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Improvement of memory deficits and A β clearance in aged APP23 mice treated with a combination of anti-A β antibody and LXR agonist

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Abstract

Passive A β vaccination has shown significant effects on amyloid pathology in pre-depositing APP mice but the results in older mice are inconsistent. A therapeutic effect of LXR and RXR agonists consisting of improved memory deficits and A β pathology has been demonstrated in different AD mouse models. Here, we report the effect of a combination of N-terminal A β antibody and synthetic LXR agonist T0901317 (T0) on AD-like phenotype of APP23 mice. To examine the therapeutic potential of this combination the treatment of mice started at 11 months of age, when amyloid phenotype in this model is fully developed, and continued for 50 days. We show that A β immunization with or without LXR agonist restored the performance of APP23 transgenic mice in two behavior paradigms without affecting the existing amyloid plaques. Importantly, we did not observe an increase of brain microhemorrhage which is considered a significant side effect of A β vaccination. Target engagement was confirmed by increased ABCA1 and APOE protein level as well as increased APOE lipidation in soluble brain extract. In interstitial fluid (ISF) obtained by microdialysis we demonstrate that immunization and T0 significantly reduced A β levels, indicating an increased A β clearance. We found no interaction between the immunotherapy and T0, suggesting no synergism, at least with these doses. The results of our study demonstrate that anti-A β treatments can ameliorate cognitive deficits in APP mice with advanced AD-like phenotype in conjunction with a decrease of A β in brain interstitium and increase of APOE lipidation without affecting the existing amyloid plaques.

Keywords

APP23 mice; A β immunization; LXR agonist; ApoE lipidation; Abca1; fear conditioning; radial water maze; microdialysis; interstitial fluid; amyloid plaques

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Introduction

Alzheimer's disease is a late-onset dementia characterized by the presence of amyloid β ($A\beta$) plaques, neurofibrillary tangles, and cognitive decline. According to amyloid cascade hypothesis $A\beta$ has a critical role in the pathogenesis of AD [1]. Therefore from a therapeutic point of view, $A\beta$ -targeted immunotherapy is considered one of the most promising approaches [2]. In animal models, $A\beta$ immunotherapy has shown significant effects on both amyloid pathology and cognition, however, human trials have been disappointing. In patients, immunized with $A\beta$ or treated with passive immunization via administration of various $A\beta$ -specific antibodies, $A\beta$ levels and plaques in brain were decreased. However, this did not prevent the progressive cognitive decline [3,4]. Important side effects of both passive and active $A\beta$ -immunization in patients were reversible vasogenic edema [4], meningoencephalitis, and brain microhemorrhage which led to suspension of some clinical trials [5]. The lack of effect on memory and the adverse effects in humans have not precluded further development of $A\beta$ -targeted therapeutic approaches, including immunization.

Liver X receptors (LXR) and Retinoic X Receptors (RXR) are ligand-activated transcription factors that act as permissive heterodimers. Agonists for LXR and RXR were shown to ameliorate amyloid pathology and improve cognitive decline [6–10]. The effect of LXR and RXR agonists on AD-like phenotype in model mice was attributed to the increased expression of *Abca1* and *ApoE* that collectively increase ApoE lipidation and stability, and consequently enhance $A\beta$ clearance [10–12].

In this study we tested the effect of a synthetic LXR agonist - T0901317 (T0) on the phenotype of human APP - transgenic mice immunized with anti- $A\beta$ antibody. We reasoned that T0 will further increase $A\beta$ clearance induced by passive immunization, ameliorate memory deficits and diminish the adverse side effects of the vaccination. In order to determine the effect of T0 on these adverse side effects we chose to work with the APP23 mouse model, which develop pronounced CAA, considered a predisposing factor for hemorrhages.

Materials and Methods

Unless documented otherwise, all reagents and plastics were purchased from Fisher Scientific.

Animals

Mouse Strains—The study fully conformed to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals and was approved by the University of Pittsburgh Institutional Animal Care and Use Committee. APP23 transgenic mice (C57BL/6 background) express human APP751 familial Swedish AD mutant (APPK670N, M671L), and the expression of the transgene is driven by the murine Thy-1 promoter, restricted to neurons [13]. Wild-type C57BL/6 mice were crossed to APP23 mice to generate APP23 hemizygous mice and wild-type littermates used in this study. For each experiment, 10–11 month old male and female mice were randomly assigned to the experimental groups. Age-

and gender matched non-transgenic littermates were used as controls for behavior experiments.

Treatment

Passive immunization—11 month old male and female APP23 mice were passively immunized weekly by intraperitoneal injections of 0.5 mg/per mouse of anti-A β mouse monoclonal HJ3.4 IgG antibody that recognizes mid N-terminal domain of A β . Anti-A β antibody was purified from the supernatant of a hybridoma cell line clone HJ3.4 (generously provided by Dr. Dave Holtzman) using Prepacked HiTrap column (GE Healthcare). Age- and gender-matched control APP23 mice received weekly intraperitoneal injections of 0.5 mg of a control mouse IgG.

