Robust amyloid clearance in a mouse model of AD provides novel insights into the mechanism of Aβ immunotherapy

Allan Wang1,2, Pritam Das6, Robert C. Switzer III7, Todd E. Golde8, and Joanna L. Jankowsky2,3,4,5

1Department of Psychology, Rice University, Houston, TX
2Department of Neuroscience, Baylor College of Medicine, Houston, TX
3Department of Neurosurgery, Baylor College of Medicine, Houston, TX
4Department of Neurology, Baylor College of Medicine, Houston, TX
5Department of Huffington Center on Aging, Baylor College of Medicine, Houston, TX
6Department of Neuroscience, Mayo Clinic Florida, Jacksonville, FL
7NeuroScience Associates, Knoxville, TN
8Department of Neuroscience, McKnight Brain Institute, Center for Translational Research in Neurodegenerative Disease, University of Florida, Gainesville, FL.

Abstract

Many new therapeutics for Alzheimer’s disease delay the accumulation of Aβ in transgenic mice, but evidence for clearance of pre-existing plaques is often lacking. Here we demonstrate that anti-Aβ immunotherapy combined with suppression of Aβ synthesis allows significant removal of antecedent deposits. We treated amyloid-bearing tet-off APP mice with doxycycline to suppress transgenic Aβ production before initiating a 12 week course of passive immunization. Animals remained on doxycycline for 3 months afterwards to assess whether improvements attained during combined treatment could be maintained by monotherapy. This strategy reduced amyloid load by 52% and Aβ42 content by 28% relative to pre-treatment levels, with preferential clearance of small deposits and diffuse Aβ surrounding fibrillar cores. We demonstrate that peripherally administered anti-Aβ antibody crossed the blood-brain barrier, bound to plaques, and was still be found associated with a subset of amyloid deposits many months after the final injection. Antibody accessed the brain independent of plasma Aβ levels, where it enhanced microglial internalization of aggregated Aβ. Our data support a mechanism by which passive immunization acts centrally to stimulate microglial phagocytosis of aggregated Aβ, but is opposed by the continued aggregation of newly secreted Aβ. By arresting the production of Aβ, combination therapy allows microglial clearance to work from a static amyloid burden towards a significant reduction in plaque load. Our findings suggest that combining two therapeutic approaches currently in clinical trials may improve neuropathological outcome over either alone.

Keywords

Alzheimer’s disease; amyloid; immunotherapy; transgenic mouse; microglia; tetracycline transactivator
Introduction

The amyloid hypothesis for Alzheimer’s disease predicts that slowing the accumulation of Aβ before damage ensues will slow the progression of cognitive decline (Hardy and Higgins, 1992; Hardy, 2009). Given this, considerable effort has been devoted to developing drugs that reduce production of Aβ or enhance its clearance from the brain. Patients with AD carry extensive amyloid neuropathology (Forsberg et al., 2008; Okello et al., 2009; Wolk et al., 2009), thus therapies aimed at Aβ may be most successful if they can remove pre-existing deposits. While many studies have documented the functional consequences of exposure to soluble Aβ aggregates (Ondrejcak et al., 2009; Shankar and Walsh, 2009; Palop and Mucke, 2010), their insoluble aftermath is not benign. Amyloid deposits cause physical and functional changes in local neurons that impact long-range communication in the brain (Le et al., 2001; D’Amore et al., 2003; Stern et al., 2004; Tsai et al., 2004; Busche et al., 2008; Kuchibhotla et al., 2008; Meyer-Luehmann et al., 2008). Plaque formation also incites a dramatic immune response which may further contribute to neuronal dysfunction (Jucker and Heppner, 2008). Thus, an ideal treatment for AD would not only prevent further Aβ aggregation, but also clear pre-existing amyloid.

