Published in final edited form as:

J Neuroimmunol. 2015 January 15; 278: 90–99. doi:10.1016/j.jneuroim.2014.12.007.

Co-stimulation with TNF receptor superfamily 4/25 antibodies enhances *in-vivo* expansion of CD4+CD25+Foxp3+ T cells (Tregs) in a mouse study for active DNA A β 42 immunotherapy

Doris Lambracht-Washington and Roger N. Rosenberg

Department of Neurology and Neurotherapeutics, Alzheimer's Disease Center, University of Texas Southwestern Medical Center, Dallas, Texas, USA

Abstract

The study was designed to test DNA A β 42 immunization in mice as alternative approach for possible active immunotherapy in Alzheimer patients. As results, we found polarized Th2 immune responses, efficient A β 42 antibody levels, and disappearance of antigen specific T cells. *In-vivo* TNFRSF4/25 antibody co-stimulation enhanced A β 42 specific T cell responses with initial Th2 expansion and subsequent development of A β 42 specific CD4+CD25+Foxp3+ cells. It showed that Th2 biased responses due to gene gun immunizations propagate the development of regulatory T cells. In conclusion, full-length DNA A β 42 immunization into skin results in a regulatory response with minimal risk of inflammation and autoimmunity.

Keywords

TNFRSF4/25 antibody co-stimulation; DNA A β 42 immunization; Alzheimer disease; Th2/Treg differentiation; skin immunity

1. Introduction

We are interested in characterizing the immune response in an immunotherapeutic approach for Alzheimer disease using DNA A β 1–42 immunization. Following the complications due to inflammatory T cell responses in a clinical trial, in which AD patients received active full-length A β 42 peptide immunizations (AN1792), many therapy approaches are now concentrating on the B cell epitope or using passive immunizations to avoid negative side effects (Mangialasche et al. 2010, Carillo et al. 2013, Lambracht-Washington and Rosenberg, 2013). We are pursuing immunization with a DNA encoding three copies of full-length A β 1–42 peptide for several reasons: 1) The immune response elicited by DNA immunization differs significantly from A β 1–42 peptide induced immune responses, 2) The immunization with full-length DNA A β 1–42 provides the basis for a wider epitope spectrum

© 2014 Elsevier B.V. All rights reserved.

Corresponding author: Doris Lambracht-Washington, PhD, doris.lambracht-washington@utsouthwestern.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

and 3) We found the disappearance of antigen specific T cells and propose the development of a regulatory T cell response (Lambracht-Washington et al. 2011, 2012). It might be beneficial for several reasons when a complete immune response, encompassing B and T cell responses, is addressed within a potential vaccine. B cell epitopes are important for the antibody specificity but antigen specific T cell help improves antibody production and antigen specific regulatory T cells modulate the immune responses.

Antibody treatment with agonistic antibodies binding to receptors of the TNF superfamily, TNF Receptor Superfamily 4 (TNFRSF4) and TNFRSF25, had been described to expand CD4+CD25+Foxp3+ regulatory T cells (Tregs) in mice *in-vivo*. The published data showed expansion of CD4+CD25+Foxp3+ cells in the context of co-stimulation or vaccination with a peak value at 5 to 6 days which was the half-life of the antibodies (Schreiber et al. 2010 and 2012, Wolf et al. 2012, Khan et al. 2013). Thus, the possible expansion of antigen specific Tregs cells with this type of co-stimulation with DNA A β 42 immunization prompted us to further characterize the T cell site. We emphasize that we used this antibody treatment only as a tool to enable the characterization of the T cell response in our model and to demonstrate potential benefits of a Th2/regulatory immune response. Characteristic features of the immune responses following DNA A β 42 immunization were enhanced due to the co-stimulation which contributed to the overall clarity of the data shown here.

Immunizations and immunotherapy have a broader clinical use, for instance immunizations against a bacterial antigen provide protection from disease caused by the respective organism, or in the context of autoimmunity it has been shown that immunizations which result in expansion of Tregs can reduce the outcome of an inflammatory event. Immunizations with tumor antigens have been shown successful in treating cancer patients in prevention of reoccurring cancer cells. In the context of treatment for AD, it is important to state that the presence of an immune response had been shown to be successful in removing amyloid plaques from brain, but that the humoral immune response also led to brain microhemorrhage and an accompanying cellular immune response led to inflammation (Orgogozo et al. 2003, Fox et al. 2005, Gilman et al. 2005).

Our results support the development of Tregs in the skin with a DNA A β 42 immunization protocol. TNFRSF4/25 ab injection stimulates T cells in general and depending on which type of immune response was activated with the in parallel performed immunization, increased Th1, Th2, or mixed Th1/Th2 will be found. In our immunization protocols in combination with the gene gun mediated immunization into the skin we found increased numbers of CD4+CD25+Foxp3+ cells and IL-10 cytokine secretions which did not occur with the A β 1–42 peptide immunizations and TNFRSF ab co-stimulation. These findings highlight that the development of a regulatory immune response is enhanced in Th2 biased responses characteristic for our model of DNA A β 42 immunization and is further propagated with the immunization into the skin as site for the initial antigen contact. Immune responses to self-antigen in peripheral tissue and particularly the skin can lead to the priming of regulatory memory as shown by several investigators (Rosenblum et al. 2011, Gratz et al. 2014, Sanchez Rodriguez et al. 2014).

2. Material and methods

2.1. Mice

Experiments were performed in 3 to 8 month old male and female BALB/c-Foxp3-EGFP mice (Stock # 006772, Jackson Laboratories, Bar Harbor, Maine). Mice used in the individual sets of experiments were age and gender matched with three to four mice/group. Animal use for this study was approved by the UT Southwestern Medical Center Animal Research Committee.

2.2. Immunizations and antibody treatment

DNA vaccinations with a two plasmid DNA system (an activator plasmid encoding Gal4 transcription factor and a responder plasmid encoding three copies of human full-length A β 1–42) and A β 42 peptide immunizations were performed as described previously (Lambracht-Washington et al. 2009, 2011). For the DNA vaccinations, a new vector system with a Kanamycin resistance gene was used in the ratio of 0.75 μ g activator plasmid: 1 μ g of responder plasmid. This new vector has been tested thoroughly to assure identical performance to the vector we had used before (Qu et al. unpublished). For Treg expansion a combination of DR3 (clone 4C-12) and OX-40 (clone OX-86) abs (20 μ g of each ab per mouse, LEAF purified low endotoxin, azide free, Biolegend) was mixed in PBS and given by i.p. injection directly following the DNA immunization. Two times immunized mice received two intradermal DNA immunizations (total of 4 μ g DNA bound to 4 mg gold particles/immunization) in a biweekly interval delivered via gene gun and one i.p. antibody injection following the second immunization; three times immunized mice received three DNA immunizations in biweekly intervals followed by one ab injection following the third immunization, respectively. Three groups of mice received i.p. injections of a IL-4 neutralizing ab (clone 11B11, 100 μ g, mouse, BioXcell) together with the TNFRSF abs. Naïve mice in the control groups received i.p. injections of isotype control abs, or i.p. injections of the TNFRSF4/25 abs.