LXR ligand treatment was performed as before [9] with minor modifications: LXR agonist T0901317 (T0) (Cayman Chemical) was dissolved in dimethyl sulfoxide (DMSO) as vehicle, mixed with double distilled water and milled control diet (LabDiet 5P76) was reconstituted to a concentration of 0.025% DMSO and 0.0175% T0. Consequently, the milled diets were divided into daily portions, briefly dried and stored at -20°C at which time animals were feed fresh food daily at a dose of 25 mg T0/ kg body weight. Chow for the vehicle-treated group was prepared likewise, containing only the vehicle DMSO.

All treatment continued for 50 days and at the end mice were subjected to behavior testing and immediately perfused at the average age of 12.2 mo.

Radial Arm Water Maze

Two day radial arm water maze (RWM) was used to measure the ability of mice to form a spatial relationship between a safe, but hidden platform and visual cues surrounding the maze [11]. The RWM consisted of six arms (20 cm wide, 40 cm long, 8 cm high walls above the water) and a central area (30 cm diameter), filled with water (temperature, $21 \pm 1^{\circ}\text{C}$) to a level 1 cm above the hidden platform (10 cm diameter). All animals were handled for 2 min for 2 consecutive days before behavioral testing. Acquisition testing was performed over 2 consecutive days with mice trained in groups of five or six. Each day, a mouse received two 6 trial blocks and a final 3 trial block (total of 15 trials per day) with a 30 min rest between blocks. During day 1 of training, a visible platform (flag projecting 6 cm from the platform) was used during trials 1, 3, 5, 7, 9, and 11 to define the rule of a safe platform. All other trials consisted of animals finding the location of a hidden platform. Animals were allowed 60 s to find the platform and 20 s to rest on it. Mice that failed to find the platform were led there by the experimenter and allowed to rest there for 20 s. All animals in a group completed the trial before proceeding, providing a 5 min inter-trial interval. The start location was changed for each trial and the platform location was changed between groups. Following acquisition phase, visible platform training was performed to measure swim speed, motivation, and visual acuity. Briefly, all arms and distal visual cues were removed and the platform was marked with a flag projecting 6 cm above the surface of the water. Testing then proceeded for 15 trials as documented for the acquisition trial except the position of the platform was moved while the start position remained constant throughout training. Performance was recorded with an automated tracking system

(AnyMaze; Stoelting) during all phases of training. During the acquisition phase, total number of incorrect arm entries and time errors were combined for the overall performance of an animal during a trial. An incorrect arm entry was defined as the entry of 50% of the animal's body into an arm that did not contain the hidden platform. A time error was defined as the failure of an animal to enter an arm after 15 s elapsed. For the 15 daily trials, performance during 3 consecutive trials was averaged into a block (total of 5 blocks per day). During the open pool task of training, speed and latency to the platform were used to compare the performance between genotypes.

Contextual fear conditioning

Contextual fear conditioning (Stoelting) was performed following the RWM as before [11]. Briefly, mice were placed in a conditioning chamber for 2 min before the onset of a tone [conditioned stimulus (CS), duration of 30 s, 85 dB sound at 2800 Hz]. In the last 2 s of the CS, mice were given a 2 s, 0.7 mA footshock through the bars on the floor of the chamber, and this cycle was repeated twice. Finally, the mice were allowed to remain in the chamber for 30 s before being returned to their housing cages. Contextual fear was evaluated 24 h after training by measuring freezing behavior for 5 min in the original chamber before mice were returned to their housing cages. Freezing behavior, defined as the absence of movement except for that needed for breathing, was scored using AnyMaze software (Stoelting). Cued fear learning was assessed 24 h after contextual testing by placing mice in a novel context (gray walls were replaced with black-and-white stripped walls) for 2 min, after which they were exposed to the CS for 3 min, and freezing behavior was measured. All chambers were cleaned between animals with 70% ethanol. Data are represented as percentage freezing during all stages of testing.

In vivo Microdialysis

For microdialysis experiments APP23 were treated with the same dose of HJ3.4 (5 mg/mouse), T0 (25 mg/kg/day) or a combination of both, while control mice received control mouse IgG. The duration of these treatments was 15 days. *In vivo* microdialysis to assess brain interstitial fluid (ISF) A β 40 and A β 42 in the hippocampus of awake, freely moving mice was performed as previously described [14–16]. Briefly, mice were anesthetized with Avertin (250 mg/kg, i.p.), head shaved, and placed into a stereotaxic frame (Lab Standard, Stoelting). An incision was made exposing the dorsal aspect of the skull and the skull leveled. A bore hole (0.75 mm) was made above the left hippocampus (coordinates: –3.1 mm from bregma, 2.5 lateral), as well as the right aspect of the skull for placement of an anchoring screw. An AtmosLM Guide Cannula (PEG-6, Eicom) was then stereotaxically inserted into the left hippocampal formation (dura mater –0.8 mm) and secured using binary dental cement. An AtmosLM Dummy Cannula (PED-6, Eicom) was then inserted into the guide cannula and secured with a plastic cap nut. The animal was removed from the stereotaxic frame and placed into a clean cage with access to food and water ad libitum. A 1,000 kDa MWCO membrane microdialysis probe (AtmosLM Microdialysis probe, PEP-6-4, Eicom) was conditioned with a 1 s dip in ethanol and connected to a push-pull *in vivo* microdialysis setup. The inlet-port of the microdialysis probe was connected to the push channel (CMA 402 Syringe Pump, CMA Microdialysis AB) of the system using Teflon tubing (inner diameter 0.1 mm) and the outlet-port was connected to the pull channel of the

