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Effects of single and combined immunotherapy approach targeting amyloid β protein and α-synuclein in a dementia with Lewy bodies–like model

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Abstract

Introduction—Immunotherapeutic approaches targeting amyloid β (Aβ) protein and tau in Alzheimer's disease and α-synuclein (α-syn) in Parkinson's disease are being developed for treating dementia with Lewy bodies. However, it is unknown if single or combined immunotherapies targeting Aβ and/or α-syn may be effective.

Methods—Amyloid precursor protein/α-syn tg micewere immunized with AFFITOPEs® (AFF) peptides specific to Aβ (AD02) or α-syn (PD-AFF1) and the combination.

Results—AD02 more effectively reduced Aβ and pTau burden; however, the combination exhibited some additive effects. Both AD02 and PD-AFF1 effectively reduced α-syn, ameliorated degeneration of pyramidal neurons, and reduced neuroinflammation. PD-AFF1 more effectively ameliorated cholinergic and dopaminergic fiber loss; the combined immunization displayed additive effects. AD02 more effectively improved buried pellet test behavior, whereas PD-AFF1 more effectively improved horizontal beam test; the combined immunization displayed additive effects.

Discussion—Specific active immunotherapy targeting Aβ and/or α-syn may be of potential interest for the treatment of dementia with Lewy bodies.

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Keywords

Immunization; Alzheimer's disease; Dementia with Lewy bodies; Immunotherapy; α-synuclein; AFFITOPE®

1. Introduction

In the last few years, it has become apparent that the pathology of Alzheimer's disease (AD) and Parkinson's disease (PD) overlap [1–7] and that Lewy body disease (LBD) is a part of this spectrum. LBD is a heterogeneous group of diseases that includes PD, Parkinson's disease with dementia, and dementia with Lewy bodies (DLB). In DLB, there is degeneration of the neocortex, limbic system, and striatonigral system [8–10] with accumulation of α-synuclein (α-syn) [11–13], Aβ [14,15], tau [16–18], and TDP-43 in the limbic system [19–21]. Moreover, Aβout 75% of patients with AD have Lewy body-like α-syn aggregates in the amygdala [22,23], and the pathology of AD and α-syn overlaps in cortical regions in DLB, familial AD, and Down syndrome (DS) cases [24,25].

Along these lines, we have previously shown that when crossing mutant amyloid precursor protein (APP) transgenic (tg) mice with mice overexpressing human α-syn under the PDGFβ promoter, the α-syn pathology and motor deficits worsen [14]. Likewise, in mThy1-APP crossed with mThy1-α-syn tg mice, we found that amyloid β (Aβ) cross-seeds α-syn resulting in oligomer formation and greater degeneration in the neocortex, hippocampus (CA3), cholinergic and nigrostriatal systems when compared with single tg mice [26,27]. These double tg mice displayed extensive Aβ, α-syn, and endogenous mouse Tau pathology and behavioral deficits mimicking some aspects of DLB [26,27]. Consistent with our findings, other studies have shown that introducing the mutant human α-syn transgene into the 3xTg-AD mouse model results in accelerated cognitive decline associated with a dramatic increase of human Aβ, tau, and α-syn pathologies [15]. However, as with other tg models of neurological disease, results must be interpreted with caution when translating to sporadic AD and DLB, since in these disorders increased levels Aβ, tau, α-syn might be the result of alterations in clearance rather than due to overexpression [28]. Development of alternative models that express physiological levels of human APP and α-syn are underway [29], and it will be of interest to evaluate the interactions of these proteins under more basal conditions. Nonetheless, it is worth noting that individuals with DS (with duplication of the APP gene) and some familial form of parkinsonism (with α-syn multiplication) where these proteins are overexpressed display in later stages of life with AD [30] and DLB [31,32]. Interestingly in older cases with DS, in addition to Aβ and tau there is also α-syn accumulation with the formation of Lewy-like bodies in the limbic system [24].

Together, these observations suggest that neurodegenerative disorders of the aging population share common pathways, leading to the progressive accumulation of several proteins including Aβ, tau, α-syn, and TDP43 (among others) and that these proteins might interact, leading to the formation of combined oligomers that potentiate toxicity to selective neuronal populations [9,11,33]. At the present time, most therapeutic experimental studies have focused at targeting one of these aggregates at a time; however, given that AD and DLB

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are poly-proteinopathies, it might require targeting simultaneously more than one protein or pathway [34]. Interestingly, previous immunotherapy studies have shown that reducing Aβ decreases tau accumulation [15,35] and vice versa [36]. Moreover, we have previously shown that combining active and passive immunotherapy strategies targeting α-syn with anti-inflammatory approaches ameliorates behavioral and neurodegenerative pathology in transgenic models of PD [37] and multiple system atrophy [38]. For these studies, we utilized a next-generation specific active immunotherapy technology with small pep tides, or AFFITOPEs® (AFF) [39]. These are short immunogenic (B-cell response) peptides that are too short for inducing a T-cell response (autoimmunity) and do not carry the native epitope but rather a sequence that mimics the original epitope (e.g., oligomeric α-syn) [39]. Based on this methodology, PD-AFF1 was found to be the most effective for targeting α-syn and advanced to phase I clinical trials in PD and multiple system atrophy [37] and is now moving to phase II trials. A similar approach was used for targeting Aβ utilizing AFF peptides such as AD02 [40,41]. Although AD02 is no longer in clinical development, it represents a useful tool compound for research purposes, given the capacity to trigger the production of titers against Aβ aggregates [41]. Taking into consideration the limitations with this vaccine approach and with translating findings in transgenic mouse models of AD and DLB to the clinic, the main objective of this study was to provide proof of concept data as to the potential enhanced effects of immunizing with AD02, PD-AFF1, or the combination in APP/α-syn double tg mouse model that mimics some aspects of DLB with the prospect of evaluating in the near future in more physiological model.

2. Materials and methods

2.1. Peptide production and formulation

Peptides (AFFITOPEs®) were synthesized by FMOC solid phase peptide synthesis (EMC Microcollections GmbH, Tuebingen, Germany). The AFFITOPEs® contain either an additional C-terminal cysteine residue (AD-AFFITOPE, AD02) or an additional N-terminal cysteine residue (PD-AFFITOPE1, PD-AFF1) and are conjugated to the carrier protein keyhole limpet hemocyanin (KLH; Biosyn GmbH, Fellbach, Germany) using N-gamma-maleimidobutyryl-oxysuccinimide ester (Thermo Scientific, Rockford, USA). AFFITOPE®-KLH conjugates were adsorbed to aluminum hydroxide (Alum, Brenntag, Frederikssund, Denmark) as an adjuvant. These two AFFs were selected based on previous studies showing that AD02 and PD-AFF1 displayed greater selectivity and specificity in recognizing Aβ and α-syn aggregates with in vivo studies. Moreover, these studies have shown that antibodies generated by AD02 and PD-AFF1 cross into the central nervous system, recognize Aβ and α-syn, and reduce behavioral and neuropathological deficits in APP [40] and α-syn tg mice [37].

2.2. Titer determination by ELISA

Standard enzyme-linked immunosorbent assay (ELISA) technology was used to measure levels of AFFITOPE® induced antibodies in plasma [40]. Plasma samples from individual animals undergoing repeated immunization were obtained at multiple time points. Substrates used included human (BACHEM, CH) Aβ1–42 (5 μg/ml), recombinant human and mouse α-syn (2 μg/ml, rPeptide), the peptide α-syn 110–130, and AFFITOPE peptide-bovine

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serum albumin (BSA) conjugates (1 mM). In addition, irrelevant peptides such as Aβ11–20 (C-terminally coupled to BSA to mimic the BSA conjugation performed for AD02) and Aβ35–40 (N-terminally coupled to BSA to mimic the BSA conjugation performed for PD-AFF1) were used to assess treatment-dependent background reactivity to the linker of the conjugate or to BSA. Optical density (OD) was measured at 405 nm using a micro-well reader (Tecan, CH). For determination of titers against the respective peptide, $OD_{max}/2$ was calculated.

2.3. Transgenic mouse models and active immunization protocol

For these experiments, mThy1-APP tg (Line 41) [42] and mThy1-α-syn (Line 61) [43,44] were crossed to generate double APP/α-syn tg [26]. This model develops behavioral deficits [45], neurodegeneration and accumulation of Aβ and α-syn, and aggregates in cortical and subcortical regions [46], mimicking aspect of DLB [14,47].

A total of 52 mice were included; of them, 12 non-tg and 40 APP/α-syn tg mice (5-month-old at the start of the experiment) were used. Aged-matched non-tg mice littermates were treated with adjuvant alone, while the APP/ α-syn tg mice (n 5 10 per group) were treated with adjuvant alone, AD02, PD-AFF1, or the combination of AD02/PD-AFF1. Mice received biweekly to monthly subcutaneous injections for 5 months. The dose used for immunizing the animals was 30 μg peptide containing 0.1% Alum. In the group receiving both compounds in combination, both were applied using 30 μg peptide containing 0.1% Alum at alternating flanks, respectively. Mice were bled, and antibody titers were monitored by ELISA analysis. At the end of the studies, mice were tested for behavioral effects.

At the conclusion of experiments, mice (around 10month-old) were sacrificed and brains were extracted, and the right hemibrain was post-fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) (pH 7.4) at 4C for 48 h for neuropathological analysis, while the left hemibrain was snap-frozen and stored at 270C for subsequent protein extraction. All experiments were approved by the Institutional Animal Care and Use Committee at the University of California, San Diego, and were performed according to NIH recommendations for animal use.

