deletion whereas high levels lead to clonal anergy in antigen specific T cells (Redmond et al. 2005). Another possible reason might be the lack of the third signal necessary for CD8 differentiation which involves stimulation via receptors for inflammatory cytokines like IL-12 and IFN α (Obar and Lefrancois, 2010) which are missing in the DNA-immunized mice due to highly polarized Th2 immune response. CD4 T cell help is required for an antigen-specific cytotoxic T lymphocyte (CTL) response (CD8 T cell effector cells) and these T cells might be again missing in the mice immunized with the DNA A β 42 trimer construct (Maecker et al. 1998). Last but not least, it is possible that the endosomal targeting sequence attached to the DNA construct (Qu et al. 2010) directs the protein exclusively to the MHC class II presentation pathway so that only CD4 T cells are activated.

In conclusion, the results presented clearly show that an initial T cell response as indicated by the isotype switching of the antibodies in DNA A β 42 trimer-immunized mice after the third immunization fades with continued immunization intervals as we were not able to show an increased T cell reactivity to full-length A β 42 or T cell effector functions as measured by cytokine secretion at the later time points. Thus, the risk for inflammatory reactions of the immune system against the self antigen A β 42 in brain is likely minimal and makes this particular immunization approach an attractive candidate for future clinical testing.

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Fig. 1.

Illustration of the CFSE assay used to analyze T cell proliferation. **a** Cells analyzed were in the live lymphocyte gate P1. *Dot plot* showing the dilution of the dye CFSE by cell division on the *x* axis (FL1) and the gating (Gate R1) on the antibody positive cells on the *y* axis (FL4). Antibody negative cells are localized in the *bottom* of the graph and the antibody positive cells are found in the *upper portion* of the graph. 34.6% of the analyzed cells were CD4 positive. Cells which have divided have lower fluorescence intensity which moves these cells to the *left hand side* of the graph. **b** Histogram showing CFSE-labeled cells from gate R1. Marker indicates the percentage of divided cells. 46.4% of the CD4 positive cells have divided in cell culture. **c** Cell proliferations after analysis with FCS3 express software (De Novo). *Grey histogram* indicates the undivided cells; *black histograms* indicate six daughter generations of cells. Shown is the analysis from three times peptide-immunized mice and re-stimulation with A β 1-42 peptide

Fig. 2.

Analysis of the T cell proliferations and humoral immune response in 1 $\times$, 3 $\times$, and 6 $\times$ immunized mice. Proliferation of CD4 and CD8 T cells from one, three, and six times immunized mice (A β 42 peptide and DNA A β 42 trimer) as assessed by CFSE dilution. Percentage of cell proliferation was analyzed in the life cell gate, followed by further gating on CD4 and CD8 positive cells and compared for the medium control and the A β 42 peptide re-stimulated cell cultures. The left hand side of the graphs show CD4 T cell proliferation, the right hand side of the graphs show CD8 T cell proliferation. Pooled splenocytes from three A β 42 peptide-immunized mice (*closed circles*) and three DNA A β 42 trimer-immunized mice (*open circles*) were analyzed in triplicates for proliferative responses after in vitro re-stimulation with A β 1-42 and A β 10-26 peptides and compared to medium controls. (2A) Proliferation of CD4 and CD8 T cells found in one time immunized mice, (2B) Proliferation of CD4 and CD8 T cells in three times immunized mice, (2C) Proliferation of CD4 and CD8 T cells in six times immunized mice. Anti-A β 42 antibody production in response to A β 42 peptide and DNA A β 42 trimer immunization: Shown were OD readings for one (2D), three (2E), and six (2F) immunization time points from three mice immunized with peptide (*closed circles*) or DNA A β 42 trimer (*open circles*), respectively. Antibody isotypes (IgG1, IgG2a, IgG, IgM) were indicated on the x axis

Fig. 3.

Epitope studies, precursor frequencies, and cytokine analysis by ELISPOT. **a** Comparison of the T cell proliferation in response to full-length $A\beta$ 1-42 peptide and the T cell epitope peptide, $A\beta$ 10-26: proliferation of CD4 and CD8 (*striped bars*) T cells from three times immunized mice ($A\beta$ 42 peptide (*black bars*) and DNA $A\beta$ 42 trimer (*white bars*) was assessed by CFSE dilution and compared for the medium control and the $A\beta$ 42 or $A\beta$ 10-26 peptide re-stimulated cell cultures. **b** CD4 T cell precursor frequencies in three times $A\beta$ 42 peptide and DNA $A\beta$ 42 trimer-immunized mice. Analyses were performed using the software program FCS express. **c–f** ELISPOT analysis for the cytokines IFN γ , IL-2, IL-5, and IL-17: pooled splenocytes from three $A\beta$ 42 peptide-immunized mice and three DNA $A\beta$ 42 trimer-immunized mice (1, 3, and 6 immunization time points) were analyzed in triplicates for cytokine secretion after in vitro re-stimulation with $A\beta$ 1-42 and $A\beta$ 10-26 peptides and compared to medium controls. *Black bars* indicate $A\beta$ 42 peptide-immunized mice; *white bars* indicate DNA $A\beta$ 42 trimer-immunized mice