system (ERP-10 peristaltic pump, Eicom) using FEP tubing (inner diameter 0.25 mm) through a liquid swivel (inner diameter 0.45 mm, Eicom). All tubing was flushed with water and blocked for 3 hr at a rate of 10 μ l/min with artificial CSF containing 0.15% BSA (aCSF) (in mM: 1.3 CaCl₂, 1.2 MgSO₄, 3 KCl, 0.4 KH₂PO₄, 25 NaHCO₃, 122 NaCl, pH 7.35) that was prepared on the day of use and filtered through a 0.1 μ m syringe filter. The setup was blocked with aCSF for another 60 min once the microdialysis probe was put on line. Mice were then briefly anesthetized with isoflurane, probes were inserted into the hippocampus through the guide cannula, the mouse fitted with a harness, and placed into an enclosure that allows for free movement (Instech). Following probe implantation, the flow rate was set at 10 μ l/min for 180 min to prevent clogging of the microdialysis membrane. To measure A β 40 and A β 42, microdialysis probes had a constant flow rate of 1.0 μ l/min and samples were collected every 90 min using a refrigerated fraction collector (CMA Microdialysis AB). The mice were kept under constant light conditions for the remainder of the experiment. The levels of A β 40 and A β 42 in each sample were determined by ELISA as described below. At the conclusion of the microdialysis experiment, animals were perfused as described below, and 30 μ m brain sections were stained with cresyl violet to confirm probe placement.

Animal Tissue Processing

Mice were anesthetized with Avertin (250 mg/kg of body weight, i.p.) and blood was drawn from the heart. [11]. The mice were perfused transcardially with 25 ml of cold 0.1 M PBS, pH 7.4. Brains were rapidly removed and divided into hemispheres, with one of the hemispheres being dissected into the cortex and hippocampus. These parts were snap-frozen on dry ice, while the other hemisphere was drop fixed in 4% phosphate-buffered paraformaldehyde at 4°C for 48 h before storage in 30% sucrose.

Histology and Immunohistochemistry

All procedures were as reported previously [11]. Histoprep-embedded hemibrains were cut in the coronal plane at 30 μ m sections and stored in a glycol-based cryoprotectant at -20°C until staining. Sections were selected 700 μ m apart, starting from a randomly chosen section ~150 μ m caudal to the first appearance of the CA3 and dentate gyrus.

X-34 staining: sections mounted on slides were washed in PBS for 10 min and stained with 1,4-bis(3-carboxy-4-hydroxyphenylethenyl)-benzene (X-34) (100 μ m) for 10 min. Following the staining, sections were washed in water, incubated in 0.2% NaOH in 80% unbuffered ethanol for 2 min, washed again in water, and soaked in PBS for 10 min. *6E10 staining:* another set of adjacent sections were immunostained with 6E10 antibody [11] (SIG-39340; Covance). Briefly, free-floating sections were blocked for endogenous peroxidases, avidin-biotin quenched, antigen retrieval was performed with 70% formic acid, and tissue was blocked with 3% normal goat serum. Sections were then incubated in 6E10 biotin-labeled antibody (1:1000) at room temperature for 2 h before being developed with Vectastain ABC Elite kit and a DAB substrate (Vector Laboratories). Microscopic examination was performed using a Nikon Eclipse 80i microscope (20 $\times$ magnification). For quantitative analysis, staining in the cortex and hippocampus was defined as the percentage area covered by X-34 or 6E10 positivity using MetaMorph 7.0 software (Molecular

Devices). Furthermore, the numbers of plaques in the cortex and hippocampus were manually counted and displayed as number of plaques per section.

Quantification of Cerebral Hemorrhage was performed on 30- μ m sections as before [17]. The sections were mounted on microscopic slides rinsed with PBS, incubated in Perl's solution, equal parts of 2% potassium ferrocyanide and 2% hydrochloric acid, and rinsed again. The sections were counterstained with Nuclear Fast Red (Vector Laboratories) for 5 min, rinsed with PBS, and coverslipped. All sections were scanned at 20 $\times$ and focal hemorrhages analyzed and counted at 100 $\times$. The numbers of Perl's Berlin blue-stained clusters of hemosiderin staining (or Prussian blue-positive sites) were counted on all sections, and the average number of sites per section was quantified. A set of five equally spaced sections throughout the neocortex, hippocampus, and thalamus were examined, and the number of positive profiles was determined and averaged to a per section value.