2.4. Round beam test

The APP/α-syn tg mice display behavioral alteration including motor and learning deficits [14]. The round beam test allows for the assessment of gait and balance impairments through distance traveled in an allotted amount of time over a round beam placed horizontally. As previously described [48], three consecutive trials, 1 min each, were run in one day. The total forward distance traveled and the numbers of foot slippages were recorded. Speed on the beam was calculated as distance traveled/time, and errors on the beam were calculated as foot slips/distance traveled.

2.5. Adhesive removal test, buried pellet test, and activity in the open field

In DLB and PD, somatosensory and smell deficits have been reported to occur early in the disease in association with alterations in the olfactory/limbic axis. Olfactory testing has been considered for screening and early detection relevant to developing protective treatments. In

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this context, it has been suggested that somatosensory and olfactory testing could be useful in determining the therapeutic potential of compounds and eventual translation to the clinic [49]. We have shown before that the mThy1-α-syn tg display somatosensory [45] and olfactory deficits [49]. Therefore, as previously described, we evaluated the effects of vaccination in APP/α-syn tg by using the adhesion removal test (somatosensory) [45] and buried pellet test (olfaction) [49]. To evaluate activity, the mice were tested in the open field apparatus, and data were collected using a Kinder SmartFrame Cage Rack Station activity monitor system (Kinder Scientific, Poway, CA), in 3-D space using a 7 × 15 beam configuration. Data collection began when an animal was placed in the test chamber. Animals were evaluated for 10 min.

2.6. Immunoblot and analysis of Aβ by ELISA

Left hemibrains were homogenized and divided into cytosolic and membrane fractions as previously described [50,51]. For immunoblot analysis, 20 mg of total protein per lane was loaded on 4–12% Bis-Tris sodium dodecyl sulfate polyacrylamaide gel electrophoresis and blotted onto polyvinylidene fluoride membranes. To determine the effects of immunotherapy in levels of Aβ and α-syn, blotted samples from immunized α-syn tg mice were probed with antibodies against Aβ (82E1, 1:1000, IBL), full-length α-syn (1:1000, Syn1, BD), and phosphorylated Tau (pTau) (PHF-1 courtesy of Dr. P. Davies). Incubation with primary antibodies was followed by species-appropriate incubation with secondary antibodies tagged with horseradish peroxidase (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA), visualization with enhanced chemiluminescence, and analysis with a Versadoc XL imaging apparatus (BioRad, Hercules, CA). Analysis of β-actin (Sigma) levels was used as a loading control. The level of human Aβ42 was determined using Human Aβ42 ELISA kit (KHB3441, Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instruction. The cytoplasmic and particulate mouse brain homogenates were diluted in sample dilution buffer (final 50 mg per sample). Optical signals at 450 nm were read on SpectraMax E5 plate reader (Molecular Devices, San Jose, CA).

2.7. Immunohistochemistry and image analysis

Analysis of Aβ, α-syn, and pTau accumulation was performed in serially sectioned vibratome sections from vaccinated non-tg and APP/α-syn tg mice by incubating the sections overnight at 4°C with monoclonal antibodies against Aβ (82E1 1:1000, IBL), α-syn (1:250, Syn1, BD), and pTau (PHF-1, courtesy of P. Davis). Antibodies against Iba1 (1:1000, Wako, Richmond, VA) and glial fibrillary acidic protein (GFAP) (1:1000, Millipore, Temecula, CA) were used to examine the effects of immunization on microglial and astroglial cell activation, respectively. To evaluate the effects of active immunotherapy on neurodegeneration, vibratome sections were analyzed with antibodies against the pan-neuronal marker NeuN (1:1000, Millipore, Temecula, CA), the cholinergic cell marker choline-acetyl transferase (ChAT) (1:1000, Millipore, Temecula, CA), and the dopaminergic cell marker tyrosine hydroxylase (TH) (1:1000, Millipore, Temecula, CA). Primary antibody incubation was followed by biotinylated secondary incubation (1:100, Vector Laboratories, Inc., Burlingame, CA), Avidin-Biotin Complex (1:200, ABC Elite, Vector), and developed by incubation with diaminobenzidine for sections incubated with antibodies against α-syn, pTau, NeuN, TH, ChAT, Iba-1, and GFAP. Sections immunolabeled with the 82E1 antibody

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(Aβ) were visualized with FITC (1:250, Vector). Sections were developed with diaminobenzidine and analyzed with a bright field digital BX50S Olympus microscope; serial digital images were analyzed with the ImageJ program (NIH) to determine counts and levels of immunoreactivity [48,52,53]. The numbers of NeuN (neocortex and hippocampus), ChAT (nucleus basalis), and TH (substantia [S.] nigra) immunoreactive neurons were estimated using unbiased stereological methods [54]. Hemisections containing the neocortex, hippocampus, striatum, and SN were outlined using an Olympus BX51 microscope running StereoInvestigator 8.21.1 software (Microbrightfield). Grid sizes for the hippocampal CA3 and CA1 pyramidal layers were as follows: 300 ! 300 mm and the counting frames were 50 × 50 μm, respectively. The average coefficient of error for each region was 0.09. Sections were analyzed using a 100X 1.4 PlanApo oil-immersion objective. A 5-μm-high dissector allowed for 2-μm top and bottom guard zones. For the basal forebrain cholinergic neurons, grids were set as previously described [55], and for the S. nigra dopaminergic neurons, total counts in the right hemibrain were estimated as previously described [56].

2.8. Single and double immunofluorescence analysis and confocal microscopy

Double labeling analysis was performed to determine the effects of the single and combined specific active immunotherapy approach on the clearance of α-syn. For this purpose, vibratome cut sections were double labeled with antibodies against Iba-1 (1:250) and α-syn (1:250, Syn1, BD) detected with secondary antibodies tagged with FITC and with an anti-Iba-1 antibody to detect microglia was detected with Tyramide Red amplification system (Thermo Fisher). For immunofluorescence analyses, sections were imaged with a Zeiss 63X objective on an Axiovert 35 microscope (Zeiss, Jena, Germany) with an attached MRC1024 laser scanning confocal microscope system (BioRad) [43]. Confocal images were analyzed with the ImageJ program as previously described [57] to determine the percent area of the neuropil covered by Aβ immunoreactive material and to ascertain the percent of Iba-1–positive cells colocalizing with α-syn [26]. All sections were processed simultaneously under the same conditions, and experiments were performed in triplicate to assess the reproducibility of results.

2.9. Experimental design and statistical analysis statistical analysis

All analyses were performed using GraphPad Prism (version 5.0) software.

All experiments were done blind-coded and in triplicate. Values in the figures are expressed as means ± standard error of the mean. To determine the statistical significance, values were compared using one-way analysis of variance (ANOVA) with Dunnett's post hoc test as indicated in each figure legend. Adjusted *P* values are reported for specific comparisons in the Results section. Each analysis had 47 degrees of freedom. The differences were considered to be significant if the *P* values were less than 0.05. The terms "additive effects" and "synergistic effects" are used to describe the reported statistical differences between individual immunizations and the combine immunization. While with "additive effects" the combined therapy is equal or close to the effects of the individual vaccine with synergism, the combined effects are greater than the sum. Moreover, a synergistic effect might be when

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the statistical effects are greater than the expected combined statistical effect of the two individual immunizations.

3. Results

3.1. Selection of Aβ and α-syn AFFITOPEs®

Selection and primary analysis of the two AFFITOPE®s used in this studies targeting either Aβ (AD02) or α-syn (PD-AFF1) has been described previously [37,38,40]. Of note, both types of active immunotherapies proved to be eliciting antibodies selective for aggregated forms of the targeted molecules, Aβ and α-syn, and were eliciting differential responses as compared to active immunotherapy consisting of peptide sequences of the native Aβ and α-syn molecules (i.e.: Aβ1–6-KLH and α-syn -peptide-KLH [37,38,40]. The effects elicited by the two AFFITOPEs® included a lack of cross-reactivity to the full-length APP or sAPPa [40] and a lack of antibodies reacting against the neuroprotective α-syn homolog β-syn [37,38]. In addition, both compounds induced an antibodies response specific for human Aβ or α-syn [37,38,40]. Moreover, application of both AFFITOPEs® did not lead to induction of Aβ or α-syn specific T-cells [37,40].

3.2. Analysis of immune reaction following repeated AD and PD AFFITOPE® application

Analysis of AFFITOPE® -induced antibodies in AD02, PD-AFF1, or AD02/PD-AFF–treated double tg animals revealed strong, comparable IgG titers towards the peptide(s) used for immunization (Fig. 1A) in all three groups. As previously reported, both compounds also show the Ability to induce antibodies reacting to the targeted epitope on the two molecules presented as BSA conjugates (Fig. 1B, C), as well as to full-length (fl), mainly monomeric, human (h) Aβ and α-syn (Fig. 1D, E). As expected, AFFITOPE®-induced antibodies showed a higher reactivity to peptide-BSA conjugates carrying the short epitopes targeted by the induced antibodies in all three AFFITOPEs® treated groups as compared to their respective hFl proteins (hFl-Aβ or hFl-α-syn). Increased binding to BSA conjugates could indicate a selectively higher reactivity against aggregated forms of the targets (Aβ and α-syn) and could thus be considered as a confirmation of aggregate selectivity of AFFITOPE®-induced antibodies previously shown using several different analyses [37,38,40] in this mouse model. Neither AD02 nor PD-AFF1- or AD02/PD-AFF1-induced plasma samples reacted with irrelevant control peptides such as Aβ11–20 offered as BSA conjugates (Fig. 1F). Of note, the AD02/ PD-AFF1 combination did not change the immunogenicity and did not lead to a change in IgG titers toward both peptides used for immunization as compared to single AFFITOPE®-treated groups. Similarly, no differences in IgG titers toward the targeted epitopes or recombinant proteins between single and combined AFFITOPE®-treated animals could be detected. Nonspecific responses, as detectble by ELISA against irrelevant control peptides, did not change as well. In addition, the IgG responses triggered by the 2 single AFFITOPEs® and the combination of both followed the same kinetics *in vivo*.