2.3. Antibody levels in plasma

ELISA assays for ab levels in mouse plasma and ab isotypes were done as described previously (Lambracht-Washington et al. 2009) with the commercially available anti-A β 17–24 mab 4G8 (Covance) as standard. The IgG1/IgG2a ratio as an indicator of a Th1 versus Th2 profile of the humoral immune response was calculated from the OD values in ELISAs in which biotinylated anti-IgG isotype antibodies were used to detect the anti-A β antibodies.

2.4. Flow cytometry

Splenocytes were directly labeled with the following abs: CD4 (FITC, PE, and APC), CD25 (clone PC61, PE and APC), GITR (PECy7), CD127 (PE), DR3, OX-40 (BD biosciences, Biolegend). Intracellular Gata-3 (PE), Helios (APC) staining was used to determine Th2 and Treg polarization/activation, respectively. Foxp3 positive cells were analyzed without staining in FL1 for the EGFP labeled transgene in the used mouse strain. Fluorescence was measured using an Accuri C6 Flow Cytometer (Ann Arbor, MI) and analyzed with CFlow

Plus and FCS Express Version 3 software programs. Gates and markers were set based on the respective isotype ab control staining.

2.5. T cell stimulation in cell culture

Mouse splenocytes (5×10^6 cells/ml) were cultured in 24-well plates and stimulated with soluble anti-CD3 ab (0.5 μ g/ml) or A β 1–42 peptides (10 μ g/ml). Cells and culture supernatants were harvested at 48 h, 72 h, 96 h, and 120 h for ab staining and/or cytokine ELISAs.

2.6. Cytokine analysis by ELISA and ELISPOT

Cytokine assays were performed as described previously (Lambracht-Washington et al. 2009, 2011). ELISA and ELISPOT assays for murine IL-4, IL-9, IL-10, IL-17, and IFN γ (eBioscience, San Diego, CA) were performed with pooled splenocytes in triplicate wells according to standard procedures. Spots were counted with an automated ELISPOT plate reader (Bioreader 5000, Biosys, Karben, Germany). The IL-10 ELISPOT was performed with an extended incubation time of 96 h.

2.7. Statistics

For statistics (unpaired t test with two tailed *p* values, non-parametric Mann-Whitney test, column statistics) we used GraphPad Prism version 6.02 software for Windows (San Diego, CA, www.graphpad.com). *P*-values of ≤ 0.05 were considered significant.

3. Results

3.1. TNFRSF co-stimulation leads to expansion of CD4+CD25+Foxp3+ cells *in-vivo*

We propose a regulatory T cell response in active full-length DNA A β 42 immunized mice (Lambracht-Washington et al. 2011, 2012), and to further characterize the T cell immune responses, we used the TNFRSF antibodies, TNFRSF4 (CD134, clone Ox86) and TNFRSF25 (clone DR3), to target and expand CD4+CD25+Foxp3+ T cells. These two TNFRSF antibodies have been described to provide co-stimulatory effects in immunizations and in particular in the immune responses following secondary antigen contact (Schreiber et al. 2012). For detection of Tregs (CD4+CD25+Foxp3+) we used BALB/c-Foxp3-EGFP mice in which all cells expressing the fork head transcription factor Foxp3 are genetically labeled and show Enhanced Green Fluorescence. At the 6th day following immunization and ab injections, which had been described in the literature as the peak day for Treg expansion (Schreiber et al. 2012), mouse splenocytes were analyzed by flow cytometry for expansion of specific CD4 T cell populations. As shown in Figure 1, the comparison of splenocytes analyzed directly from naïve mice, three times DNA A β 42 immunized mice, and three times DNA A β 42 immunized mice which received also the ab injections, the CD4+CD25+Foxp3+ T cell population (Tregs) increased from 9.33% (mean $\pm$ SEM: $11.6\% \pm 1.95\%$) in naïve mice, to 16.74% ($15.7 \pm 0.61\%$) in the $3 \times$ DNA immunized mice, to 26.43% ($22.8 \pm 0.46\%$) in the DNA immunized and TNFRSF ab injected mice within the CD4+ T cells (**1A and B**). In additional mouse groups we found that the TNFRSF ab injection also induced expansion of CD4+CD25+Foxp3+ cells in peptide immunized mice and naïve mice in the directly *ex-vivo* analyzed cells (last two histograms, **1C**). However, cells from these mice did not

secrete IL-4 and IL-10 (see 3.3.). After 72 h in-vitro A β 42 peptide re-stimulation in culture, CD4+CD25+Foxp3+ had expanded in all groups of mice (upper right quadrant of the contour plots) except the mice which had received the TNFRSF abs injection only without any immunization. Importantly, an expansion of CD4+CD25+Foxp3- (T effector cells, Teffs) was found only in the mice which had received the A β 42 peptide immunization indicating the presence of antigen specific effector T cells which proliferate in response to the antigen stimulation in culture (1D). The positive control cultures, anti-CD3 ab re-stimulation *in-vitro*, lead to a strong T cell proliferation in all mouse groups shown and can be seen in both populations of CD4+CD25+ T cells (1E), Tregs as well as Teffs. In mice which had received TNFRSF abs co-stimulation a substantial number of cells remained in the CD4+CD25- population ($32.5\% \pm 5.2\%$, non-polarized CD4 T cells).

3.2. TNFRSF antibody co-stimulation leads to increased levels of A β 42 antibodies in plasma with no change in the ab isotype patterns

We determined for all mouse plasma samples the levels of A β 42 antibodies as well as the isotype composition to identify a Th2 biased immune response in the DNA immunized mice versus a mixed immune response in the peptide immunized mice as we have described before (Lambracht-Washington et al. 2009, Qu et al. 2007 and 2010). In Figure 2A the anti-A β 42 IgG ab levels were shown from two times immunized mice. Highly significant were the differences between A β 42 peptide and DNA A β 42 immunized mice with and without co-stimulation ($p = 0.0006$ and 0.0001 , Mann-Whitney test, Figure 2A). Increases in ab levels in the comparison of A β 42 peptide immunizations and A β 42 peptide immunization together with the TNFRSF ab co-stimulation were not significant ($p = 0.805$, Mann-Whitney test). For the DNA A β 42 immunized mice the co-stimulation did lead to a significant increase of the antibody response ($p = 0.0032$, Mann-Whitney test). After two DNA immunizations mice had increased levels of 143.5 ± 17.18 μ g (SEM) A β 42 specific IgG antibodies per ml plasma ($n=26$) compared to 71.6 ± 16.81 μ g ($n=19$). Importantly, the Th2 signature was not changed (2B). While the peptide immunized mice had IgG1/IgG2a ratios around 1, all of the DNA A β 42 immunized mice had a mean IgG1/IgG2a ratio of 8.45 and with TNFRSF co-stimulation the IgG1/IgG2a ratios had a mean value of 52.17. Even in mice which had received anti-IL4 ab injections to prevent Th2 signaling directly following the DNA immunizations, anti-A β 42 antibodies were of the IgG1 isotype with an IgG1/IgG2a ratio of 10.25 ± 3.045 ($n=8$). The decrease in the antibody levels in the mice which had received the anti-IL4 ab injection was not significant in the comparison to the identical immunized mouse group of 2 $\times$ DNA A β 42/TNFRSF ab co-stimulation without the IL-4 ab treatment ($p = 0.1042$, Mann-Whitney test).