Western blotting and ELISA

Tissue homogenization—Frozen cortices and hippocampi were homogenized together [11] in tissue homogenization buffer (250 mM sucrose, 20 mM Tris base, 1 mM EDTA, 1 mM EGTA, 1 ml per 100 mg tissue) and protease inhibitors (10 μ g/ml leupeptin, 10 μ g/ml aprotinin and 10 μ g/ml AEBSF) with a glass dounce.

Soluble and insoluble brain fractions—An aliquot of the homogenate was spun at 100,000 $\times$ g for 1 hr and supernatant was assigned as “soluble brain fraction” and used to assess soluble A β and ApoE levels. To extract insoluble A β , the remaining pellet was resuspended in 70% formic acid, sonicated for 30 sec, and spun at 100,000 $\times$ g for 1 hr.

A β ELISA—ELISA was performed essentially as in [11]. Briefly, for A β ELISA 6E10 was used as the capture antibody and anti-A β 40 (G2-10 mAb) and anti-A β 42 (G2-13 mAb) monoclonal antibodies conjugated to horseradish peroxidase (Genetics Company) were used as detection antibodies. The final values of A β were based on comparison with A β 40 and A β 42 peptide standard curve (Bachem Biosciences) using linear regression analysis, normalized to total protein concentration in the sample, and expressed as pmoles/mg.

ELISA for mouse apoE—We used polyclonal anti-ApoE antibody from Calbiochem (#178479) as the capture and biotin-labeled polyclonal anti-ApoE antibody from Meridian (#K7401 B) as the detection antibody. The assay was developed with HRP-labeled streptavidin (Fitzgerald Industries International Inc.). The final values of apoE were based on comparison with serial dilution of normal mouse serum standard curve using linear regression analysis, normalized to total protein concentration in the sample, and expressed as ng/mg. ApoE knockout mouse was used as a negative control.

Western blotting (WB)—Extracts containing 50–100 μ g of total protein were mixed with Tris/glycine loading buffer, loaded, and electrophoresed on 10 % Tris/glycine gels (Invitrogen). Gels were transferred to nitrocellulose membranes, incubated with respective primary antibodies followed by secondary antibodies conjugated to horseradish peroxidase and processed for visualization by enhanced chemiluminescence Plus-ECL (PerkinElmer).

Native PAGE was performed as before [18] using a protocol provided by Dr. C. Wellington group with slight modification. Briefly, samples from ISF and TBS brain extract were mixed with 6× non-denaturing loading buffer and resolved on 6% non-denaturing Tris-HCl polyacrylamide gels. Wild type and ApoE serum samples were used as positive and negative controls respectively. Native protein standards from GE (#17-0445-01) were used as markers. As primary antibody we used polyclonal anti-ApoE antibody from Calbiochem (#178479) at 1:3,000 dilution.

RIPA extraction—To detect Abca1, ApoE, Carboxy terminal fragments of APP (CTF α/β) protein extracts were prepared by 1:1 dilution by volume of the initial homogenate with 2 X RIPA buffer in the presence of protease inhibitors and WB were performed as before [11]. Abca1 was detected using monoclonal antibody, ab7360 (Abeam), ApoE using M-20 polyclonal antibody (Santa Cruz Biotechnology), CTF β with polyclonal C8 antibody (provided by Matthias Staufenbiel, Novartis); β -Actin was used as a loading control for all WB and detected with monoclonal antibody, A5441 (Sigma). The relative band intensities were quantified by densitometry (Image Quant version 5.2, Molecular Dynamics).

A β Dimer ELISA was performed as in Esparza et.al [19] with modifications in the labeling of detection antibody. Briefly, unlabeled monoclonal antibody clone HJ3.4 was used as capture antibody at 10 μ g/ml to coat 96 well Nunc MaxiSorp plates (overnight at 4°C). HJ3.4 antibody was labeled with biotin (Innova Biosciences) and used as detection antibody (at a concentration 0.5 μ g/ml, incubated 4 hours at room temperature). The assay was developed with HRP-labeled streptavidin (1:15,000 for 1 hour at room t). The final values of A β dimer were based on comparison with standard curve of A β ₁₋₄₀Ser26Cys dimer using linear regression analysis, normalized to total protein concentration in the sample, and expressed as ng/mg. To determine the specificity of the assay we used A β 40 monomer disaggregated as we have shown previously [20], dissolved in the same buffer as A β dimer. Because the combination of unlabeled HJ3.4/ biotin-labeled HJ3.4 did not recognize A β 40 monomer (in contrast to the standard ELISA described above) we conclude that our dimer ELISA is specific for A β dimer.

Oligomer standard was prepared as previously published [21] with modifications. Briefly, synthetic A β ₁₋₄₀Ser26Cys was purchased from Keck Biotechnology Center (Yale University, New Haven, CT). A β ₁₋₄₀Ser26Cys was purified by reverse phase HPLC and various fractions were analyzed for purity by LC-MS. Pure fractions with correct mass were pooled and the solution (acetonitrile/water/TFA) pH was increased to 9.0 by addition of sodium hydroxide. To this solution 10 μ M copper chloride was added to accelerate disulfide formation. Aliquots were analyzed by LC-MS at regular time points. Homo-dimers of A β were purified by reverse phase HPLC to remove unmodified peptide and copper chloride. Pure dimer peptide fractions were pooled, lyophilized and used in subsequent experiments. A β dimer was dissolved in Guanidinium HCl and passed through Superdex 200 (GE health Sciences) equilibrated with PBS. A β dimers elute as oligomers under these conditions.

