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Combinatorial Vaccine against Complement Factor C5a and Amyloid Beta: A New Therapeutic Approach in Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) is the most common neurodegenerative disease characterized by neuronal loss due to amyloid beta (Aβ) aggregations, neurofibrillary tangles, and prominent neuroinflammation. Inflammatory processes in AD primarily occur in response to misfolded and aggregated proteins, or mislocalized nucleic acids and reactive microglia. Prolonged chronic neuroinflammation is thought to reinforce neuronal cell dysfunction and cell death. In our previous study we demonstrated that the interference with the pro-inflammatory mediator C5a by AFF1 vaccine at an early stage of disease is able to reduce microglia activation and amyloid plaque burden which is accompanied by ameliorated memory deficiency in Tg2576 mice, a mouse model of AD. In a follow-up study we tested the effect of a combinatorial vaccine targeting neuroinflammation by C5a and Aβ aggregates, two detrimental processes in AD. The amyloid plaque burden in the brain of Tg2576 mice was significantly reduced upon vaccination by the monovalent anti-C5a (AFF1) as well as anti-Aβ (AD02) vaccine, however, the combinatorial AFF1/AD02 vaccine showed a clear additive beneficial effect. Moreover, contextual memory in Tg2576 mice was significantly improved by the combinatorial AFF1/AD02 vaccine when compared to monovalent or control vaccines. Thus, targeting two neuropathological processes such as neuroinflammation and Aβ aggregation may represent a new and promising approach for the treatment of AD.

Keywords: AFFITOPE® vaccine; Tg2576 mice; Neuroinflammation; Amyloid aggregation; Microglia activation

Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disorder characterized by cognitive dysfunction and progressive memory loss. The complex disease pathology includes three prominent pathological hallmarks: extracellular plaques composed of Aβ peptide aggregates [1], intracellular neurofibrillary tangles of tau protein [2], and chronic neuroinflammation [3].

The most commonly accepted mechanism underlying AD pathology until recently has been the amyloid hypothesis [4,5]. It postulates that overproduction of Aβ peptides and their subsequent pathological aggregates play a central role in AD pathogenesis. Hence for decades, the development of therapeutic approaches was focused on modifying the Aβ levels by preventing its aggregation, hindering its production, and/or enhancing its degrading activity [6]. Several therapeutic approaches that targeted Aβ aggregation were tested in clinical trials, however by now, none of them showed consistent improvements in AD patients [6,7]. Recently monoclonal antibodies (mAb) against Aβ have advanced into late clinical trials as reviewed in Liu et al. [8], but the most advanced mAbs Bapineuzumab [9] and Solanezumab [10] failed to meet their clinical endpoints in phase III clinical studies. However, efforts still go on and only recently a newly developed mAb showed very promising results. Aducanumab that selectively targets aggregated Aβ was found to reduce cerebral Aβ in a dose- and time-dependent manner accompanied by a slowing of clinical decline in a phase Ib clinical trial [11]. Further large scale studies are necessary to demonstrate if Aducanumab is able to halt or reverse cognitive decline in AD patients.

In contrast to the passive immunotherapy approaches the development of active immunization targeting Aβ is limited. Only few vaccines have advanced to phase II clinical development [12].

Using our proprietary AFFITOME®-technology we developed the anti-Aβ peptide vaccine AD02 [13]. The administration of AD02 to transgenic mouse model of AD was able to reduce the cerebral amyloid burden, and the associated neuropathological alterations, and improved the cognitive functions [13].