In a long term approach in which mice had received six DNA A β 42 immunizations with TNFRSF ab injections with every second immunization (3 $\times$), the IgG1/IgG2a ratio was 71.73 ± 25.58 , and the respective six times A β 42 peptide/ three times TNFRSF immunized mice had an IgG1/IgG2a ratio of 1.265 ± 0.135 (data not shown).

3.3. Enhanced IL-4 (Th2 signature cytokine) secretion in splenocytes from DNA A β 42 immunized mice injected *in-vivo* with TNFRSF4 and -25 antibodies

Cell cultures from the differently immunized mouse groups were analyzed for cytokine secretion with IFN γ , IL-4 and IL-10 ELISPOT and the respective ELISA assays as these are signature cytokines for Th1 (IFN γ), Th2 (IL-4) and Th2/Treg (IL-10) responses. A representative result for cells from two times immunized mice (secondary antigen contact) is shown in Fig 3A. The described T cell epitope for BALB/c mice (*H2^d*) is A β 6–20 (Cribbs et al. 2003). Consistent with our previously published results we did not find IFN γ secreting cells in DNA A β 42 immunized mice (Lambracht-Washington et al. 2011) and injection of the TNFRSF antibodies and respective co-stimulation of the antigen specific T cells did also not lead to IFN γ secretion (Fig. 3A). In contrast to our published results describing no T cell activation in DNA A β 42 immunized mice with continued immunizations in B6SJLF1 mice (Lambracht-Washington et al. 2011), we found here using DNA immunization plus TNFRSF ab co-stimulation in Balb/c mice and analyses of cytokine pattern at early immunization time points (2 $\times$ and 3 $\times$), high numbers of IL-4 producing cells (Fig. 3A, 3B). The number of IL-4 secreting cells is highly increased from cells of DNA A β 42/TNFRSF ab immunized mice already in the medium controls. We found 184 ± 4 IL-4 secreting cells per 10^6 cells in the medium controls and 151 ± 34.93 spots per 10^6 cells in the A β 6–30 re-stimulated cultures. On the other hand, in splenocytes from A β 42 peptide/TNFRSF ab immunized mice we found lower numbers of IL-4 secreting cells in the medium controls (33 ± 1 spots per 10^6 cells) which increased to 138.3 ± 40.5 spots per 10^6 cells upon stimulation with A β 6–30 peptide. Antigen specificity of the IL-4 secreting cells in the DNA A β 42 immunized mice can be concluded from the comparison to naïve mice which had received the TNFRSF ab injections as these cells did not secrete IL-4 upon peptide re-stimulation (Fig. 3A). A similar result was found when we analyzed and compared the immune responses in one time, two times, and three times DNA A β 42 immunized mice in combination with a one-time TNFRSF ab injection directly after the final immunization (Fig. 3B). The two and three times DNA immunized mice with TNFRSF co-stimulation showed increased numbers of IL-4 secreting cells (159.7 ± 18.28 spots/ 10^6 cells and 220.7 ± 1.33 spots/ 10^6 cells), while the one times DNA immunized mice with TNFRSF co-stimulation showed ELISPOT counts comparable to the one times DNA immunized mice without antibody injection (10.67 ± 4.17 spots/ 10^6 cells and 11.67 ± 4.63 spots/ 10^6 cells, respectively). Thus, the TNRRSF ab injections were effective on secondary antigen contact boosting the respective immune responses (Th2 bias in DNA immunized mice) but not during the priming processes.

3.4. Enhanced IL-10 cytokine secretion in splenocytes from DNA A β 42 immunized mice

These findings suggested a possible link between IL-4 producing Th2 cells and the generation of regulatory T cell immune responses. We had shown previously that splenocytes from DNA A β 42 immunized mice secrete the immunomodulatory cytokine IL-10 *in-vitro* after A β 42 peptide re-stimulation (Lambracht-Washington et al. 2009), and therefore we analyzed next IL-10 production in cells from the TNFRSF co-stimulated mice. The cytokine IL-2 had been shown as a limiting factor for Treg expansion and survival and to show an influence of IL-4 on the level of IL-10 producing cells, we studied in parallel cell cultures in

which the cells were incubated with IL-2 plus A β 1–42 peptide to cultures with IL-4 plus A β 1–42 peptide. Cells from naïve mice produced no IL-10 (Fig. 3C). Cells from three times and two DNA immunized mice combined with the TNFRSF ab injection produced high levels of IL-10 under all culture conditions ranging from 2927 ± 117.1 pg/ml in the medium control for cells from 2 $\times$ DNA/TNFRSF Ab immunized mice to 4611 ± 48.2 pg/ml in supernatants from cells of 3 $\times$ DNA/TNFRSF Ab immunized mice which had been re-stimulated for 120 h with A β 1–42 peptide plus IL-4. While cells from 1 $\times$ A β 42 peptide/TNFRSF ab immunized mice produced no IL-10 in medium or A β 42/IL-2 cultures, they made 1071 ± 24.6 pg IL-10/ml medium after re-stimulation with A β 42/IL-4. Cells from 1 $\times$ DNA A β 42/TNFRSF ab immunized mice produced almost twice as much, 1751 ± 77 pg IL-10/ml, and splenocytes from 1 $\times$ DNA A β 42 immunized mice produced 1649 ± 20.9 pg IL-10/ml medium (p values in the comparison to the peptide immunized mice 0.0011 and < 0.0001 , unpaired t test, respectively). Since Th9 cells are also potent producers of IL-10 we analyzed splenocytes supernatants from the immunized mice in parallel for IL-9 secretion by ELISA. No IL-9 production was found (data not shown).

IL-10 was also found in the respective ELISPOT assays and we found significant better results with an extended time frame of 96 h. Cells from 2 $\times$ DNA/TNFRSF ab immunized mice, 2 $\times$ DNA/isotype control ab immunized mice and naïve/isotype control ab injected mice were tested in this assay with a 48 h incubation time as well as an 96 h incubation time. In both assays we found that IL-4 in the cell culture medium lead to IL-10 secreting cells only in the DNA immunized mice but not in naïve mice. While the spot counts were not significantly different between medium wells and IL-4 wells after 48 h, these numbers were significant after an extended 96 h incubation time ($p = 0.0315$ and 0.0052 , unpaired t test, respectively, Fig. 3D).