Protein Concentration—Bradford assay was used to determine protein concentrations of all samples. Briefly diluted sample was combined 1:1 by volume with 40% Bradford reagent dye (Bio-Rad) and absorbance at 595 nm was read on a microplate reader. Concentration

was determined by comparison to bovine serum albumin standard curve using linear regression analysis.

Statistical Analysis

Results were reported as means $\pm$ S.E.M. Statistical significance of differences between mean scores during acquisition phase of training in the RWM were assessed with two-way repeated measures ANOVA (General Linear Model/RM-ANOVA) and Tukey post hoc test for multiple comparisons using Genotype/Treatment and Trial Block Number as sources of variation. For the rest of the results statistical significance of differences between the means were assessed using one-way analysis of variance (one-way ANOVA) and Dunnett's post-test, and *t*-test. To examine the interaction between anti-A β and LXR treatment we used two-way ANOVA. All statistical analyses were performed in GraphPad Prism version 4.0 and differences considered significant when $p < 0.05$.

Results

Passive A β immunization and LXR treatment restore cognitive performance in APP23 mice

To examine if an LXR agonist adds to the effect of passive immunization, 11 month old APP23 mice were passively immunized with N-terminal anti-A β antibody clone HJ3.4 plus or minus LXR ligand T0901317 (T0). We specifically chose mice at this age when AD phenotype is fully developed and the goal was to examine the therapeutic potential of the combined treatment. Control mice were treated with the same dose of mouse IgG plus or minus T0. APP23 mice were dosed for 50 days and at the end were subjected to behavior testing. As shown on Fig. 1A, contextual fear conditioning paradigm demonstrated that an immunization plus or minus LXR agonist were efficient in restoring the performance to level of non-transgenic mice (one-way ANOVA, $p < 0.05$). To determine if there was an interaction between HJ3.4 antibody and LXR treatments the data were analyzed by two-way ANOVA. The results were tested for interaction by two-way ANOVA and we found that there was an interaction between these two variables with a significant single effect of LXR but not of immunization. Cued test showed no statistical difference in the performance of the mice. It is well known that in rodents, amygdala damage severely impairs both cued and context fear conditioning [22], whereas lesions of the hippocampus impair context conditioning but have little effect on cued conditioning [23–25]. The fact that hippocampus-lesioned animals can still learn during the contextual phase suggests that the hippocampus' involvement in the acquisition of the context-foot shock association [26]. The results from two-way ANOVA for cued testing demonstrate that there is no interaction between HJ3.4 antibody application and LXR treatment but there was a significant main effect of LXR.

The same mice were tested in RWM paradigm which confirmed that HJ3.4 antibody and LXR agonist improved spatial memory deficits in a statistically significant manner. Analysis by two-way repeated measures ANOVA shows that there is a significant main effect of treatment (Fig. 1C). The performance on the last trial block of RWM (Fig. 1D) demonstrates that anti-A β treatment and LXR restored memory to the level of non-transgenic controls ($p < 0.01$). The conclusion from these experiments is that passive immunization and LXR

treatment are equally effective in restoring memory deficits when applied alone with no additive effect when used in combination.

Amyloid plaques are unaffected by A β immunization or LXR agonist

Following the behavior testing amyloid pathology was examined in the same mice. First, to visualize the compact amyloid plaques we used X-34 staining. Fig. 2 demonstrates that neither treatment affected % area (Fig. 2A and B) or number of the compact amyloid plaques (Fig. 2C and D) in hippocampus and cortex. As seen from panels A and C, LXR treatment showed a strong trend toward decrease in hippocampus but the difference was insignificant. The data were also examined by two-way ANOVA and we did not observe an interaction or main effects of any of the treatments.

We used anti-A β staining with 6E10 antibody to assess an effect on the level of diffuse plaques in hippocampus and cortex (Fig. 2E and F). We observed that LXR caused a strong trend towards a decrease of % plaques area in hippocampus (panel E) and cortex (panel F) but the difference was insignificant. Analysis by two-way ANOVA for % plaques area demonstrated that there was no interaction between the treatments however there was a significant main effect of LXR ligand in hippocampus (see figure legend). Similar effect of T0 on A β plaques in the hippocampus but not in the cortex was previously reported by Riddell et al. [27].

Because the passive anti-A β immunotherapy reportedly increased the microhemorrhages in the brain, we used Prussian Blue staining and counted the number of microhemorrhages in our mice. The data demonstrate that there was no difference between the number of hemorrhages when A β -immunized (0.2 ± 0.12) or to A β +T0 treated mice (0.3 ± 0.16) were compared to controls (0.21 ± 0.16).