Since AD is considered to be a multifactorial disease, not only Aβ aggregation, but many over-lapping processes contribute to neuronal degeneration and cognitive loss, and especially the role of neuroinflammation was strengthened in the pathogenesis and exacerbation in AD in the last years [14-16]. Aβ aggregates cause direct cytotoxicity and self-propagation, but also a constant, self-sustaining inflammatory environment by prolonged activation of astroglial [17,18] and microglial cells [19]. Activation of microglia by Aβ and complement is supposed to promote the excessive release of proinflammatory cytokines [20], chemokines [21,22], and further complement components [23,24], as well as the release of reactive oxygen and nitrogen species [25,26], altogether leading to dysfunction and loss of synapse signaling [27]. Pro-inflammatory mediators provoke a number of stress conditions which, in turn, can enhance

APP production and processing to amyloid peptides (Aβ 1–42) [28-31]. Thus, this uncontrolled and prolonged activation of the immune system in the brain goes beyond physiological control and eventually might result in a detrimental rather than beneficial effect on the CNS. Amyloid deposits itself trigger the upregulation of the complement system, as well as activated astrocytes and microglia may exacerbate the secretion of complement factors by secreting cytokines [32-34]. In different mouse models for AD the proinflammatory complement factor C5a and its receptor have been found to be up-regulated in microglia in the immediate surroundings of cerebral amyloid plaques [35]. Fonseca *et al.* demonstrated that the treatment with an antagonist of C5aR (PMX205) resulted in a reduction of pathological markers and improved memory skills in two different mouse models of AD [36]. In our previous study we showed that immunization against the proinflammatory complement factor C5a by AFF1 vaccine is able to interfere with microglia activation and thus neuropathology in Tg2576 mice, a model of AD [37]. AFF1 treated mice did not only show a reduced number of activated microglia in the hippocampal region, but also improved contextual learning and memory skills [37]. Moreover, cerebral amyloid plaque load was diminished when vaccinated at an early stage of the disease [37].

This study clearly demonstrated that active immunotherapy against C5a can ameliorate neuroinflammation and contextual learning in AD-like disease, indicating that neuroinflammation may be directly involved in memory decline.

Altogether, we assume that targeting both pathways neuroinflammation and Aβ aggregation at the same time is a reasonable approach and could lead to optimal efficacy in the treatment of AD.

Materials and Methods

Animals

Tg2576 mice on a 129S6 genetic background (Taconic Farms, USA; 129S6/SvEvTac) which carry a transgene coding for the 695-amino acid isoform of the human AD amyloid precursor protein (APP) and the Swedish mutation (KM670/671NL) were used as a model of AD [38]. It is reported that memory deficits in this model start at an age of 6 months and at 9-12 months amyloid plaques in the cortex and hippocampus become apparent. Experiments were performed in accordance with the Austrian Animal Experiments Act (TVG2012) under the approval numbers: LF1-TVG-22/0102011. Blood was taken in regular intervals, plasma was prepared and used for ELISA assays. At the study end mice were sacrificed and the brains were collected and fixed in 4% paraformaldehyde (PFA, Sigma Aldrich, USA) for immunohistochemical analyses.

Vaccine preparation and immunization scheme

The proprietary AFFITOME® technology [39] was used to develop short immunogenic peptides (AFFITOPE®s), which mimic either the C-terminal epitope of murine C5a [37] or the N-terminal epitope of human Aβ [13]. Vaccines were prepared as described previously [37].

Tg2576 mice were immunized with AFF1 (n=23) and AD02 (n=17) as a mono vaccine, the combinatorial vaccine AFF1/AD02 (n=11), and the control vaccine (n=14) lacking the antigenic peptide moiety. The study included different cohorts and immunizations started at different time points. AD02 vaccine was injected s.c. 6 times in monthly intervals starting at the age of 6 months whereas AFF1 followed a slightly different immunization scheme with 4 times biweekly and three times monthly intervals, in total 7 s.c. injections starting at the age of 8 months. Control mice (n=14) were treated with a vaccine lacking the antigenic peptide moiety following the immunization scheme of AD02.

ELISA

Induced antibody titers against the antigenic peptide moiety of AFF1, AD02, and AFF1/AD02 combinatorial vaccine were determined by ELISA as described previously [37].

Contextual memory test

Contextual learning and memory of AFF1, AD02, and the combinatorial AFF1/AD02 vaccine treated Tg2576 mice were determined by a contextual fear conditioning test and compared to control and wt Tg2576 mice as described previously [37].