Antibody titers were measured in APP/α-syn tg mice for the injected peptides (Fig. 1G, H). Titers in each group were increased after the first injection and reached maximum at week 4 compared to vehicle-treated non-tg and APP/α-syn tg mice. No significant differences were

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observed in PD titer levels between mice immunized with PD-AFF1 or the combined AD02/PD-AFF1 immunotherapy nor in AD02 titer levels between mice immunized with AD02 and the combined AD02/PD-AFF1 immunotherapy (Fig. 1G, H). Antibody titer levels were also measured for recombinant Aβ (Fig. 1I) and α-syn (Fig. 1J). In mice immunized with PD-AFF1 or AD02/PD-AFF1, there was a maximal titer level achieved by week 8 which decreased over time but was still clearly detectable by the end of the experiment (Fig. 1I, J). Aβ titer levels in mice immunized with AD02 or AD02/ PD-AFF1 achieved a maximal titer level by week 4 (Fig. 1I, J). Again, Aβ titer levels were detectable over time, but differences between the AD02 alone group and the combination group in regards to Aβsolute titers were observed (Fig. 1I, J).

3.3. Effects of single and mixed immunization with AD02 and PD-AFF1 at reducing the accumulation of protein aggregates in APP/α-syn tg mice

Next, we characterized the effects ofthe immunizations on the levels of accumulation of Aβ in the brains of APP/α-syntg mice. Vehicle-treated APP/α-syn tg mice had a significant Aβ immunoreactivity in both the neocortex and hippocampus compared to vehicle-treated non-tg mice (Fig. 2A–C). Immunization with either AD02 or PD-AFF1 lead to a significant decrease in Aβ aggregates in both the neocortex (Fig. 2A, B, *P* value <.0001) and hippocampus (Fig. 2A, C, *P* value <.0001), although the PD-AFF1 was less effective than the AD02 immunization in both the neocortex (*P* value = .0267; Fig. 2B) and hippocampus (*P* value = .0324; Fig. 2C). Importantly, there was an additive effect in the AD02/PD-AFF1 combined immunization where the Aβ aggregates were significantly decreased compared to the PD-AFF1 alone in both the neocortex (Fig. 2A, B; *P* value <.0001) and hippocampus (Fig. 2A, C, P value <.0001). Similarly, we then characterized the effects of the specific active immunotherapy on α-syn immunoreactivity in the neocortex and hippocampus. As expected, compared with vehicle-treated non-tg mice, vehicle-treated APP/α-syn tg mice had a significant increase in α-syn immunoreactivity in both the neocortex (Fig. 2D, E, P value <.0001) and hippocampus (Fig. 2D, F, P value <.0001), which was significantly decreased by each immunization. Interestingly, the combined AD02/PD-AFF1 immunization approach did not result in an additional decrease in α-syn levels (Fig. 2D–F) as it did for the Aβ immunoreactivity (Fig. 2A–C).

We have shown that levels of pTau immunoreactivity are increased in the APP tg [58] and α-syn tg [59]. Given the interest in the role of pTau in AD and DLB, we also characterized the effects on pTau of the single and combined immunization in the APP/α-syn tg mice. Compared with vehicle-treated non-tg mice, vehicle-treated APP/α-syn tg mice had a significant increase in pTau immunoreactivity in both the neocortex (Fig. 2G, H, *P* value < .0001) and hippocampus (Fig. 2G, I). pTau immunoreactivity was significantly decreased by AD02 (*P* value < .0001) and AD02/PD-AFF1 (*P* value <.0001) and to a lesser extent by PD-AFF1 (*P* value = .0470) in the neocortex (Fig. 2G, H). In the hippocampus, pTau immunoreactivity was decreased by the AD02 (Fig. 2G, I, *P* value = .0011) and AD02/PD-AFF1 combined approach (Fig. 2G, I, *P* value <.0001), but not the PD-AFF1 alone (Fig. 2G, I; *P* value = .5689).

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Immunoblot analysis was used to confirm our immunohistochemical findings. Soluble Aβ protein was significantly increased in vehicle-treated APP/α-syn tg mice compared to non-tg mice (Fig. 3A, B, *P* value = .0001). Immunization with either AD02 or AD02/PD-AFF1 significantly decreased the levels of soluble (Fig. 3A, B; *P* values = .0050 and .0021, respectively) and insoluble (Fig. 3A, B; *P* values = .0338 and .0015, respectively) Aβ protein compared to vehicle-treated APP/α-syn tg mice; however, immunization with PD-AFF1 had no significant effect on soluble (*P* value = .7828) or insoluble (*P* value = .9586) Aβ protein levels (Fig. 3A, B). Next, levels of α-syn were analyzed, as expected both soluble (P value 5 .0001) and insoluble (P value 5 .0004) α-syn protein levels were significantly increased in vehicle-treated APP/α-syn tg mice compared to vehicle-treated non-tg mice (Fig. 3C, D). Interestingly, immunization with AD02 or PD-AFF1 alone did not significantly reduce levels of soluble protein (*P* values = .06 and .26, respectively); in contrast, the combined AD02/PD-AFF1 vaccine significantly reduced soluble α-syn (*P* values = .0146). Overall levels of insoluble α-syn were significantly increased in the vehicle-treated APP/α-syn tg mice compared to vehicle-treated non-tg mice (*P* value = .0001) (Fig. 3C, D). Treatment with AD02, PD-AFF1, and the combination resulted in a significant decrease (*P* values = .0014, .0006 and .0014 respectively) compared to vehicle APP/α-syn tg (Fig. 3C, D). Next, we analyzed the levels of endogenous Tau and found that levels of mouse pTau protein were significantly increased in vehicle-treated APP/α-syn tg mice compared to vehicle-treated non-tg mice (Fig. 3E, F, *P* values = .0001). Immunization with AD02, PD-AFF1, or AD02/PD-AFF1 significantly reduced both soluble (*P* values = .0001, for each) and insoluble pTau protein levels (*P* values = .0002, .049, and .0001, respectively) compared to vehicle-treated APP/α-syn tg mice (Fig. 3E, F). Finally, to further confirm the Aβ protein results by an independent method, ELISA analysis was performed. Consistent with the immunocytochemistry and Western blot, by ELISA soluble and insoluble Aβ42 was significantly increased in vehicle-treated APP/α-syn tg mice compared to non-tg mice (Fig. 3G, H) (*P* value = .0002 and .00001 respectively). Immunization with either AD02 or AD02/PD-AFF1 significantly decreased the levels of soluble Aβ42 (Fig. 3G; *P* values = .0489 and .04, respectively) and insoluble (Fig. 3H; *P* values = .032 and .0013, respectively) Aβ42 protein compared to vehicle-treated APP/α-syn tg mice; however, immunization with PD-AFF1 had no significant effect on soluble (*P* value = .9) or insoluble (*P* value = .9) Aβ42 protein levels (Fig. 3G, H). In summary, AD02 was more effective than PD-AFF1 in reducing Aβ and pTau burden, with the combination exhibiting some additive effects. AD02 and PD-AFF1 were similarly effective at reducing the α-syn with no additive effects of the combination.

3.4. Effects of vaccination with single and combined AD02 and PD-AFF1 on selective neuronal degeneration in APP/α-syn tg mice

We have previously shown that combined overexpression of APP and α-syn in biogenic mice results in selective loss of pyramidal neurons in deeper layers of the neocortex and CA3 of the hippocampus [27]. Moreover, while single tg mice show preservation of cholinergic neurons in the nucleus basalis and TH-positive cells in the S. nigra (but not the degeneration of axons), the double tg mice display neurodegeneration in these brains regions [14,27]. To evaluate the effects of the immunization in these selected neuronal populations, immunocytochemistry with antibodies against NeuN, ChAT, and TH and stereological

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analysis were performed. The number of NeuN-positive neurons was significantly decreased in the neocortex (*P* values = .0001) and hippocampus (CA3) (*P* value = .0084) of vehicle-treated APP/α-syn tg mice compared to vehicle-treated non-tg mice (Fig. 4A–C). Immunization with AD02, PD-AFF1, or AD02/PD-AFF1 significantly normalized the number of neurons in the neocortex to comparable levels (Fig. 4A, B; *P* values = .0001 for each). The AD02 immunotherapy (*P* value = .0229) and AD02/PD-AFF1 (*P* value = .045) immunotherapy rescued the loss of neurons in the hippocampus (Fig. 4A, C) compared to vehicle-treated APP/α-syn tg mice. As expected, compared to non-tg mice, the vehicle-treated APP/α-syn tg mice displayed loss of ChAT neurons in the nucleus basalis of Meynert (*P* value = .01) and fibers in the hippocampus (*P* value = .01) (Fig. 4D–F). Immunization with PD-AFF1 (*P* value = .0243) or AD02/PD-AFF1 (*P* value = .0145), but not AD02 (*P* value = .1552) rescued neurodegeneration of the cholinergic system of vehicle-treated APP/α-syn tg mice in the nucleus basalis of Meynert (Fig. 4D, E). Immunization with AD02 (*P* value = .0098), PD-AFF1 (*P* value = .0324), or AD02/PD-AFF1 (*P* value = .0004) rescued neurodegeneration of the cholinergic fibers in the hippocampus (Fig. 4D, F). Likewise, compared to non-tg mice, the vehicle-treated APP/α-syn tg mice displayed loss of TH neurons in the S. nigra (*P* value = .001) and fibers in the striatum (*P* value = .001) (Fig. 4G–I). Interestingly, neurodegeneration in the S. nigra was unaffected by treatment with AD02 (*P* value = .9246) but was rescued by PD-AFF1 (*P* value = .030) and AD02/PD-AFF1 (*P* value = .04) (Fig. 4G, H); while immunization with PD-AFF1 (*P* value = .0123) or AD02/PD-AFF1 (*P* value = .0005), but not AD02 (*P* value = .2893), was effective at reducing neurodegeneration of TH-positive fibers (vehicle-treated APP/α-syn tg mice vs. vehicle-treated non-tg mice; *P* value = .0003) in the striatum (Fig. 4G, I).