A β immunization or LXR agonist do not affect soluble and insoluble A β

To determine the level of soluble and insoluble amyloid, A β was extracted from the cortices and hippocampi first with TBS followed by formic acid. As shown on Fig. 3A and B, there was no change in the levels of insoluble and soluble A β . Furthermore, there was no interaction between the treatments. Because A β oligomers are reported to affect memory we also measured their level using dimer specific ELISA as recently published by Esparza et al. [19]. As shown on Fig. 3C there was no statistical difference between the mean levels of soluble A β oligomers in brain parenchyma. Furthermore, we did not find an interaction between treatments when the data were re-analyzed by two-way ANOVA.

LXR agonist treatment increases ABCA1 level and APOE lipidation in the brain of treated mice

Target engagement was confirmed by examining protein expression level of known LXR targets - ABCA1 and APOE. As shown on Fig. 4A and D, T0 treatment significantly increased ABCA1 level in anti-A β +LXR and LXR groups. The level of APOE was measured by ELISA (Fig. 4B) and was increased significantly only in mice treated with T0 (anti-A β +LXR and LXR groups).

We also determined that there is no effect on APP processing by examining carboxy-terminal fragments (CTF α/β), products of α - or β -secretase. As shown on Fig. 4C and a representative picture on Fig. 4D, neither treatment affected the level of APP proteolytic fragments.

Finally we examined the level of ApoE lipidation using soluble brain extract (TBS extraction) run on a native PAGE as before [9]. As shown on Fig. 4E LXR agonist treatment increased ApoE lipidation in comparison with IgG and anti-A β treatments. On the left side of the picture is shown soluble brain extract from ApoE^{ko} mice as negative control. In conclusion, LXR agonist increases the level of Abca1 and ApoE proteins as well as ApoE lipidation.

Immunization and T0 treatment reduce A β level in interstitial fluid

To determine the level of A β in interstitial fluid of the brain groups of mice underwent the same dose of each treatment as described above but for a shorter time: 15 instead of 50 days. Interstitial fluid was obtained by microdialysis of freely moving mice as in our previous studies. For these measurements we used microdialysis probe with a larger pore-sized membrane (1000 kD) which allows higher molecular weight A β oligomers to enter the perfusate. As shown on Fig. 5A and B, immunization alone or in combination with LXR agonist decreased the level of A β 40 and A β 42, however, the difference was statistically significant only for A β 42. As shown on Fig. 5B the combination and the single treatments significantly decreased A β 42 level. We used two-way ANOVA to determine if there was an interaction between immunization and LXR agonist treatment. We found that there is no interaction between immunization and T0 treatments regarding A β 40 and A β 42 levels. However, for A β 42 there was significant single effect of A β immunization [F(1,7)=6.42, p=0.034] and T0 ligand [F(1,7)=23.56, p=0.0018].

We also examined the levels of A β -dodecamer (or so called A β *) in ISF using Western blotting as before [28, 29]. Our data suggest that neither treatment affected ISF A β * (data not shown). Furthermore, we also measured the level of A β using dimer-specific ELISA and as shown on Fig. 5C neither treatment affected A β dimer level.

Lastly, since LXR [9, 30, 31] and RXR ligands [8, 16] increase APOE lipidation, we examined the lipidation state of APOE in ISF using native gel electrophoresis performed as in Fitz et al [9], and in brain homogenates as in the previous section. We revealed that both single T0 and the combination of T0 and anti-A β immunization increased APOE-native complexes in ISF (Fig. 5D). On the left side of the picture is shown mouse serum for comparison. As visible, ApoE complexes in serum migrate slightly higher than ApoE native complexes in ISF that is consistent with the higher level of lipidation in periphery. The conclusion from the native gel shown on Fig. 5D is that both single T0 and the combination of T0+anti-A β immunization increased ApoE-native complexes in ISF.

Discussion

Passive and active A β immunization is the most promising treatment for AD. However, as discussed in a recent review [32], at present there are considerable challenges. For active A β

vaccination the weakened immune state of the elderly should be taken into consideration due to increased risk of autoimmune reactions [32]. The most significant obstacles for the passive immunization are the limited penetrance of antibodies into the brain and the increased side effects such as brain microhemorrhages that led to the termination of previous clinical trials. Furthermore, it is not entirely understood how the passive immunizations clears A β plaques. It has been suggested that the antibodies enter the brain and can activate A β phagocytosis by microglia. However, the concentration of anti-A β antibodies in the brain is usually less than 0.1 % of their plasma level [32] suggesting that other mechanisms are possible. One such mechanism is the so called “peripheral sink” by which A β is cleared through BBB because of a brain to plasma gradient. However, N-terminal anti-A β antibodies such as the one used in this study were reported to increase microhemorrhages particularly in old mice with considerable CAA like APP23[33]. This suggests that clearance apparatus via BBB can be easily jammed especially in old mice or AD patients with higher amyloid load leading to deleterious side effects. Thus a combination of A β immunization with another drug[34] or a small molecule[35] could be an effective way to improve the positive outcome and decrease side effects.