Immunohistochemistry and image analysis

Immunohistochemistry and image analyses are described previously in Landlinger *et al.* [37].

Statistical analysis

All values were evaluated for homoscedasticity and normality assumption using both Kruskal-Wallis and Shapiro-Wilk normality tests. To determine statistical significance of more than two groups, values were compared using one-way ANOVA with the Dunn's test (non-parametric test). The p values ≤ 0.05 were considered significant and are expressed as $^{*}p<0.05$ and $^{**}p<0.001$ and $^{***}p<0.0001$.

Results

Combinatorial vaccine against C5a and Aβ by AFF1/AD02 vaccine in a mouse model of AD

Short immunogenic peptides, which mimic either the C-terminal epitope of murine C5a [37] or the N-terminal epitope of Aβ [40] were selected and formulated. These vaccines where then used for an immunization therapy in human APP transgenic Tg2576 mice, a mouse model of AD-like disease. Tg2576 mice received either AFF1 vaccine (n=23), AD02 (n=17), or both vaccines (n=11). All vaccines either applied as mono vaccines or as a combinatorial vaccine were able to induce high and specific titers against the respective antigenic peptide moieties (Figure 1).

AFF1/AD02 vaccine ameliorates memory deficiencies

Memory deficits in untreated Tg2576 mice start at the age of 6 months. In order to investigate the impact of AFF1/AD02 combinatorial vaccine on the progression of AD-like cognitive decline we performed a contextual fear conditioning test. This method assesses the time freezing in a 2 minute interval as an indicator for contextual learning and memory to recall the shocks they received on the previous day. Control treated mice showed an almost complete loss of contextual learning and memory with only 10% time freezing (Figure 2). Compared to control, AFF1 and AD02 vaccinated mice showed improved memory skills with 32% and 22% time freezing, respectively, although the latter group does not reach statistical significance (Figure 2). However, the combinatorial vaccine AFF1/AD02 reached 46% time

freezing indicating a highly statistically significant increase in memory skills (p<0.0001) (Figure 2).

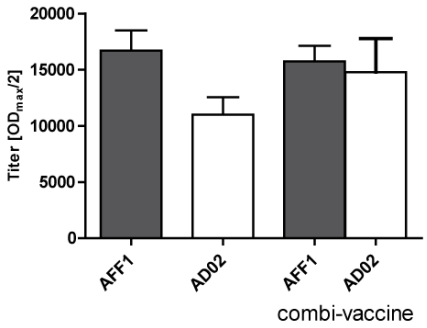

Figure 1: AFF1, AD02, and AFF1/AD02 combinatorial vaccines induce target specific antibody response in Tg2576 mice. Tg2576 mice were either immunized with AFF1 (n=23), AD02 (n=17) or AFF1/AD02 (n=11) combinatorial vaccine. Plasma samples collected after the last immunization (week 28) were analyzed by ELISA and the mean IgG antibody titers (OD$_{max}$/2) against the injected peptide AFF1 and AD02 are presented. All data points represent the group means ± SEM of n animals.

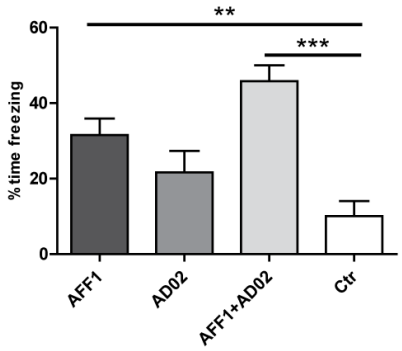

Figure 2: Contextual memory is significantly improved following AFF1 and AFF1/AD02 combinatorial vaccination. At the age of 15 months a contextual fear conditioning test was performed with AFF1 (n=23), AD02 (n=17), AFF1/AD02 (n=11) and control (n=14) immunized Tg2576 mice. Mice were exposed to electric foot shocks and the % time freezing in a 2 minute interval was assessed which reflects the memory skills to recall these shocks. Bars represent the group means ± SEM of n animals. The p-value was determined using 1way ANOVA test followed by a Dunn's Multiple Comparison Test (non-parametric test). The p-values are expressed as $^{*}p$<0.05 and $^{***}p$<0.0001.