In summary, AD02 and PD-AFF1 were able to ameliorate the degeneration of pyramidal neurons in the neocortex and CA3 region of the hippocampus with no additive effects. However, PD-AFF1 was more effective than AD02 at ameliorating the loss the cholinergic and dopaminergic system with additive effects of the combination.

3.5. AD02 and PD-AFF1 reduced markers of neuroinflammation and promoted microglial clearance in APP/α-syn tg mice

Next, we investigated the effects of the specific active immunotherapy in neuroinflammation. Compared to the non-tg controls, the vehicle-treated APP/α-syn tg mice displayed significant astrogliosis increase in both the neocortex (Fig. 5A, B, *P* value = .0001) and hippocampus (Fig. 5A, C; *P* value = .0001). Immunization with AD02, PD-AFF1, or AD02/PD-AFF1 combined approach significantly decreased astrogliosis in both the neocortex (Fig. 5A, B, *P* values = .0001 for each comparison) and hippocampus (Fig. 5A, C, *P* value = .0001 for each comparison). Similarly, microgliosis was significantly increased in vehicle-treated APP/α-syn tg mice compared to vehicle-treated non-tg mice (*P* value = .0001 in both the neocortex and hippocampus) (Fig. 5D–F). Immunization with AD02, PD-AFF1, or AD02/PD-AFF1 reduced microgliosis in both the neocortex (Fig. 5D, E, *P* values = .0001 for each comparison) and hippocampus (Fig. 5D, F, *P* values = .0001 for each comparison).

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Finally, to evaluate the effects of combined immunotherapy on α-syn clearance by microglia, colocalization imaging studies were performed for α-syn (green; Fig. 5G) and microglia (red; Fig. 5G). As expected, in the vehicle-treated non-tg mice, no α-syn was detected in microglia (Fig. 5G, H). In contrast, in vehicle-treated APP/α-syn tg mice, there was a modest subset of microglia with α-syn accumulation (Fig. 5G, H). There was a significant increase in the percent colocalization between α-syn and microglia in vehicle-treated APP/α-syn tg mice compared to vehicle-treated non-tg mice (Fig. 5H; *P* value = .0044). Immunization with AD02 (*P* value =.0002), PD-AFF1 (*P* value = .0002), or AD02/PD-AFF1 (*P* value < .0001) further increased the colocalization of α-syn with microglia compared to vehicle-treated APP/α-syn tg mice (Fig. 5G, H). Importantly, there was an additive effect of the AD02/PD-AFF1 immunization which had a significantly higher percent colocalization between the microglia and α-syn protein compared to the PD-AFF1 single immunization (Fig. 5G, H; *P* value = .0186). In brief, both AD02 and PD-AFF1 reduced astrogliosis and microgliosis with no additive effects of the combination; however, enhanced clearance of α-syn by microglia was detected when AD02 and PD-AFF1 immunotherapies were combined.

3.6. Active immunotherapy with AD02 and PD-AFF1 improves behavioral deficits in APP/α-syn tg mice

Finally, we evaluated if the improvements in neuropathological markers in the APP/α-syn tg mice were accompanied by functional effects on behavioral evaluations. Motor testing in the round beam (Fig. 6A–B) showed that at 10 months of age, the vehicle-treated APP/α-syn tg mice had significantly more errors (*P* value < .0001) and traveled a significantly shorter distance (*P* value = .0016) compared to vehicle-treated non-tg mice. Immunization with AD02, PD-AFF1, or AD02/PD-AFF1 significantly decreased at comparable levels the number of errors in the APP/α-syn tg mice (Fig. 6A, *P* values = .0001, .0004, and .0037, respectively) but did not significantly increase the distance traveled (Fig. 6B, *P* values = .4207, .2804, and .7136, respectively).

Next, we assessed somatosensory and olfactory function using the buried pellet test and the adhesion removal test. While the buried pellet test has been shown to sensitively detect early olfactory deficits in synucleinopathy models [49], the adhesion removal test has been shown to be sensitive to cortical deficits following a stroke in the mice [60] and to dopaminergic and cholinergic deficits in synucleinopathy models [45]. Moreover, these tests were used as surrogates to learning and memory tests such as the water maze that turned out to be difficult to execute in the aged APP/ α-syn tg. In the buried pellet test, vehicle-treated APP/α-syn tg mice took significantly longer time to find the buried pellet compared to vehicle-treated non-tg mice (P value 5 .0330) (Fig. 6C). Immunization with AD02/PD-AFF1 (P value 5 .0443), but not AD02 (P value 5 .1444) or PD-AFF1 (P value 5 .8127), significantly decreased the amount of time for the mice to find the buried pellet (Fig. 6C). In the adhesive removal test, vehicle-treated APP/α-syn tg mice took significantly longer time to detach the tape compared to vehicle-treated non-tg mice (*P* value = .0016) (Fig. 6D) and reaction time (*P* value = .0010) (Fig. 6E) with no differences among the groups in success rate (Fig. 6F). Immunization with AD02 or PD-AFF1 significantly reduced the removal and reaction time (*P* value = .034; *P* value = .005) and the combination of AD02/PD-AFF1 has additive effects

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(*P* value = .0022) (Fig. 6D, E). In summary, both AD02 and PD-AFF1 improved motor deficits in the double tg mice, with no additive effects of the combination; however, in somatosensory and olfactory tests, the combination of AD02 and PD-AFF1 was more effective than the individual vaccination.

4. Discussion

While previous studies have investigated the cross-talk effects of immunization against Aβ on Tau [61] or conversely the effects of Tau immunization on Aβ in 3xTg-AD mice [36], our study explored the effects of specific active immunotherapy against Aβ and α-syn individually or in combination on APP/α-syn tg mice. We showed that immunization against Aβ with AD02 was more effective than immunization against α-syn with PD-AFF1 in reducing Aβ and mouse pTau burden in APP/α-syn tg mice with the combination displaying some additive effects. Moreover, AD02 was as effective as PD-AFF1 at reducing α-syn. However, both AD02 and PD-AFF1 ameliorated the degeneration of pyramidal neurons, while PD-AFF1 was more effective at preventing the loss of cholinergic and dopaminergic cells with additive effects of the combination that was reflected in reduced neuroinflammation and improved behavioral performance.

Together, these results indicate that targeting Aβ in addition to reducing Aβ and Tau also has consequences in the accumulation of other proteins relevant to the pathogenesis of DLB such as α-syn. Moreover, our results indicate that the combined specific active immunotherapy has some additive effects and that immunization targeting α-syn has effects on Tau aggregation and to a lesser extent on Aβ. This is in agreement with other studies suggesting that Aβ might be a trigger for the aggregation of other proteins involved in neurodegeneration such as Tau [62] and α-syn [14] and supports the notion that blocking Aβ in double tg mouse model might have the added benefit of reducing the aggregation and toxicity of α-syn and Tau aggregation [14,63]. Along the same lines, a recent study showed that targeting Tau oligomers with a specific antibody blocks α-syn accumulation and toxicity in an α-syn tg mouse model, suggesting that α-syn and Tau might also interact [64].

The mechanisms through which the single or combined specific active immunotherapy might reduce the accumulation of Aβ, α-syn, and Tau are complex and might depend on the timing of the therapeutic intervention [34,65]. Multiple mechanisms of action have been proposed depending on if the antibodies are targeting intracellular or extracellular Aβ, α-syn, and Tau. For intracellular protein aggregates, we have previously shown that immunotherapy targeting α-syn might trigger clearance via autophagy [66], block c-terminus truncation [67], facilitate trafficking of multivesicular bodies containing aggregated proteins via the endosomal sorting complex required for transport pathway to the lysosome [68,69], attenuate inflammation [37,70], or reduce oligomerization and accumulation [65,71–74].

For extracellular proteins, antibodies might block seeding [75,76], prion-like propagation [76], block oligomer receptors [77], and interfere with protein-protein interactions or transsynaptic transmission [57]. For Aβ, it is possible that this protein might directly interact with Tau and α-syn in the surface of membranes [26,78–80], in intracellular compartments

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or in the cytosol following leakage from endosomes [14,26,81]. These proteins can also interact in the extracellular compartments [82–85] given recent evidence that not only Aβ [86] is released into the extracellular space but also Tau [87–89] and α-syn [90,91] resulting in the formation of heterodimers [26,92]. The beneficial effects of antibodies against Aβ (and α-syn) might be associated with blocking both intracellular and extracellular interactions between Aβ and α-syn (and Tau) and by promoting the microglial clearance of these proteins [38,52,93].