To further specify the effect of IL-4 on IL-10 secretion we performed DNA immunizations and treated groups of mice with an anti-IL4 ab together with the TNFRSF ab injections. A representative result is shown in Fig. 3E. While we did not find significant changes for the numbers of IL-4 secreting cells (Fig. 3E) and IL-4 levels in cell cultures (data not shown) for cells from mice with (166.8 ± 13.7 spots) or without (205.8 ± 7.3 spots) the IL-4/TNFRSF ab injections (Fig. 3E, upper graph), we found significant lower levels of IL-10 secreting cells in mice which had received the anti-IL4/TNFRSF ab injections (Fig. 3E, lower graph, 41 ± 10.3 spots compared to 78.2 ± 3.8 spots, $p = 0.0286$ Mann-Whitney). Again, cells from mice which had received 2 A β 42 peptide immunizations and TNFRSF ab injections produced IL-4 but no IL-10 (122.3 ± 15.7 and 19.7 ± 4.6 spots, respectively).

In mice which had received repeated A β 42 peptide immunizations in combination with TNFRSF ab injections, we found no IL-10 producing cells (3F). 6 $\times$ DNA A β 42 immunized mice had significant higher levels of IL-10 producing cells in cultures which had been re-stimulated with A β 42 peptide compared to the medium controls ($p = 0.0149$, unpaired t test). Addition of the cytokine IL-4 alone did not increased the numbers of spots compared to the medium control but the combination of IL-4 and A β 42 peptide lead to increased numbers compared to medium or A β 42 peptide alone. Cells which had been cultured with A β 42 peptide and anti-IL4 ab had highly significant reduced numbers of IL-10 producing cells ($p = 0.0023$, unpaired t test).

3.5. Co-stimulation lead to *in-vivo* and *in-vitro* proliferation and expansion of CD4+CD25+Foxp3+ cells in the DNA immunized mice

In-vivo cell proliferation was analyzed by intracellular staining for Ki67, a nuclear protein accessible only in dividing cells. Using this method we compared directly dividing CD4+Foxp3⁻ and CD4+Foxp3⁺ in the differently immunized mouse groups. Our gating strategy for the analyses of *in-vivo* divided Ki67⁺ cells is shown in **4A**. CD4⁺ cells were divided into Foxp3⁻ and Foxp3⁺ cells which were then further analyzed for staining with Ki67 as marker for recently divided cells. Percentages of Ki67⁺ CD4⁺ Foxp3⁺ and Ki67⁺CD4⁺Foxp3⁻ for the differently immunized mice (n=4 per group) were shown in **4B**. In naïve mice we found that 18.1% ± 0.58 of the CD4⁺Foxp3⁻ and 5.72 % ± 0.20 of the CD4⁺Foxp3⁺ cells were Ki67⁺ and thus proliferating. Percentages for the Ki67⁺CD4⁺Foxp3⁺ were variable between groups. Higher percentages of Ki67⁺CD4⁺Foxp3⁺ than Ki67⁺CD4⁺Foxp3⁻ T cells was found for the DNA immunized mice, 23.48% ± 0.91 in the 2× DNA/isotype control ab immunized mice, 44.73% ± 0.43 in the 2× DNA/TNFRSF abs immunized mice, and 42.23% ± 1.98 in the 2× DNA/TNFRSF abs which had received a neutralizing anti-IL4 ab injection. A significant reduction of Ki67⁺CD4⁺Foxp3⁺ from the other TNFRSF ab treated groups was found for the 2× Aβ42 peptide immunized mice with 28.83% ± 3.05 cells ($p = 0.286$ Mann Whitney). Instead, in these mice we found a strong increase of Ki67⁺CD4⁺Foxp3⁻ (Teff) cells (34% ± 2.4) in comparison to groups which had received DNA immunizations (20.28% ± 1.23), DNA immunizations plus TNFRSF ab (24.7% ± 2.02), and naïve mice which had received the TNFRSF ab injection only (28.88% ± 3.05, p values of 0.0267, 0.0147, and 0.0023 respectively student t test).

When we compared numbers and percentages of CD4+CD25+Foxp3⁺ and CD4+CD25+Foxp3⁻ T cells from splenocyte cultures after 72 h in medium and with peptide re-stimulation we observed that the Aβ42 peptide stimulation resulted in an increase of Tregs in DNA immunized mice and in an increase of Teffs in peptide immunized mice similar to the *in-vivo* findings described above (Fig. 4). In Figure 5 comparisons from 2× and 4× DNA and peptide immunized mice with and without TNFRSF ab co-stimulation are shown. In two times DNA immunized mice we found a significant increase of Tregs in the Aβ42 cultures compared to medium only ($p = 0.0286$ Mann-Whitney), the difference for Tregs in the peptide immunized mice between medium and Aβ42 peptide medium was not significant. Instead, in the peptide immunized mice the difference in Teffs in the Aβ42 cultures compared to medium only was significant ($p = 0.0286$ Mann-Whitney). Furthermore, the difference in Tregs between Aβ42 re-stimulated cultures from DNA or peptide immunized mice was significant. These findings were confirmed in cultures from mice which had received immunizations with TNFRSF ab co-stimulation. The total numbers were up due to the co-stimulation and the differences in Treg percentages between DNA and peptide immunized mice after peptide re-stimulation and Teffs in peptide immunized mice was again significant, showing that the TNFRSF co-stimulation has not changed the immune response signatures. In four times immunized mice which had received the TNFRSF ab co-stimulation the differences found in the percentages of Tregs from DNA immunized mice and peptide immunized mice were highly significant. After 72 h culture in Aβ42 containing medium DNA immunized mice had much higher percentages of Tregs compared to peptide

immunized mice ($p = 0.0065$, Mann Whitney) and again the increase in Teffs in the peptide immunized mice compared to DNA immunized mice was highly significant ($p = 0.0022$, Mann Whitney).