Multiple anti-Aβ therapeutics are currently in development or clinical trials. Drugs designed to decrease Aβ production include β and γ-secretase inhibitors, γ-secretase modulators, and α-secretase activators (De Strooper et al., 2010) Aggregation inhibitors and plaque busters are being tested to improve Aβ clearance (Golde et al., 2010). Considerable effort has focused on inactivating Aβ through immunotherapy by active vaccination against the peptide or passive immunization with anti-Aβ antibodies (Brody and Holtzman, 2008). Each of these approaches attenuates the progression of amyloid formation in APP transgenic mice (Abramowski et al., 2008; Garcia-Alloza et al., 2009). However, only direct intracranial delivery of anti-Aβ antibody – a route of administration unlikely to be used in a clinical setting - has been definitively shown to clear pre-existing deposits (Bacsikai et al., 2001; Wilcock et al., 2003; Oddo et al., 2004; Brendza et al., 2005; Maeda et al., 2007).

Pre-clinical studies have not yet evaluated the possibility of combining multiple anti-Aβ therapies in the treatment of AD. Combination treatments have become the standard of care for several diseases, including HIV/AIDS and cancer. We have previously shown that two models of monotherapy now in clinical trials – suppression of Aβ synthesis (Jankowsky et al., 2005) and passive immunization with anti-Aβ antibodies (Levites et al., 2006a; Levites et al., 2006b) - could arrest but not reverse amyloid pathology in transgenic mice. We reasoned that reducing the production of Aβ prior to passive antibody transfer might allow one treatment to improve efficacy of the other. We now demonstrate that this combination approach results in significant amyloid clearance from animals harboring substantial preexisting pathology.

Materials and Methods

Mice

Two tet-responsive APP transgenic lines, tetO-APPswe/ind lines 102 and 107, over-expressing similar levels of APP were used for study (Jackson Laboratories #7051 and 7052 (Jankowsky et al., 2005)). Each line was mated to CaMKIIα-tTA line B (Jackson Laboratories #3010 (Mayford et al., 1996)) and resulting double transgenic male offspring were mated with wild-type females (C57BL/6J for line 102 and F1 C3B6 for line 107) to produce experimental cohorts.
**Doxycycline administration**

Long-term treatment at 6 mo (line 107): Doxycycline (dox) treatment was started at 6 mo of age and continued until harvest either 2 wk or 6 mo later. Dox was administered via chow containing 200 mg/kg of antibiotic (#S3888 or #F4845; BioServ, Frenchtown, NJ)

Long-term treatment at 18 mo (line 107)—Animals were reared on dox by feeding medicated chow to mating pairs. Offspring were maintained on dox until 12 mo of age, then switched to un-medicated chow to induce transgene expression for 6 mo before being returned to dox from 18 mo until harvest.

Short-term treatment at 7 mo (line 102)—Dox was started at 7 mo of age and continued until harvest 3.5 wk later. Because line 102 is more responsive to dox than line 107, chow formulation was reduced to 50 mg/kg antibiotic (#F5903; BioServ).

**Antibody injections**

Two weeks after starting on dox, mice received the first of 2 or 12 weekly i.p. injections containing 500 μg of mouse IgG, either as purified anti-Aβ1-16 monoclonal IgG2a antibody Ab9 (Levites et al., 2006b) or as non-specific mouse IgG (#SLM66, Equitech Bio, Kerrville, TX.).

**Tissue Harvest**

Animals were perfused with PBS, and the isolated brains hemisected along the midline. One hemisphere was frozen for Aβ analysis; the other hemisphere was immersion-fixed for 48 hr at 4° C in 4% paraformaldehyde.

**Plasma Sampling**

Whole blood samples were collected by submandibular bleed prior to treatment and again after 2 wk on either dox or regular chow. Blood was harvested into EDTA-charged tubes and plasma isolated by centrifugation.

**ELISA**

Hemi-forebrain samples were prepared for analysis of Aβ levels by two-step sequential extraction using 2% SDS followed by 70% formic acid as previously described (Jankowsky et al., 2007). Plasma samples were used neat. Aβ levels were determined by end-specific sandwich ELISAs using mAb 2.1.3 for capture (human Aβx-42 specific) and HRP-conjugated mAb Ab9 (human Aβ1–16 specific) for detection, or mAb Ab9 for capture and HRP-conjugated mAb 13.1.1 (human Aβx-40 specific) for detection (Levites et al., 2006a; Levites et al., 2006b). All values were calculated as pmol per g based on the initial weight of brain tissue. Serum concentrations were baseline corrected by subtracting the average non-specific signal detected in TTA single transgenic mice.