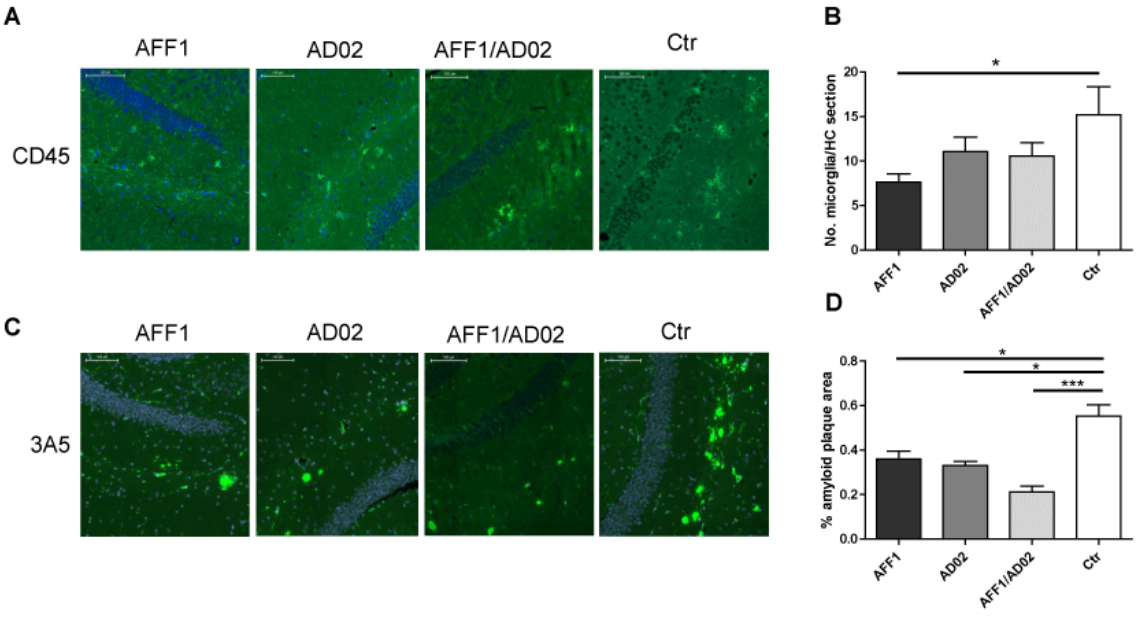

Figure 3: Immuohistological evaluation of activated microglia and amyloid plaque load in the brain of AFF1, AD02 as well as AFF1/AD02 vaccinated Tg2576 mice. **(A)** Brain sections of 15 months old Tg2576 mice immunized with either AFF1 (n=21), AD02 (n=17), AFF1/AD02 (n=11) or control (n=14) vaccines were stained for CD45-positive microglia cells. **(B)** The average number of CD45^{high} cells in the hippocampal region of all sections. **(C)** Amyloid plaques were stained by the 3A5 mAB (in house) and representative images are presented. **(D)** The percentage of amyloid area of the total brain sections all containing the hippocampal region was calculated by the eDefiniens Software. Bars represent the group means ± SEM of n animals. The p-value was determined using 1way ANOVA test followed by a Dunn's Multiple Comparison Test (non-parametric test) and expressed as $^{*}p$<0.05 and $^{***}p$<0.0001.

AFF1/AD02 vaccine ameliorates microgliosis and strongly reduces the amyloid plaque burden

We showed in our previous studies that anti-C5a vaccination (AFF1) was able to reduce microgliosis in the hippocampus accompanied by less cerebral Aβ pathology in the brain of 15 months old Tg2576 mice when compared to control [37]. It was also reported that Tg2576 mice immunized with anti-Aβ vaccines (AD01 and AD02) had significantly less amyloid deposits compared to control [13].