3.6. Comparison of transcription factors Foxp3, Helios, and Gata-3 in the differently immunized mice

It had been reported that expression of the transcription factor Helios is a marker of T cell activation (Akimova et al. 2011), and we used this marker here to analyze Treg activation. Our results show high numbers of CD4+Foxp3+Helios+ cells in splenocytes from DNA immunized mice which had received the TNFRSF ab co-stimulations (Fig 6A, middle graph). Based on the comparison of CD4+Foxp3+Helios+, CD4+Foxp3+Helios-, CD4+Foxp3-Helios+, as well as CD4+Foxp3-Helios- cell subsets from naïve mice and DNA A β 42 immunized mice, we observed that the Tregs in DNA immunized mice have derived from a CD4+Foxp3- cell population. The percentages of the CD4+Foxp3- cells were significantly lower in the DNA immunized mice ($p = 0.0013$) and a substantial shift was found in the DNA immunized mice after TNFRSF ab co-stimulation further reducing the CD4+Foxp3+ cell population ($p < 0.0001$) which lead to the observed expansion of CD4+Foxp3+Helios+ cells (6A and B). In regard to the Th2 polarization following DNA immunizations, we analyzed another transcription factor, Gata-3, together with Foxp3 in freshly isolated splenocytes from the immunized mice (6C and 6D). In these experiments we found that naïve mice as well as the 2 $\times$ DNA immunized mice had comparable numbers of CD4+Gata-3+Foxp3- cells ($p = 0.5860$) and CD4+Gata-3+Foxp3+ cells ($p = 0.2245$), while the numbers of CD4+Gata-3-Foxp3+ cells showed a non-significant difference which was consistent with the expansion of Foxp3+ cells after DNA immunization ($p = 0.0305$). After TNFRSF ab co-stimulation we found a significant decrease in the CD4+Gata-3+Foxp3- population ($p = 0.0012$) and marked increases in the CD4+Gata-3+Foxp3+ ($p = 0.0049$) as well as the CD4+Gata-3-Foxp3+ ($p = 0.0001$) population compared to the DNA immunized group of mice. No differences were found for the CD4+Gata-3-Foxp3-subsets (p values of 0.9262, 0.8109, and 0.7278 respectively). These data provide evidence that the Tregs found here might come from a CD4+Gata-3+Foxp3- cell population.

In the comparison of Helios and Gata-3 expressing CD4 T cells from DNA immunized mice and naïve mice we found increased numbers of Helios-Gata3+ cells in the DNA immunized mice which is consistent with the Th2 polarization (5E and 5F). The marked increase of CD4+Helios+Gata3- cells in mice which had received two DNA immunizations together with the TNFRSF ab co-stimulation *in-vivo* was derived from the Helios-Gata-3+ population and is again consistent with the development of Tregs from a CD4+Gata-3+Helios- T cell population.

4. Discussion

We are interested in the development of a non-inflammatory and effective immunotherapy approach as therapy to prevent Alzheimer disease. In our model of active full-length DNA A β 1-42 immunization into the skin of wild-type mice, a down regulation or unresponsiveness of antigen specific T cells at later immunization time points occurred, in

support of the presence of a regulatory immune response (Lambracht-Washington et al. 2011, 2012). We are currently testing the development of a regulatory T cell response in a triple transgenic AD mouse model (B6;129-Psen1 Tg(APP^{Swe},tauP301L)1Lfa/Mmjax) to supplement the data. A strong Th2 biased immune response due to the gene gun immunizations into skin (Weiss et al. 2002) persisted with the co-stimulatory effect of TNFRSF4/25 ab injections. The Th2 signature with high IgG1/IgG2a ratios remained constant. Two factors were important for our findings: One is the polarized Th2 response, and the second is the immunization site, as it had been shown that antigen exposure in the skin does lead to the priming of a regulatory immune response towards the respective antigen (Clark and Kupper, 2007, Dudda et al, 2008, Rosenblum et al. 2011, Gratz et al. 2014, Sanchez Rodriguez et al. 2014).

Regulatory T cells are in general only present in very low frequencies and therefore an expansion of these cells with the TNFRSF ab injections was initiated (Schreiber et al. 2012). The results from the use of the agonistic TNFRSF4/25 ab treatment showed two- to three-fold expansions of CD4+CD25+Foxp3+ T cells in combination of DNA A β 42 immunization compared to isotype ab treated naïve mice in the Balb/c mice shown as well as B6SJLF1 mice (data not shown). The differences in Foxp3+ cell numbers analyzed directly *ex-vivo* are representative for the nature of the DNA immunization elicited T cell response, as it has been shown that fully differentiated cells (Th1 or Th2) will not express Foxp3 upon activation and that only a small percentage of T cells from the neutral undifferentiated T cell pool will become Foxp3 positive (Davidson et al. 2007, Pillemer et al. 2009). The high numbers of CD4+CD25+Foxp3+ cells in all mouse groups which had received the TNFRSF ab co-stimulation indicates Treg expansion but not antigen specificity.

Important results in regard to the antigen specificity of the Tregs came from two sets of data: The analysis of Ki67+ positive CD4+CD25+Foxp3+ cells in the different mouse groups and the comparison of numbers and percentages of Tregs and Teffs in the different mouse groups following *in-vitro* culture with medium or A β 42 peptide re-stimulation. Ki67 positivity is an indicator that the respective T cell had interacted with the antigen within a five-day time window (Booth et al. 2010, Yonkers et al. 2011, Sanchez Rodriguez et al, 2014). This time frame follows exactly the experimental design as we immunized the mice and injected the TNFRSF antibodies five days prior to the Ki67 staining of freshly isolated splenocytes. While we found high numbers of Ki67+ Tregs in all the DNA immunized mouse groups, we found the opposite for A β 42 peptide/TNFRSF ab immunized mice with significantly higher numbers of Ki67+ Teffs. Thus, both T cell subsets were antigen specific and had seen the antigen five days prior to the staining: the A β 42 specific Tregs in DNA immunized mice and the A β 42 specific Teffs in the peptide immunized mice. From the comparison of increasing cell numbers in A β 42 peptide re-stimulated cultures with the medium control cultures, we were also able to show expansion of Tregs in the DNA immunized mouse groups while we found expansion of Teffs in the peptide immunized mouse groups.

Analyses of several transcription factors were done to distinguish whether the Tregs come from a natural (thymic derived) Treg pool or are an induced Treg subpopulation originating from CD4+CD25+Foxp3- cells. From the results presented it was concluded that Tregs

found after DNA A β 42 immunization might be induced Tregs. While the differences for levels of the transcription factors, Foxp3, Helios, and Gata-3, between naïve T cells and T cells from DNA immunized mice were not significantly different, the TNFRSF co-stimulation changed the balance and made the assumed pathway more evident. In all three comparisons, the cell populations which were Foxp3⁻ (or Helios-respectively) decreased in size, indicating that the Foxp3⁺ cells were induced from this pool. In the comparisons containing also Gata3, the decreased cell populations were CD4⁺Gata3⁺Foxp3⁻ or CD4⁺Gata3⁺Helios⁻ indicating a Th2 T cell phenotype. Since the most expanded cell populations were CD4⁺Gata3⁻Foxp3⁺ or CD4⁺Gata3⁻Helios⁺, we conclude that the Tregs derived from CD4⁺Foxp3⁻ T cells via a CD4⁺Gata3⁺Foxp3⁻ intermediate state in agreement with a close balance for transcription factors and cytokines between Th2 and Treg T cell subsets (Chapoval et al. 2010).