For the present study, we have shown that single and combined specific active immunotherapy against Aβ and α-syn reverses pathological and functional alterations in a mouse model of DLB, suggesting that therapeutic development for this synucleinopathy might be of interest. However, given recent evidence that the combined accumulation of these proteins is not unique to DLB but might be relevant to AD, applications of this approach might be extended to other neurodegenerative disorders. In patients with AD, levels of soluble α-syn are elevated in brain and fluids, differentiating AD from PD [94,95]. Aβ and α-syn cooperated to block SNARE-vesicle fusion [96], and recent studies showed that in APP tg mice, α-syn infusion reduces amyloid plaque formation but enhances synaptic degeneration [97]. This finding might appear contradictory at first glance; however, it is worth noting that the authors showed that soluble α-syn interfered with the fibrillation of Aβ, α-syn, and Tau in a combinatorial manner might be of therapeutic value for AD and DLB. However, given the record of low translatability of studies in tg mice to AD patients in clinical trials, immunotherapy results in overexpressing models should be taken with caution. In the future, comparable studies will be necessary in models mimicking more closely sporadic forms of AD and DLB including recently developed APP and α-syn knock in rodent models expressing these molecules at physiological levels. Moreover, translation of experimental results in rodent models will require having comparable biomarkers that mimics the progression of these neurodegenerative disorders along the lines of what has been shown with positron emission tomography imaging and cerebrospinal fluid indicators for Aβ and Tau in patients with AD; unfortunately reliable α-syn biomarkers for DLB are not yet available. Having an equivalent set of imaging and/or fluid biomarkers in APP/α-syn rodent models comparable to humans could allow target engagement studies with more careful pharmacodynamics and pharmacokinetic studies in the rodent models that will better inform design of clinical trials including dose response and sample size.

Given the various mechanisms involved, in recent years, the concept of combinatorial therapeutics for synucleinopathies and other neurodegenerative disorders has emerged [34]. Combinatorial therapy, for example, includes mixing of different modalities of immunotherapy, simultaneously targeting more than one protein (as tested in the present study), immunotherapy with small molecules, neurotrophic factors, stem cells, or anti-inflammatory drugs. For example, we recently showed that combining immunization against α-syn with cellular immunotherapy targeting T-regs cells was more effective by modulating immune responses and microglial activation [98]. Likewise, combining passive immunization with single-chain antibodies against α-syn oligomers with the anti-inflammatory compound lenalidomide was more effective at rescuing the neuropathology in a different synucleinopathy model of multiple systems atrophy [70,99]. In conclusion, taking into account the advantages and pitfalls of currently available tg models, this study suggests

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that both individual and combined specific active immunotherapy targeting Aβ and α-syn might be potentially suitable for the treatment of AD and DLB.

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RESEARCH IN CONTEXT

1. Systematic review: The literature was reviewed using PubMed and Google Scholar. Immunotherapeutic approaches targeting Aβ and tau in Alzheimer's disease (AD) and α-synuclein in Parkinson's disease are being developed. It is unknown if single or combined immunotherapies targeting Aβ and/or α-synuclein are effective in models of dementia with Lewy bodies. These relevant citations are cited.
2. Interpretation: Our findings suggest that specificactive immunotherapy targeting Aβ and/or α-synuclein might be of potential interest for the treatment of dementia with Lewy bodies. This hypothesis is consistent with nonclinical and clinical findings currently in the public domain.
3. Future directions: The manuscript proposes a framework for the generation of new hypotheses and the conduct of additional studies, including further understanding (1) mechanisms through which α-synuclein contributes to AD pathogenesis; (2) asynuclein regulation as a therapeutic target for AD; (3) combined specific active immunotherapy against Aβ and α-synuclein for therapeutic treatment of AD; and (4) intervention timing.

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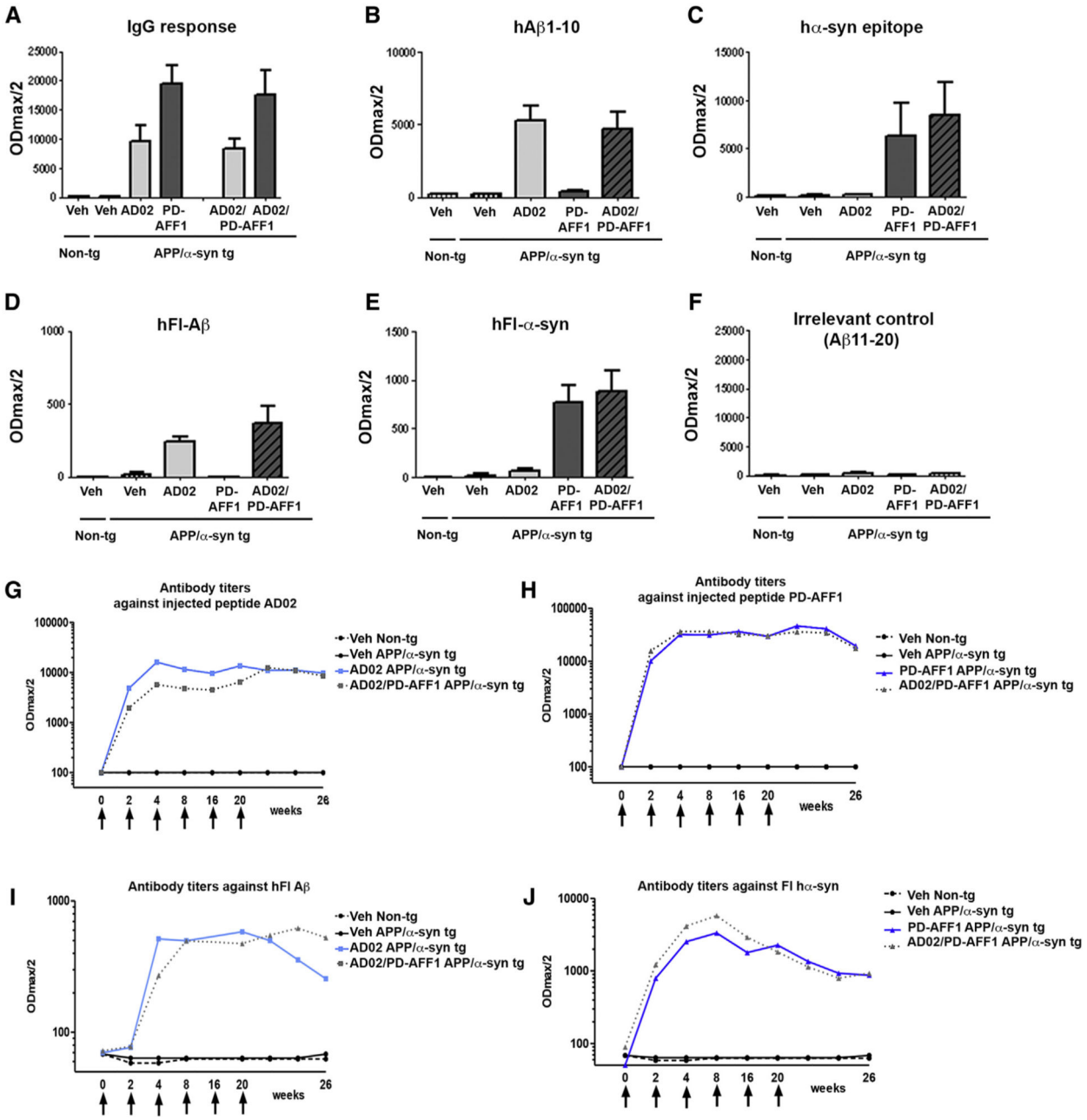

Fig. 1.

Analysis of the immune response and titers following injection of AD02, PD-AFF1, and a combination of both AFFITOPE®s in APP/α-syn double transgenic mice. APP/α-syn double transgenic mice and wild-type littermates (ALUM only) were subcutaneously injected 6 times with AD02 (n = 11), PD-AFF1 (n = 10), and a combination of both conjugate AFFITOPE®s (n = 9) adsorbed to aluminum hydroxide (ALUM) or with PBS-ALUM alone (n = 11 double tg mice, n = 12 wild-type littermates). Plasma was taken at monthly intervals and at sacrifice. Samples were analyzed for their concentration of IgG Aβs against specific peptides at scarification. Values depicted are the titer calculated as $OD_{max}/2$ (at 405 nm) plus standard error of the mean unless otherwise stated. (A) IgG response

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toward the respective immunizing peptide (AD02: anti AD02 [light gray bar], PD-AFF1: anti PD-AFF1 [dark gray bar], AD02/PD-AFF1 combination: anti AD02 [light gray bar], anti PD-AFF1 [dark gray bar]). (B) Reactivity toward human Aβ1–10 after immunization with AD02, PD-AFF1, and the combination of AD02 and PD-AFF1 (AD02/PD-AFFw1 combination). (C) Reactivity toward human α-syn -epitope after immunization with AD02, PD-AFF1, and AD02/PD-AFF1. (D) Reactivity toward human full-length, recombinant Aβ after immunization with AD02, PD-AFF1, and AD02/PD-AFF1. (E) Reactivity toward human full-length, recombinant α-syn after immunization with AD02, PD-AFF1, and AD02/PD-AFF1. (F) Reactivity toward human Aβ11–20, as irrelevant control, after immunization with AD02, PD-AFF1, and AD02/PD-AFF1. (G) Antibody titers against injected AD02 peptide, (H) injected PD-AFF1 peptide, (I) human full-length recombinant Aβ, and (J) human full-lengthrecombinant α-syn. Values depicted are the titer calculated as $OD_{max}/2$ (at 405 nm). Abbreviations: APP, amyloid precursor protein; OD, optical density.