In this study we investigated the impact of a combinatorial vaccine of AFF1 and AD02 on microgliosis as well as amyloid plaque load in the brain. As expected, immuno-histochemical analysis of $CD45^{high}$ cells in the hippocampal brain region of AFF1/AD02 combinatorial vaccine treated Tg2576 mice did not result in reduced microgliosis beyond the level seen in AFF1 monovalent treatment (Figures 3A and 3B). Compared to control, anti-C5a (AFF1) vaccine, but not anti-Aβ (AD02) alone or AFF1/AD02 combinatorial vaccine, showed a statistically significantly reduced number of CD45-positive microglia in the hippocampus (Figure 3B).

For the detection of Aβ depositions in the brain of 15 months old Tg2576 mice the in-house generated Aβ-specific mAB 3A5 [40] was used. Both, AFF1 and AD02 vaccinated mice, revealed a statistically significant reduction of cerebral amyloid plaque area of the total coronal brain section of 0.36% ($p<0.05$) and 0.33% ($p<0.05$), compared to 0.55% in control immunized mice (Figures 3C and 3D). However, when we potentially interfere with amyloid plaque formation directly by targeting Aβ aggregates using AD02 vaccine and at the same time indirectly by the interference with neuroinflammation (AFF1 vaccine) cerebral Aβ pathology was even strongly reduced (0.2% *vs.* 0.55% amyloid plaque area, $p>0.0001$) indicating a clear additive beneficial effect of AFF1/AD02 vaccine (Figure 3D).

Discussion and Conclusion

In the present study we show a new therapeutic approach for the treatment of AD. The combinatorial vaccine AFF1/AD02 elicits antibodies that effectively bind to C5a and Aβ and thus significantly reduce cerebral amyloid plaque load and memory decline in a mouse model of AD.

Our intention was to develop a combinatorial vaccine for the treatment of AD where we target two neuropathological triggers, the pro-inflammatory complement activation product C5a as well as the aggregated Aβ protein. Thus, on the one hand we may interfere with enhanced microglia activation and sustained neuroinflammation, a trigger for enhanced Aβ production and aggregation, and on the other hand with Aβ protein depositions, itself a trigger mechanism for continuous neuroinflammation. Since AD is considered to be a multifactorial disease where widespread neuroinflammation, oxidative stress, mitochondrial damage, glutamate excitotoxicity, neurofibrillary tangle formation, and Aβ deposition are all processes that contribute to neuronal pathology and cognitive loss [41], a combinatorial therapeutic approach seems to be reasonable.

An early process in AD certainly is the generation of amyloid peptides (Aβ 1-42) by the increased proteolytic cleavage of amyloid precursor proteins (APP) due to currently unknown triggers. These proteins start to accumulate and aggregate especially in the hippocampal and cortical region of the brain. Consequently, microglia become activated either by a direct interaction with misfolded Aβ molecules via pattern recognition receptors (PRR), or by complement activation in the response to Aβ depositions which leads to the formation of the pro-inflammatory molecule C5a [14]. Enhanced microglia activation then leads to excessive release of pro-inflammatory cytokines [20], chemokines [21,22], and further complement components [23,24], as well as the release of reactive oxygen and nitrogen species [25,26]. These pro-inflammatory mediators cause a number of stress conditions which, in turn, are supposed to reinforce Aβ production and aggregation [28,30,31,42,43]. Thus, chronic and self-sustaining inflammatory interactions between the complement system, activated microglia, stressed neurons, and Aβ plaques occur, which ultimately lead to dysfunction and loss of synapse signaling and thus cognitive decline in AD patients [27].

Several therapeutics that target Aβ aggregation either by the means of mAbs or vaccines have been tested in clinical trials. However, even when effective in decreasing Aβ levels they did either not significantly improve cognitive outcomes [10,44] or the side effects have been too deleterious compared to little or no cognitive benefit [45]. Recently the mAb Aducanumab which is directed against the aggregated form of Aβ showed very promising results. Cerebral Aβ was reduced in a dose- and time-dependent manner accompanied by a slowing of clinical decline in a phase Ib clinical trial [11], still, further large scale studies of aducanumab in AD patients have to elucidate its beneficial potential.