In mice which had received the TNFRSF ab treatment in combination with an A β 1–42 peptide immunization, we did not find IL-10 secreting cells, and in mice which had received the ab only without accompanying immunization, we found neither IL-4 nor IL-10 secretion although the antibody injections had led to expansion of CD4⁺CD25⁺Foxp3⁺ cells. In mice which had received A β 42 peptide immunization in combination with the TNFRSF ab co-stimulation, we found IL-4 but not IL-10. The high numbers of IL-4 producing cells found in the DNA A β 42 immunized mice which received the onetime TNFRSF ab injection was a new finding, since we had found only very low cytokine secretions in DNA immunized mice in earlier studies (Lambracht-Washington et al. 2009 and 2011). In humans, IL-4 and IL-13 had been described to play an important role for the generation of CD4⁺CD25⁺Foxp3⁺ T cells from CD4⁺CD25⁻ T cells in an Ag-dependent manner (Ohshima et al. 1998, Skapenko et al. 2005, Chapoval et al. 2010) and we postulate from our findings that the polarized Th2 immune response facilitates the development of an antigen specific regulatory immune response.

In the combined treatment with TNFRSF antibodies and DNA A β 1–42 immunizations we had also been able to detect low frequencies of IL-10 secreting cells with significant differences compared to the isotype ab treated control naïve mice and A β 1–42 peptide immunized/TNFRSF ab immunized mice. IL-10 is produced by many cell types: Tregs, Th2, CD8, dendritic cells and B cells as well as a newly described Th subset, which is classified as Th9 cells and which is also found in skin (Bouaziz et al. 2008, Fujio et al. 2010, Schlapbach et al. 2014). Because the numbers of IL-10 secreting cells correlates to the increased numbers of CD4⁺CD25⁺Foxp3⁺ cells in mice which received DNA immunizations and TNFRSF ab co-stimulation, it is highly likely that the increased IL-10 production in our model was due to the increased numbers of Tregs.

5. Conclusions

From our data, we propose that after the activation and expansion of A β 42 specific IL-4 producing Th2 T cells due to the gene gun immunization, an expansion of Tregs follows. These findings are due to features characteristic for the skin as an immunization route supporting the development of regulatory memory in peripheral tissue (Rosenblum et al. 2011). We described previously the down regulation of antigen specific T cells with

continued DNA A β 42 immunizations and we have shown the absence of T cell proliferation in response to A β 42 re-stimulation in culture as well as the absence of cytokine production (Lambracht-Washington et al. 2011). We show here that gene gun immunization into the skin induces a highly polarized Th2 immune response resulting subsequently in the induction of a regulatory immune response leading finally to downregulation of Teff cells. Further, co-stimulation of T cells with the TNFRSF4/25 injection greatly increased the Treg population and provided additional clues for a link between Th2 and Treg immune responses. These findings provide the rationale for the uniqueness of active full-length DNA A β 42 vaccination compared to active A β 1–42 peptide vaccination to induce a non-inflammatory immune response and for its impact on future work on the development of an immunotherapy to treat or prevent AD.

Acknowledgments

This study was funded by grants from NIH/NIA to the Alzheimer's Disease Center (P30AG12300-19), the Rudman Partnership; Darryl Royal Foundation and McCune Foundation to RNR and an RO3 grant to DLW (R03AG042697-02).

References

- Akimova T, Beier UH, Wang L, Levine MH, Hancock WW. Helios expression is a marker of T cell activation and proliferation. *PLoS One*. 2011; 6(8):e24226. [PubMed: 21918685]
- Booth NJ, McQuaid AJ, Sobande T, Kissane S, Agius E, Jackson SE, Salmon M, Falciani F, Yong K, Rustin MH, Akbar AN, Vukmanovic-Stejić M. Different proliferative potential and migratory characteristics of human CD4⁺ regulatory T cells that express either CD45RA or CD45RO. *J Immunol*. 2010; 184:4317–4326. [PubMed: 20231690]
- Bouaziz JD, Yanaba K, Tedder TF. Regulatory B cells as inhibitors of immune responses and inflammation. *Immunol Rev*. 2008; 224:201–214. [PubMed: 18759928]
- Carrillo MC, Brashear HR, Logovinsky V, Ryan JM, Feldman HH, Siemers ER, Abushakra S, Hartley DM, Petersen RC, Khachaturian AS, Sperling RA. Can we prevent Alzheimer's disease? Secondary "prevention" trials in Alzheimer's disease. *Alzheimers Dement*. 2013; 9:123–131. [PubMed: 23411394]
- Chapoval S, Dasgupta P, Dorsey NJ, Keegan AD. Regulation of the T helper cell type 2 (Th2)/T regulatory cell (Treg) balance by IL-4 and STAT6. *J Leukoc Biol*. 2010; 87:1011–1018. [PubMed: 20335310]
- Clark RA, Kupper TS. IL-15 and dermal fibroblasts induce proliferation of natural regulatory T cells isolated from human skin. *Blood*. 2007; 109:194–202. [PubMed: 16968902]
- Cribbs DH, Ghochikyan A, Vasilevko V, Tran M, Petrushina I, Sadzikava N, Babikyan D, Kesslak P, Kieber-Emmons T, Cotman CW, Agadjanyan MG. Adjuvant-dependent modulation of Th1 and Th2 responses to immunization with beta-amyloid. *Int Immunol*. 2003; 15:505–514. [PubMed: 12663680]
- Davidson TS, DiPaolo RJ, Andersson J, Shevach EM. Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3⁺ T regulatory cells. *J Immunol*. 2007; 178:4022–4026. [PubMed: 17371955]
- Dudda JC, Perdue N, Bachtanian E, Campbell DJ. Foxp3⁺ regulatory T cells maintain immune homeostasis in the skin. *J Exp Med*. 2008; 205:1559–1565. [PubMed: 18573908]
- Fox NC, Black RS, Gilman S, Rossor MN, Griffith SG, Jenkins L, Koller M. Effects of Abeta immunization (AN1792) on MRI measures of cerebral volume in Alzheimer disease. *Neurol*. 2005; 64:1563–1572.
- Fujio K, Okamura T, Yamamoto K. The Family of IL-10-secreting CD4⁺ T cells. *Adv Immunol*. 2010; 105:99–130. [PubMed: 20510731]