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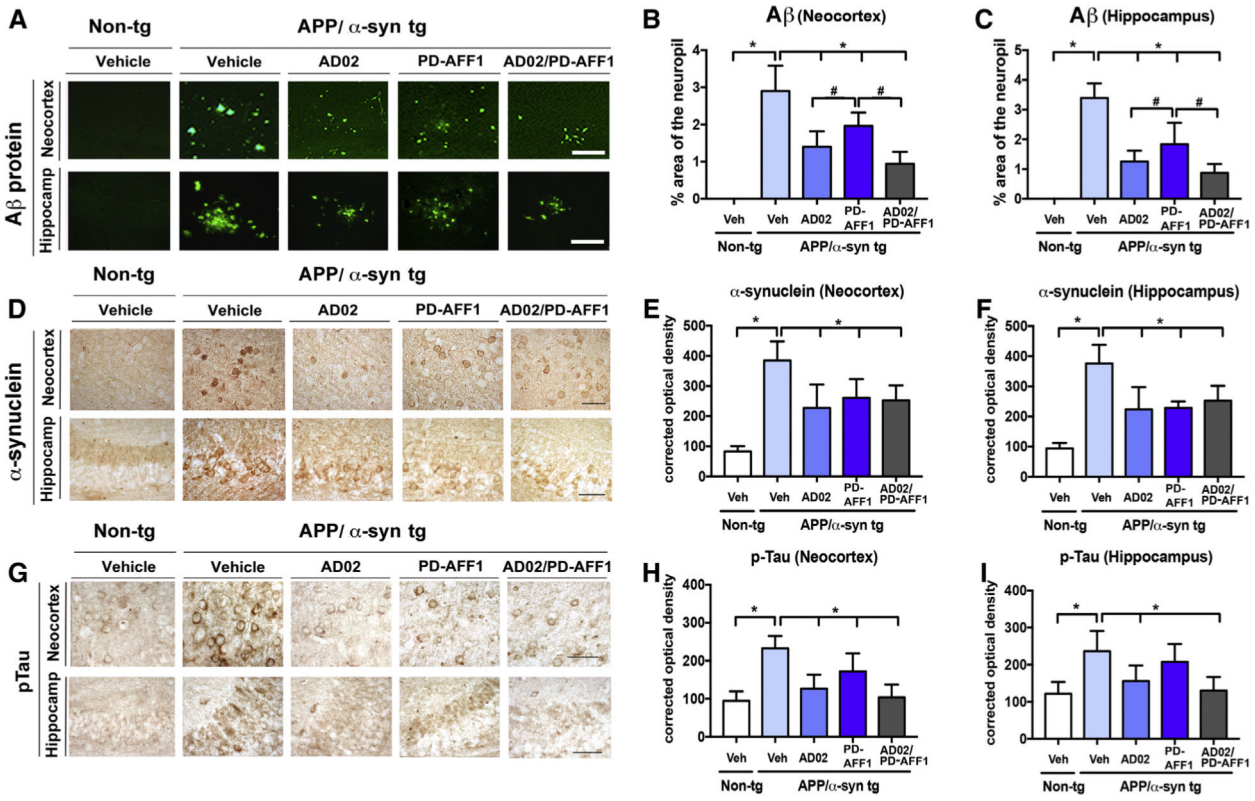

Fig. 2.

Effects of immunization on Aβ in APP/α-syn tg mice. APP/α-syn double transgenic mice and wild-type littermates (ALUM only) were subcutaneously injected 6 times with AD02 (n = 11), PD-AFF1 (n = 10), and a combination of both (n = 9) adsorbed to aluminum hydroxide (ALUM) or with PBS-ALUM alone (n = 11 double tg mice, n = 12 wild-type littermates). (A) Representative immunofluorescent images and quantitation of Aβ-ir in the (B) neocortex and (C) hippocampus. Immunizing with either of the individual peptides leads to a significant decrease in Aβ-ir in both the neocortex and hippocampus, although the PD-AFF1 was less effective than the AD02 vaccine in both the brain regions. Importantly, there was an additive effect in the AD02/PD-AFF1 combined immunotherapy where the Aβ-ir was significantly decreased compared to the PD-AFF1 treatment alone in both neocortex and hippocampus. (D) Representative photomicrographs and quantitation of α-syn-ir in the (E) neocortex and (F) hippocampus. Treatment with either of the individual peptides or the combined AD02/PD-AFF1 vaccine leads to a significant decrease in α-syn-ir in both the neocortex and hippocampus. (G) Representative photomicrographs and quantitation of pTau-ir in the (H) neocortex and (I) hippocampus. Treatment with either of the individual peptides or the combined AD02 PD-AFF1 led to a significant decrease in pTau-ir in both the neocortex and hippocampus. Scale bar (A, D, and (G) 5 40 mm. P value < .05 using ANOVA followed by Dunnett's post hoc test comparing each of the other groups with (*) vehicle-treated APP/α-syn tg mice and (#) PD-AFF1-treated APP/α-syn tg mice. Abbreviations: APP, amyloid precursor protein; pTau, phosphorylated Tau.

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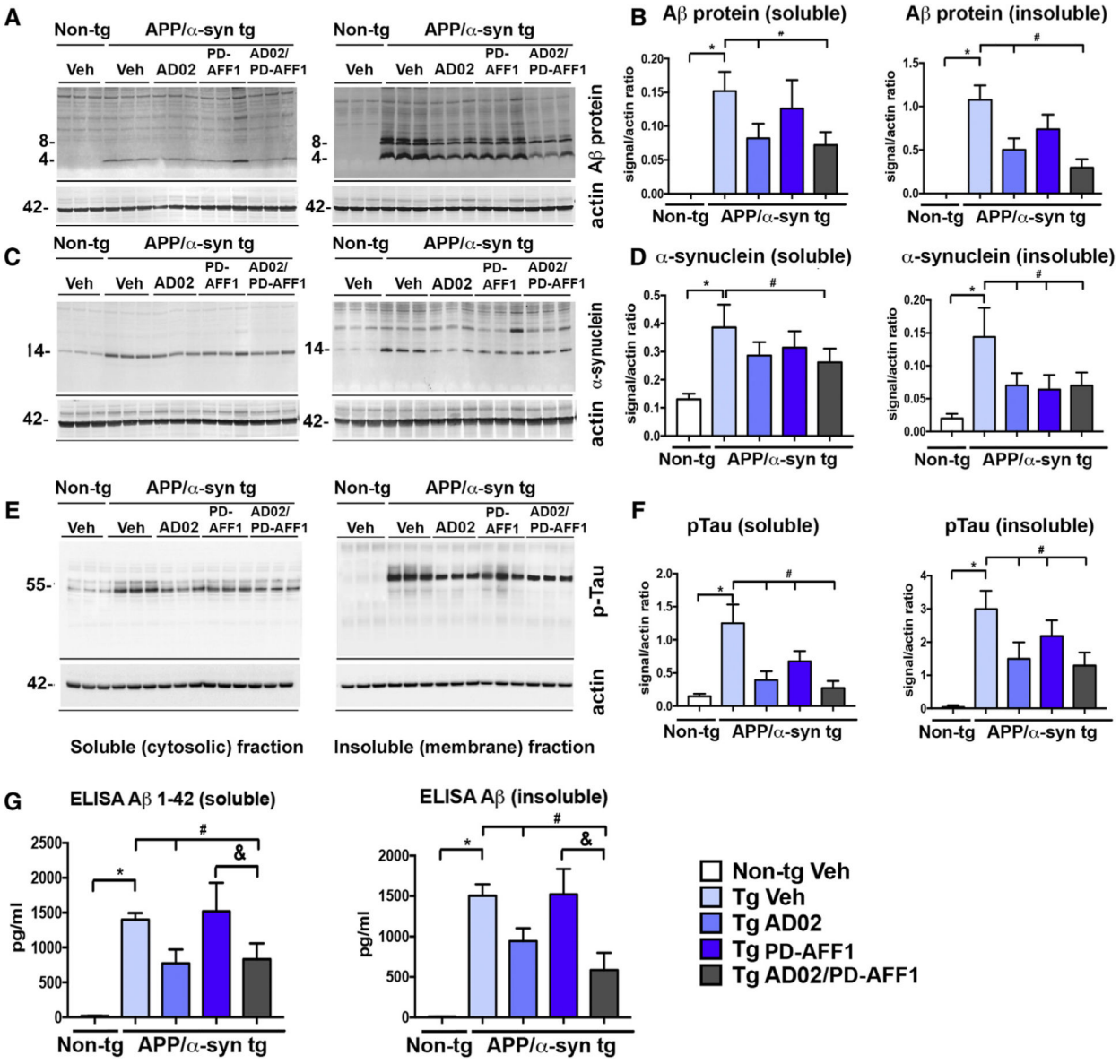

Fig. 3.