Here for the first time we examine the therapeutic efficacy of the combination of passive A β immunization and LXR agonist, T0, on the phenotype of aged APP23 mice. The assumption behind this approach is that T0 acts synergistically with A β immunization to improve A β clearance through BBB and will decrease microhemorrhages. We show that A β immunization with or without LXR agonist does not affect parenchymal amyloid in APP23 mice. This result is not surprising for several reasons. First we used older mice with already developed amyloid pathology and second, our treatment continued for 50 days instead of longer treatments reported in most studies. Passive immunization with anti-A β antibodies has been reported to decrease amyloid plaques in mice when applied at an early age before overt amyloid phenotype was developed[36]. However, this approach is mostly ineffective in removing already existing plaques[37, 38]. Importantly, we report that there was no increase of brain microhemorrhages after the vaccination. The lack of effect on microhemorrhages after the vaccination could be explained by T0 increasing A β degradation inside the brain by phagocytosis or extracellular proteases. Thus by increased A β degradation inside the brain LXR agonists can at least in part re-route some of A β directed to pass BBB. Previous data from our and other groups demonstrated that LXR ligands increase A β degradation by microglia[7, 9, 31].

Furthermore, our data demonstrate that even though there was no effect on amyloid plaques, anti-A β antibody and LXR ligand treatments improved significantly the performance in two behavior tests (see Fig. 1). There is a growing body of evidence that cognitive deficits correlate consistently with the level of soluble A β in the brain (reviewed in [39, 40]). We have recently showed that RXR agonist, bexarotene, improved memory deficits in APP mice without affecting amyloid plaques but in correlation with soluble oligomers as identified by oligomer-specific A11 antibody [10].

In the current study we observed a decrease in ISF A β 40 and A β 42 measured by ELISA following treatment with anti-A β and T0 (Fig. 5A and B). However, when interstitial A β was re-examined using an ELISA for A β dimer there was no difference among the groups

(Fig. 5C). It should be noted that there are numerous A β oligomers, detected by different approaches, and there is no definite answer which oligomeric species causes memory deficits. In addition, we did not see any monomers or low molecular A β species on Western blotting performed on ISF of APP23 mice at this age (data not shown). This suggests that our ELISA for A β 40 and A β 42 measures not only monomers but oligomers as well. This result is in agreement with a recent study by Takeda et al. that demonstrated that in older mice there are very little mono-meric and low molecular weight oligomeric A β species in ISF[15]. Another possibility is that in older mice A β oligomers in ISF are more resistant to clearance mechanism in contrast to the younger mice used in our previous studies[9, 10].

We attribute improvement in performance on the behavior test to the diminished levels of ISF A β following both immunization and T0 treatment. In terms of T0, we postulate that molecular mechanism for the effects of LXR and RXR agonists is the increased ApoE lipidation that affects ApoE binding to A β thus preventing its aggregation. As shown on Figures 4E and 5C ApoE lipidation was increased by T0 in the soluble brain fraction and ISF. Thus, by keeping A β in a soluble state ApoE facilitates A β efflux via BBB or its degradation in the brain as recently discussed in Tai et al.[41]. In support of this hypothesis are recent data with LXR and RXR ligands that demonstrated that increasing ApoE level by LXR or RXR agonists decreases A β level in ISF [8–10, 16]. Furthermore, the lack of Abca1 decreases ApoE lipidation and increases A β deposition [17, 42, 43], A β oligomers [28] and cognitive deficits [11, 28]. Furthermore, it has been suggested that ApoE lipidation is important for A β phagocytosis and A β degradation by extracellular proteases [7, 8, 16, 44].

We hypothesize that increased level of lipidated ApoE will prevent the formation of A β fibrils or larger oligomers and will increase the effect of passive immunization on A β clearance inside the brain or its efflux via BBB. The lack of side effects and the improvement of cognitive deficits are encouraging even though the present study did not show a synergistic effect of the combination.

In conclusion, our study demonstrates that passive A β vaccination and LXR agonist can ameliorate cognitive deficits in mice with advanced AD-like phenotype without affecting the existing amyloid plaques or insoluble amyloid. We also observed an increase of ApoE lipidation in the soluble brain fraction and a significant reduction of soluble A β in the brain interstitium. Importantly, there was also no increase in the side effects with regards to brain microhemorrhages. Future studies with longer duration of treatments and different time points in the progression of amyloid pathology are needed to explore the effect of LXR agonists in this or similar settings.