- Gilman S, Koller M, Black RS, Jenkins L, Griffith SG, Fox NC, Eisner L, Kirby L, Rovira MB, Forette F, Orgogozo JM. Clinical effects of Abeta immunization (AN1792) in patients with AD in an interrupted trial. *Neurol.* 2005; 64:1553–1562.
- Gratz IK, Rosenblum MD, Maurano MM, Paw JS, Truong HA, Marshak-Rothstein A, Abbas AK. Cutting edge: Self-antigen controls the balance between effector and regulatory T cells in peripheral tissues. *J Immunol.* 2014; 192:1351–1355. [PubMed: 24442443]
- Khan SQ, Tsai MS, Schreiber TH, Wolf D, Deyev VV, Podack ER. Cloning, Expression, and Functional Characterization of TL1A-Ig. *J Immunol.* 2013; 190:1540–1550. [PubMed: 23319737]
- Lambracht-Washington D, Qu BX, Fu M, Eagar TN, Stüve O, Rosenberg RN. DNA beta-amyloid (1–42) trimer immunization for Alzheimer disease in a wild-type mouse model. *JAMA.* 2009; 302:1796–1802. [PubMed: 19861672]
- Lambracht-Washington D, Qu BX, Fu M, Anderson LD jr, Stüve O, Eagar TN, Rosenberg RN. 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. *Cell Mol Neurobiol.* 2011; 31:867–874. [PubMed: 21625960]
- Lambracht-Washington D, Qu BX, Fu M, Stüve O, Eagar TN, Rosenberg RN. A regulatory immune response after DNA vaccination against Amyloid beta 42. 2012 (published abstract, AAIC 2012, Alzheimer's & Dementia Vol 8 (4), Supplement. Page P201/P1-272).
- Lambracht-Washington D, Rosenberg RN. Advances in the development of vaccines for Alzheimer's disease. *Discov Med.* 2013; 15:319–326. [PubMed: 23725605]
- Mangialasche F, Solomon A, Winblad B, Mecocci P, Kivipelto M. Alzheimer's disease: clinical trials and drug development. *Lancet Neurol.* 2010; 9:702–707. [PubMed: 20610346]
- Ohshima Y, Yang LP, Uchiyama T, Tanaka Y, Baum P, Sergerie M, Hermann P, Delespesse G. OX40 costimulation enhances interleukin-4 (IL-4) expression at priming and promotes the differentiation of naive human CD4(+) T cells into high IL-4-producing effectors. *Blood.* 1998; 92:3338–3345. [PubMed: 9787171]
- Orgogozo JM, Gilman S, Dartigues JF, Laurent B, Puel M, Kirby LC, Jouanny P, Dubois B, Eisner L, Flitman S, Michel BF, Boada M, Frank A, Hock C. Subacute meningoencephalitis in a subset of patients with AD after Abeta42 immunization. *Neurology.* 2003; 61:46–54. [PubMed: 12847155]
- Pillemer BB, Qi Z, Melgert B, Oriss TB, Ray P, Ray A. STAT6 activation confers upon T helper cells resistance to suppression by regulatory T cells. *J Immunol.* 2009; 83:155–163. [PubMed: 19535633]
- Qu B-X, Xiang Q, Li L, Johnston SA, Hynan LS, Rosenberg RN. Aβ42 gene vaccine prevents Aβ42 deposition in brain of double transgenic mice. *J Neurol Sci.* 2007; 260:204–213. [PubMed: 17574274]
- Qu BX, Lambracht-Washington D, Fu M, Eagar TN, Stüve O, Rosenberg RN. Analysis of three plasmid systems for use in DNA Abeta 42 immunization as therapy for Alzheimer's disease. *Vaccine.* 2010; 28:5280–5287. [PubMed: 20562015]
- Rosenblum MD, Gratz IK, Paw JS, Lee K, Marshak-Rothstein A, Abbas AK. Response to self antigen imprints regulatory memory in tissues. *Nature.* 2011; 480:538–542. [PubMed: 22121024]
- Sanchez Rodriguez R, Pauli ML, Neuhaus IM, Yu SS, Arron ST, Harris HW, Yang SH, Anthony BA, Sverdrup FM, Krow-Lucal E, et al. Memory regulatory T cells reside in human skin. *J Clin Invest.* 2014; 24:1027–1036. [PubMed: 24509084]
- Schlapbach C, Gehad A, Yang C, Watanabe R, Guenova E, Teague JE, Campbell L, Yawalkar N, Kupper TS, Clark RA. Human TH9 cells are skin-tropic and have autocrine and paracrine proinflammatory capacity. *Sci Transl Med.* 2014; 6(219):21.
- Schreiber TH, Wolf D, Tsai MS, Chirinos J, Deyev VV, Gonzalez L, Malek TR, Levy RB, Podack ER. Therapeutic Treg expansion in mice by TNFRSF25 prevents allergic lung inflammation. *J Clin Invest.* 2010; 120:3629–3640. [PubMed: 20890040]
- Schreiber TH, Wolf D, Boder M, Gonzalez L, Podack ER. T cell costimulation by TNFR superfamily (TNFRSF)4 and TNFRSF25 in the context of vaccination. *J Immunol.* 2012; 189:3311–3318. [PubMed: 22956587]

- Skapenko A, Kalden JR, Lipsky PE, Schulze-Koops H. The IL-4 receptor alpha-chain-binding cytokines, IL-4 and IL-13, induce forkhead box P3-expressing CD25+CD4+ regulatory T cells from CD25-CD4+ precursors. *J Immunol.* 2005; 175:6107–6116. [PubMed: 16237107]
- Weiss R, Scheiblhofer S, Freund J, Ferreira F, Livey I, Thalhamer J. Gene gun bombardment with gold particles displays a particular Th2-promoting signal that over-rules the Th1-inducing effect of immunostimulatory CpG motifs in DNA vaccines. *Vaccine.* 2002; 20:3148–3154. [PubMed: 12163266]
- Wolf D, Schreiber TH, Tryphonopoulos P, Li S, Tzakis AG, Ruiz P, Podack ER. Tregs expanded in vivo by TNFRSF25 agonists promote cardiac allograft survival. *Transplantation.* 2012; 94:569–674. [PubMed: 22902792]
- Yonkers NL, Sieg S, Rodriguez B, Anthony DD. Reduced naive CD4 T cell numbers and impaired induction of CD27 in response to T cell receptor stimulation reflect a state of immune activation in chronic hepatitis C virus infection. *J Infect Dis.* 2011; 203:635–645. [PubMed: 21220773]

Highlights

- Full-length DNA A β 42 immunization leads to Th2 immune response with efficient antibody production
- The Th2 biased immune response provides the basis for development of a regulatory immune response
- DNA A β 42 immunization leads to expansion of antigen specific Tregs *in-vivo* and *in-vitro*

Figure 1. CD4+CD25+Foxp3+ expansion *in-vivo* induced by TNFRSF25-4 antibody co-stimulation

In **A** representative Contour plots are shown from splenocytes of naïve mice, three times DNA Aβ42 immunized mice, and mice which received three DNA Aβ42 immunizations and a one-time injection of TNFRSF abs. Events were gated on CD4+ cells. CD25 staining of these cells is shown on the x axis, Foxp3 staining on the y axis. Percentages of triple positive cells (CD4+CD25+Foxp3+) were taken from the upper right quadrant UR. In **B** percentages of CD4+CD25+Foxp3+ cells from all the mice in the three mouse groups is shown. Shown are mean and SEM. Contour plots in **C** show CD4+CD25+Foxp3+ staining in freshly isolated splenocytes from mice which had received two DNA immunizations, two DNA immunizations with TNFRSF ab co-stimulation, two DNA immunizations with TNFRSF ab co-stimulation and neutralizing anti-IL4 ab injections, two peptide immunizations with TNFRSF ab injections, or naïve mice with TNFRSF ab injections only. These cells were cultured for 72 h in Aβ42 peptide containing medium (shown in **D**) or re-stimulated with a

soluble CD3 ab in the medium (shown in **E**). Data shown are representative of four performed experiments.