One of our strategies is to design an AFFITOPE®-based peptide vaccine that selectively targets the aggregated form of Aβ [13,46] in order to develop a therapeutic tool for the treatment of AD.

Another approach for the treatment of AD is to interfere with neuroinflammation. In the last decades several clinical trials were carried out to demonstrate the neuroprotective potential of nonsteroidal anti-inflammatory drugs, but many of them have shown unsatisfactory results [47-49]. Neuroinflammation outcome, driven by glial activation, depends on the context and the stage of the pathology in AD. Therefore, our strategy was to interfere with the terminal complement component C5a which seems to be an ideal target in order to prevent the detrimental anaphylatoxic effects of C5a and at the same time preserve protective complement activation events more upstream which are important for the clearance of aggregated proteins and cell debris. In our previous study we showed that anti-C5a (AFF1) vaccine is able to induce a specific immune reaction against the pro-inflammatory complement component C5a, thus preventing enhanced microglia activation in the hippocampus and reducing memory decline in a mouse model of AD [37]. Cerebral amyloid plaque load was only reduced in Tg2576 mice vaccinated with AFF1 at an early and not at a late stage of Alzheimer-like disease [37] indicating that initial Aβ depositions can be alleviated significantly by AFF1 vaccine whereas already existing plaques cannot.

In our combinatorial vaccine approach Tg2576 mice where immunized with AFF1 and anti-Aβ (AD02) vaccine starting both at an early stage of AD-like disease at 8 and 6 months, respectively. At the age of 15 months a clear additive beneficial effect was seen for AFF1/AD02 treated mice in terms of reduced amyloid plaque load in the brain as well as memory retention. By now AFF1/AD02 vaccine was only tested in a first study using a relatively small number of experimental mice. To confirm these data further studies will be necessary. Moreover, also the therapeutic effect of the combinatorial vaccine in AD-like disease at a later onset, e.g. a more pronounced stage of pathology, still needs to be elucidated. In our previous study we could show that AFF1 vaccine at the onset of 11 months, when cerebral plaque formation was already present, was able to reduce the number of activated microglia in the hippocampus and memory

decline in 15-months old Tg2576 mice compared to control [37]. Thus, one can assume that a combinatorial vaccine against C5a and Aβ at a later onset in disease may have a more pronounced beneficial effect compared to monovalent vaccines alone.

We could show excellent efficacy in preclinical studies, however, also safety issues need to be considered before entering clinical trials. All immunotherapeutic approaches which target self-proteins have to consider the activation CD4 T-cells and the formation of immune complexes and thus the issue of potential autoimmune response with life-threatening consequences [50]. Immunization with AFF1 and AD02 are designed to induce an effective B-cell response and concurrently circumvent target specific CD4 T-cell activation. Analysis by online available T cell epitope prediction algorithms did not predict any relevant T-cell epitope for the peptide moiety in AFF1 and AD02. Moreover, AFFITOPE®-based vaccines have already been tested in clinical trials and no severe adverse events occurred. Therefore, AFF1/AD02 vaccine is thought to be a safe and well-tolerated immunotherapy, which is able to elicit a specific immune response without any relevant cross-reactivity.

The combination between AFF1 and AD02 vaccine revealed a clear additive beneficial effect in terms of contextual learning and memory retention as well as in the reduction of amyloid plaque burden in the brain of Tg2576 mice compared to our previous study where we tested for AFF1 vaccine alone [37].

These promising results suggest a therapeutic potential of combinatorial vaccination not only in AD, but also for other neurological disorder like Parkinson's or Huntington disease where chronic inflammatory processes together with other triggers may play a pivotal role.

Competing Interests

Christine Landlinger, Eva Mihailovska, Gergana Galabova, and Günther Staffler are employees of AFFiRiS, the company that commercialize AFF1 AFFITOPE® vaccines described in the manuscript.

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