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Figure 1. Immunization with HJ3.4 and LXR agonist rescue memory deficits in APP23 mice
 11 month old APP23 mice were immunized with HJ3.4 antibody plus/minus LXR agonist T0 for 50 days and cognitive function was assessed with contextual fear conditioning and radial arm water maze (RWM). **A**, Contextual fear conditioning test demonstrates that treatments were efficient in improving deficits. Analysis is by one-way ANOVA, $p < 0.05$, Dunnett post-test *, $p < 0.05$ versus control IgG. Analysis by two-way ANOVA showed that there is an interaction between HJ3.4 antibody and LXR treatment $F(1,38)=5.96$, $p=0.03$. **B**, Cued test shows no statistical difference in performance. Two-way ANOVA analysis shows that there is no interaction between HJ3.4 antibody and LXR treatment $F(1,35)=3.37$, $p=0.06$. There was a significant main effect of LXR $F(1,35)=5.21$, $p=0.028$ but not of anti-A β . **C**, In RWM test treatment with HJ3.4 antibody and LXR agonist improved spatial memory deficits. Analysis by two-way repeated measures ANOVA shows no interaction between trial block and treatment $F(36,477)=0.55$, $p=0.98$. There was a significant main effects of treatment $F(4,53)=2.64$, $p=0.044$ and trial block $F(9,477)=22.03$, $p < 0.0001$. **D**, shows the performance on the last trial block of RWM demonstrating that anti-A β and LXR

restored memory to the level of non-transgenic control. Analysis is by one-way ANOVA, $p < 0.01$. Dunnett post-test *, $p < 0.05$ and **, $p < 0.01$ vs IgG treated mice. N=6–13 male and female mice per group for both experiments.

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Figure 2. A β immunization and LXR agonist treatment did not affect amyloid plaque level and insoluble amyloid in depositing APP23 mice

Amyloid pathology was assessed in APP23 mice immunized with HJ3.4 antibody plus/minus LXR agonist T0 treated for 50 days. **A, B, C and D, X-34 staining** was used to assess amyloid plaques in hippocampus (panel A and C) and cortex (panel B and D). On A and B, are presented plaques levels as % X-34 positive area and on C and D, are presented number of X-34 positive plaques. Means were analyzed by one-way ANOVA, no statistical significance for A, B, C and D. Two-way ANOVA showed no interaction and no significant

main effects in hippocampus or cortex for A, B, C and D. **E and F, 6E10 staining** was used to assess diffuse amyloid plaques in hippocampus (panel E) and cortex (panel F). On panels E and F are presented 6E10 plaques as % positive area. Means were analyzed by one-way ANOVA and there was no significant difference between treatments and control group. Two-way ANOVA showed no interaction in hippocampus and cortex, however there was a significant main effect of LXR in hippocampus [$F(1,40)=4.41$, $p < 0.05$]. For all panels $N=6-13$ mice per group.

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Figure 3. Soluble and insoluble Aβ is not affected by Aβ immunization and LXR agonist treatment

Soluble Aβ was extracted from cortexes and hippocampi by TBS followed by extraction of insoluble using formic acid, and Aβ was evaluated by ELISA. On panel **A** is shown insoluble Aβ40 and Aβ42 and on panel **B**, soluble Aβ40 and Aβ42. **C**, The level of Aβ oligomers was measured in soluble fraction using dimer-specific ELISA as described in the methods. For all panels, difference between the means were analyzed by one-way ANOVA and showed no significant change and two-way ANOVA demonstrated no interaction and no significant main effects of LXR or anti- Aβ treatments. N=6–14 male and female mice per group.

Figure 4. LXR treatment increases Abca1 and ApoE lipidation in the brain of treated mice
A, Abca1 level was measure by WB of RIPA extracts from cortex and hippocampus of treated mice. N=6 mice per group. Analysis is by one-way ANOVA, $p < 0.01$, Dunnett's post-test **, $p < 0.01$ versus control IgG. **B**, ApoE level was measured in soluble extract of cortex and hippocampus by ELISA as described in the methods. N=4–14 mice per group. One-way ANOVA, $p < 0.001$, Dunnett's post-test **, $p < 0.01$ and *, $p < 0.05$ versus control IgG. **C**, CTF α/β were measured on WB in RIPA extracts from cortex and hippocampus of treated mice. Analysis is by one-way ANOVA shows no significance, N=6 mice per group.

D, Shown are representative pictures for Abca1 and CTF α/β WB. N.S. is non-specific band.
E, Native gel electrophoresis demonstrated an increase of ApoE lipidation in TBS soluble brain extract. Native standards are shown on the left. ApoEko brain extract is shown as negative control.

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Figure 5. Immunization and LXR agonist decrease Aβ level in ISF and increases ApoE lipidation APP23 mice were treated with HJ3.4 antibody plus/minus LXR agonist T0 for 15 days. ISF was recovered by microdialysis and Aβ level determined by ELISA as described in the text. **A**, Aβ40 and **B**, Aβ42. Means were compared by one-way ANOVA and Dunnett post-test. * < 0.05 and **, p < 0.01 versus IgG. Two-way ANOVA analysis of Aβ42 levels demonstrates that there is no interaction between immunization and T0 and significant single effects of anti- Aβ F(1,7)=6.42, p=0.034 and T0 F(1,7)=.23.56, p=0.0018. **C**, The level of Aβ dimers was measured by dimer specific ELISA as described in the methods. For

A, B and C, N=3 mice per group. N.S. is non-significant. **D**, Native gel electrophoresis demonstrated an increase of ApoE lipidation in ISF after T0 treatment. For comparison, on the left is shown 1 μ J wild type mouse sera exposed for a shorter time. For all panels N=3 mice per group. N.S. is non-significant.

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