Figure 2. Comparison of A β 42 antibody levels and isotypes in plasma (dilution 1:200) from the differently immunized Balb/c mice. (A) Plasma ab concentrations: each circle represents the ab concentrations from one mouse as determined by ELISA in triplicate wells. Letters indicate the following groups: A - 2 $\times$ peptide immunized mice (light grey circles), B - 2 $\times$ peptide immunized mice with TNFRSF ab co-stimulation (light grey circles), C - 2 $\times$ DNA immunized mice (dark grey circles), D - 2 $\times$ DNA immunized mice with TNFRSF co-stimulation (dark grey circles), E - 2 $\times$ DNA immunized mice with TNFRSF co-stimulation and injection of the anti-IL4 ab (dark grey circles). The numbers in

brackets indicate the mouse numbers per group. Shown are mean values and SEM; p values (non-parametric Mann-Whitney test) are indicated by asterisks (* < 0.05 , ** < 0.01).

(B) IgG1/IgG2a isotype profile for each of the mouse groups shown in 1A. Shown are mean values and SEM.

Figure 3. Comparison of IL-4 and IL-10 cytokine secretion in the differently immunized mice
A, IFN γ and IL-4 ELISPOTs from naive mice which had received control ab injections (isotype ab, or TNFRSF ab), and mice which had received two DNA A β 42 immunizations or two A β 42 peptide immunization in combination with a one time TNFRSF ab injection were shown in medium only cultures (white circles) or after A β 6–30 peptide re-stimulation (grey circles). **B**, IL-4 ELISPOTS were shown for splenocyte cultures from naïve mice, one times peptide/TNFRSF ab immunized mice, one time DNA immunized mice, one time, two times, and three times DNA/TNFRSF ab immunized mice in response to medium (white circles) or A β 6–30 peptide (grey circles) re-stimulation. **C**, IL-10 secretion was analyzed from the differently immunized mouse groups in response to IL-2 (vertical divided circles) and IL-4 addition (horizontal divides circles) into the A β 42 peptide containing cultures after 120 h in-vitro stimulation. **D** shows the comparison of IL-10 ELISPOTS in three groups of mice (2 $\times$ DNA/TNFRSF ab, 2 $\times$ DNA/isotype ab, and naïve mice) after an 48 h incubation (upper graph) and a 96 h incubation (lower graph) in medium only cultures, or cultures containing medium plus IL-4 (black triangle) or anti-IL-4 ab (vertical divided square). **E** shows the comparison of IL-4 (upper graph) and IL-10 ELISPOTS (lower graph) in 2 $\times$ DNA immunized mice, 2 $\times$ DNA/TNFRSF ab immunized mice, 2 $\times$ DNA/TNFRSF ab/anti-IL4 ab immunized mice, 2 $\times$ peptide/TNFRSF ab immunized mice, and naïve mice which received one TNFRSF ab injection after medium (white circles) and A β 42 peptide (black

circles) re-stimulation in-vitro. In **F**, numbers of IL-10 secreting cells were compared from 6× DNA/3× TNFRSF ab immunized mice to in parallel 6× peptide/3× TNFRSF ab immunized mice. ELISPOTS counts from triplicate cultures in medium (white circles), Aβ42 containing medium (black circles), IL-4 containing medium (black triangles), medium with Aβ42 and IL-4 (vertical divided circles) and medium containing Aβ42 and anti-IL4 (horizontal divided circles) were shown. Data shown are representative for three independent experiments. In all graphs values shown are mean and SEM.

Figure 4. KI67 staining indicates expansion of A β 42 specific CD4 T cells *in-vivo*
 CD4+Foxp3+ and CD4+ Foxp3- cells from mice which had received two DNA immunization, two DNA immunizations with TNFRSF ab co-stimulation, two DNA immunizations with TNFRSF ab co-stimulation and neutralizing anti-IL4 ab injections, two peptide immunizations with TNFRSF ab injections, or TNFRSF ab injections only were subsequently stained with a Ki67 ab. The gating strategy is shown in A and the analyses of CD4+Foxp3- (black circles) and CD4+Foxp3+ (grey circles) is shown in B. Shown are mean values and SEM; significant p values (non-parametric Mann-Whitney test) are indicated by asterisks (* < 0.05).

Figure 5. Analysis of CD4+CD25+Foxp3+ and CD4+CD25+Foxp3- following 72 h in cell culture
 After 72 h culture in medium (white background) or medium containing Aβ42 peptide (grey boxed background) cells from two times (**5A**) and four times (**5B**) immunized mouse groups were stained with CD4 and CD25 abs and expansion or contractions of Tregs (white bars, grey circles) and Teffs (dark grey bars, black circles) were compared respectively. Percent marker positive cells (gated on CD4+ cells) is indicated on the y axis, mouse groups compared are given on the x axis.

Results shown are representative from four similar performed experiments. Shown are mean values and SEM; p values (non-parametric Mann-Whitney test) are indicated by asterisks (* < 0.05, ** < 0.01).

Figure 6. Analyses of intracellular staining for Foxp3, Helios, and Gata-3 in 2x DNA immunized mice with and without TNFRSF co-stimulation and naïve mice

In **A**, contour plots show the intracellular staining of CD4+ cells for Helios on the y axis and for Foxp3 on the x axis. In **B**, a grouped analysis shows the percentages of CD4+Foxp3+Helios+, CD4+Foxp3-Helios+, CD4+Foxp3+Helios-, and CD4+Foxp3-Helios-, for the three groups of mice in a total of 100% gated CD4+ cells. In **C**, contour plots show the staining of CD4+ cells for intracellular Gata-3 on the y axis and for Foxp3 on the x axis. In **D**, a grouped analysis shows the percentages of CD4+Foxp3+Gata-3+, CD4+Foxp3- Gata-3+, CD4+Foxp3+ Gata-3-, and CD4+Foxp3- Gata-3- in the CD4 gated events. In **E**, contour plots show the intracellular staining for Gata-3 on the y axis and for Helios on the x axis, and in **F** the grouped analyses from data from four separate mouse samples. Data are representative for three repeated experiments.