Immunoblot analysis of the effects of immunization in APP/α-syn tg mice. APP/α-syn double transgenic mice and wild-type littermates (ALUM only) were subcutaneously injected 6 times with AD02 (n = 11), PD-AFF1 (n = 10), and a combination of both, AD02/PD-AFF1 (n = 9) adsorbed to aluminum hydroxide (ALUM) or with PBS-ALUM alone (n = 11 double tg mice, n = 12 wild-type littermates). (A) Immunoblot and quantification of soluble and insoluble Aβ. Soluble and insoluble Aβ protein were significantly increased in vehicle-treated APP/α-syn tg mice compared to non-tg mice, and immunization with either AD02 or AD02/PD-AFF1 significantly decreased the levels of soluble and insoluble Aβ protein compared to vehicle-treated APP/α-syn tg mice. (C) Immunoblot and (D) quantification of soluble and insoluble α-syn. Soluble and insoluble α-syn protein were significantly increased in vehicle-treated APP/ α-syn tg mice compared to non-tg mice, and immunization with AD02, PD-AFF1, or AD02/PD-AFF1 significantly

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decreased the levels of soluble and insoluble α-syn protein compared to vehicle-treated APP/α-syn tg mice. (E) Immunoblot and (F) quantification of soluble and insoluble pTau. Soluble and insoluble pTau proteinwas significantly increasedinvehicle-treated APP/α-syntg mice compared to non-tgmice, and immunization with AD02,PD-AFF1,or AD02/PD-AFF1 significantly decreased the levels of soluble and insoluble pTau protein compared to vehicle-treated APP/α-syn tg mice. (G) Soluble and (H) insoluble Aβ42 protein levels measured by ELISA were significantly increased invehicle-treated APP/α-syn tg mice compared to non-tg mice. Statistical analysis was conducted using one-way ANOVA post hoc Dunnett's test for comparison with vehicle-treated APP/α-syn tg mice * = P <.05 compared to vehicle-treated non-tg mice; # = P <.05 compared tovehicle-treated APP/α-syn tg mice; and & = P <.05 when comparing PD-AFF1 vs. AD02/PD-AFF1. Abbreviations: ANOVA, analysis of variance; APP, amyloid precursor protein; ELISA, enzyme-linked immunosorbent assay; pTau, phosphorylated Tau.

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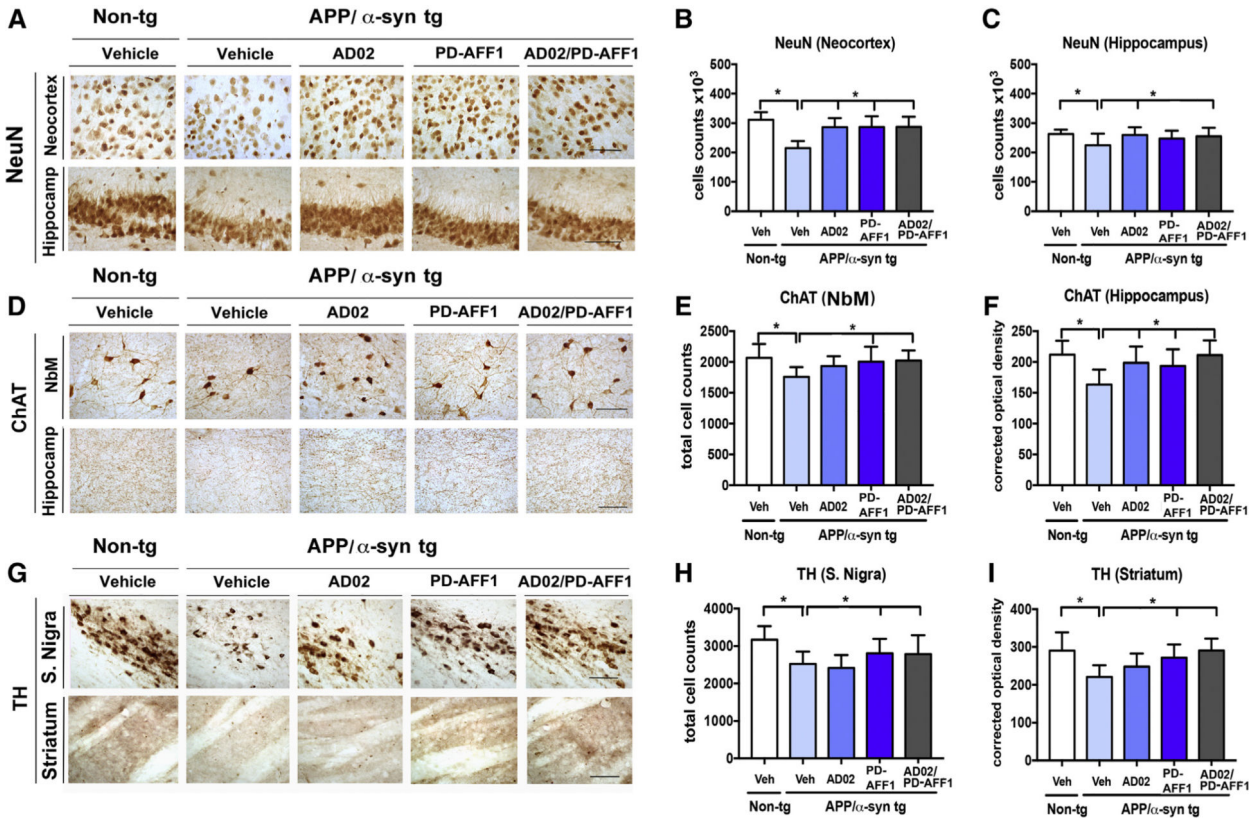

Fig. 4.

Effects of immunization on the degeneration of selectively vulnerable neuronal populations in APP/α-syn tg mice. APP/α-syn double transgenic mice and wild-type littermates (ALUM only) were subcutaneously injected 6 times with AD02 (n = 11), PD-AFF1 (n = 10), and a combination of both AD02/PD-AFF1 (n = 9) adsorbed to aluminum hydroxide (ALUM) or with PBS-ALUM alone (n = 11 double tg mice, n = 12 wild-type littermates). (A) Representative photomicrographs and quantitation of NeuN-ir in the (B) neocortex and (C) hippocampus. The number of NeuN-positive neurons was significantly decreased in the neocortex and hippocampus of vehicle-treated APP/α-syn tg mice compared to vehicle-treated non-tg mice. Treatment with either of the individual peptides or the combined AD02/PD-AFF1 immunotherapy leads to a significant increase in the number of NeuN-positive neurons in both the neocortex and hippocampus. (D) Representative photomicrographs and quantitation of ChAT-ir in the (E) neocortex and (F) hippocampus. The number of ChAT-positive neurons was significantly decreased in the nucleus basalis of Meynert (NbM) as well as the neuropil staining in the hippocampus of vehicle-treated APP/α-syn tg mice compared to vehicle-treated non-tg mice. Treatment with either of the individual peptides or the combined AD02/PD-AFF1 approach leads to a significant increase in the number of ChAT-positive neurons in the NbM and the immunoreactivity of the hippocampal neuropil. (G) Representative photomicrographs and quantitation of TH-ir in the (H) neocortex and (I) hippocampus. The number of TH-positive neurons was significantly decreased in the substantia (S.) nigra, as well as in the neuropil staining in the striatum of vehicle-treated APP/α-syn tg mice compared to vehicle-treated non-tg mice. Treatment with

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PD-AFF1 or the combined AD02/PD-AFF1 approach led to a significant increase in the number of TH-positive neurons in the S. nigra. Treatment with either of the individual peptides or the combined AP02/PD-AFF1 vaccine led to a significant increase in Th-immunoreactivity of the striatum neuropil. Scale bar 5 40 mm. *P* value <.05 using ANOVA followed by Dunnett's post hoc test comparing each of the other groups with (*) vehicle-treated APP/α-syn tg mice. Abbreviations: ANOVA, analysis of variance; APP, amyloid precursor protein; ChAT, choline-acetyl transferase;TH, tyrosine hydroxylase.

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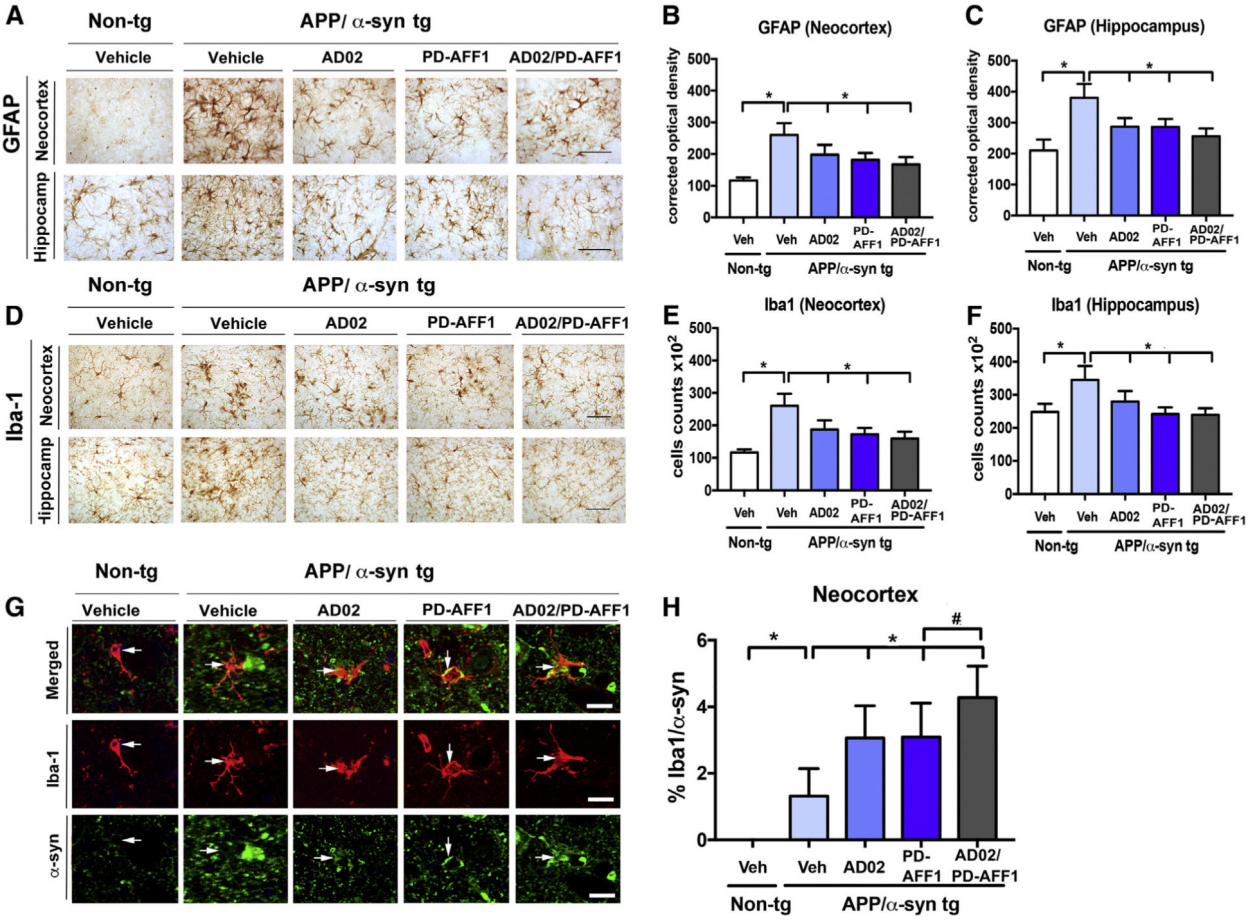

Fig. 5.

Effects of immunization on astroglial and microglial cells in APP/α-syn tg mice. APP/α-syn double transgenic mice and wild-type littermates (ALUM only) were subcutaneously injected 6 times with AD02 (n = 11), PD-AFF1 (n = 10), and a combination of both conjugate immunizations (n = 9) adsorbed to aluminum hydroxide (ALUM) or with PBS-ALUM alone (n = 11 double tg mice, n = 12 wild-type littermates). (A) Representative photomicrographs and quantitation of GFAP-ir in the (B) neocortex and (C) hippocampus. The immunoreactivity of GFAP-positive astrocytes was significantly increased in the neocortex and hippocampus of vehicle-treated APP/α-syn tg mice compared to vehicle-treated non-tg mice. Treatment with either of the individual peptides or the combined AD02/PD-AFF1 approach led to a significant decrease in the immunoreactivity of GFAP-positive astrocytes in both the neocortex and hippocampus. (D) Representative photomicrographs and quantitation of Iba-1-ir in the (E) neocortex and (F) hippocampus. The immunoreactivity of Iba-1–positive astrocytes was significantly increased in the neocortex and hippocampus of vehicle-treated APP/α-syn tg mice compared to vehicle-treated non-tg mice. Treatment with either of the individual peptides or the combined AD02/PD-AFF1 immunotherapy led to a significant decrease in the immunoreactivity of Iba-1–positive microglia in both the neocortex and hippocampus. (G) Laser scanning confocal microscopy of double-labeled sections with antibodies against Iba1 (red) and α-syn (green) to evaluate the effects of immunization on microglia clearance of α-syn and (H)

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analysis of the percent colocalization between α-syn and Iba-1–positive microglia. No α-syn was detected in microglia in vehicle-treated non-tg mice. In contrast, in vehicle-treated APP/α-syn tg mice, there was a modest subset of microglia with α-syn accumulation. Treatment with AD02 or PD-AFF1 increased the percent colocalization compared to vehicle-treated APP/α-syn tg mice. Treatment with AD02/PD-AFF1 further increased the percent colocalization compared to PD-AFF1 immunization in APP/α-syn tg mice. Statistical analysis was conducted using one-way ANOVA post hoc Dunnett's test for comparison with vehicle-treated APP/α-syn tg mice (*$P < .05$) and Tukey–Kramer test for comparing AD02/PD-AFF1 with PD-AFF1 immunizations (#P < .05). Scale bar for A and D = 40 mm, while scale bar for G 5 10 mm. Aβbreviation: APP, amyloid precursor protein.

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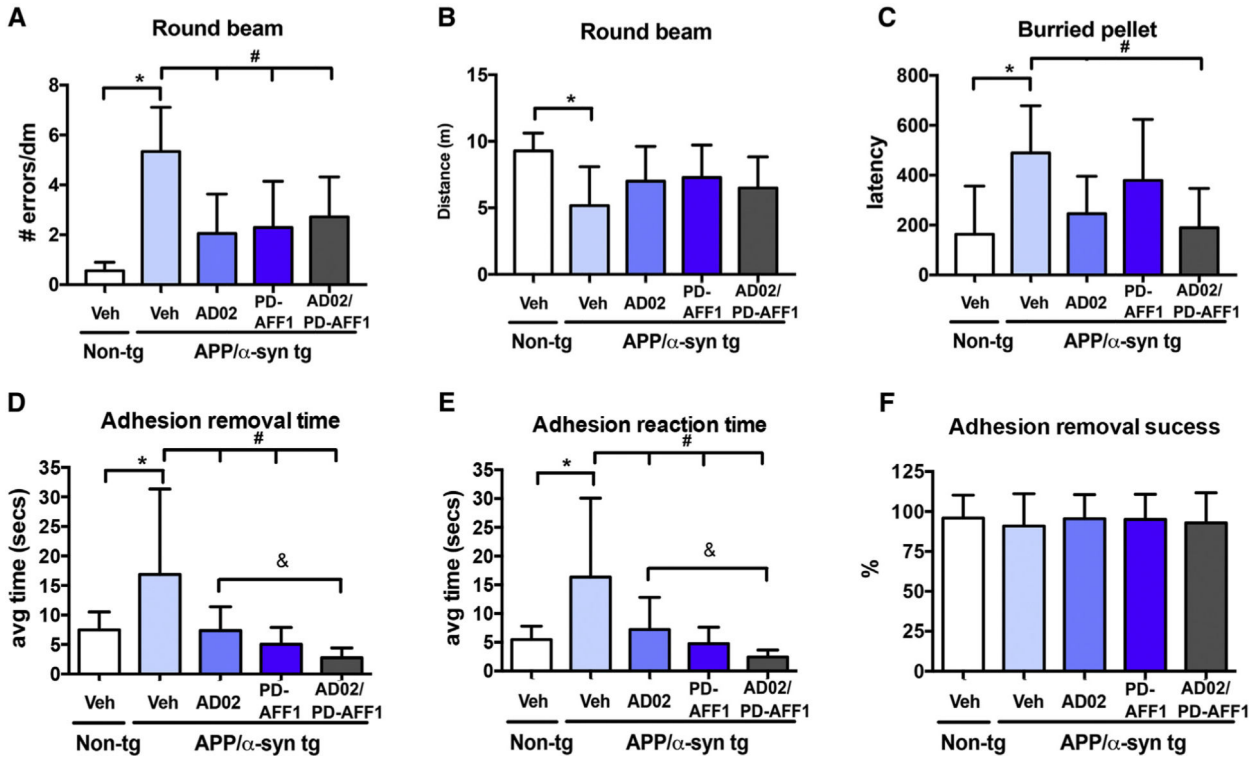

Fig. 6.

Effects of immunization on behavioral measures in APP/α-syn tg mice. APP/α-syn double transgenic mice and wild-type littermates (ALUM only) were subcutaneously injected 6 times with AD02 (n = 11), PD-AFF1 (n = 10), and a combination of both, AD02/PD-AFF1 (n = 9) adsorbed to aluminum hydroxide (ALUM) or with PBS-ALUM alone (n = 11 double tg mice, n = 12 wild-type littermates). (A) Motor testing in the round beam showed that at 10 months of age the vehicle-treated APP/α-syn tg mice had significantly more errors and traveled a significantly shorter distance compared to vehicle-treated non-tg mice. Immunization with AD02, PD-AFF1, or AD02/PD-AFF1 significantly decreased the number of errors in the APP/α-syn tg mice. (B) Distance traveled was significantly decreased in the vehicle-treated APP/α-syn tg mice, and immunization with AD02, PD-AFF1, or AD02/PD-AFF1 significantly increased the distance traveled. (C) Latency to find the buried pellet was significantly increased in vehicle-treated APP/α-syn tg mice, and immunization with AD02 or AD02/PD-AFF1 significantly decreased the amount of time for the mice to find the buried pellet. (D) Latency to remove the adhesive tape. (E) Adhesion reaction time was significantly increased in vehicle-treated APP/α-syn tg mice, and immunization with AD02, PD-AFF1, or AD02/PD-AFF1 significantly decreased the time to detach the adhesive, and the combination was better than the individual vaccine. (F) Adhesion removal success. Statistical analysis was conducted using one-way ANOVA post hoc Dunnett's test for comparing each group with vehicle-treated APP/α-syn tg mice (*P < .05) and Tukey–Kramer test for comparing vehicle-treated APP/α-syn tg versus AD02 and/or PD-AFF1 (#P <.05) or comparing single versus combination immunization (&P < .05).