**Immunoblotting**

The 2% SDS homogenates prepared for ELISA were diluted 1:1 with 2x-concentrated high-detergent RIPA buffer minus SDS (2xPBS, 1% deoxycholate, 1% NP40, 5 mM EDTA, plus protease inhibitors). Approx 25 μg of the resulting homogenate was separated on a 10.5-14% Trisglycine gel (BioRad Criterion) and transferred to nitrocellulose. The upper part of the blot was probed with anti-human APP/Aβ antibody 6E10 (1:5000, Signet #9300-02), and the lower part with an anti-SOD1 polyclonal (1:2500, Assay Designs #SOD1). Binding was detected with HRP-labeled secondary antibodies, and developed with
ECL reagent. Chemiluminescence was measured with a Fuji LAS-4000 mini CCD system and quantified using Multi Gauge software.

**Histology**

Immersion-fixed hemibrains were cryoprotected, frozen, and sectioned at 35 μm. Brains were either cut individually in the sagittal plane or embedded 32 per block in a solid matrix and sectioned coronally (MultiBrain™ processing by NeuroScience Associates, Knoxville, TN). Sections were stored in cryoprotectant at -20°C until use.

**Campbell-Switzer Silver Stain**—A detailed protocol for this stain can be found online at the NeuroScience Associates website http://www.neuroscienceassociates.com/Documents/Publications/campbell-switzer_protocol.htm

**Thioflavine-S**—Guntern-modifed thioflavine-S staining was performed as described (Jankowsky et al., 2007).

**Perls Prussian blue**—Sections were placed in a freshly prepared mixture of 2% HCl and 2% potassium ferrocyanide solution for 30 minutes at room temperature, refrigerated for 1 hour, rinsed in water, mounted, dehydrated, and coverslipped.

**Immunohistochemistry (Iba-1, CD68)**—Sections were rinsed, treated with 0.9% hydrogen peroxide in TBST (TBS + 0.02% Triton-X), and blocked with TBST plus 1.5% goat serum before overnight incubation at room temperature with primary antibody diluted in TBST (Wako Rb anti-Iba1 #019-19741, 1:15,000 or Serotec biotinylated Rt anti-CD68 #MCA1957BT, 1:400). Binding was detected with biotinconjugated secondary antibodies followed by HRP-avidin and developed with DAB. A subset of the CD68-immunostained sections were co-stained with 0.25% congo red (Sigma #C6277) and counterstained with 0.01% hematoxylin.

**Immunofluorescence (Ms IgG, Ms IgG1, Ms IgG2a, 4G8, Iba-1).**

**Ms IgG:** Sections were rinsed, blocked in TBST plus 5% donkey serum, then incubated at room temperature for 2 hr in Alexa Fluor 568-conjugated Dk anti-Ms IgG (Invitrogen #A10037) diluted 1:200 in block.

**Ms IgG + Iba-1 +Thioflavine-S:** Sections were rinsed and blocked as above, then incubated overnight at 4°C with Rb anti-Iba-1 antibody diluted in blocking solution (Wako Rb anti-Iba-1 #019-19741, 1:500). After rinsing with TBS, sections were incubated for 2 hr at room temperature with Alexa Fluor-conjugated secondary antibodies diluted in blocking solution (Invitrogen Dk anti-Rb Alexa 647 #A31573, 1:400; Dk anti-Ms IgG Alexa 568, 1:200). Sections were rinsed in TBS, then incubated in 0.002% Thioflavine-S / 1x TBS for 8 min, followed by brief rinses in 50% ethanol.

**Ms IgG1 + Ms IgG2a:** Sections were rinsed, blocked in TBST plus 5% donkey serum and 1% goat serum, then incubated at room temperature for 2 hr in Alexa Fluor 488-conjugated Gt anti-Ms IgG1 (Invitrogen #A21121) and Alexa Fluor 568-conjugated Gt anti-Ms IgG2a (Invitrogen #A21134) each diluted 1:400 in blocking solution.

**4G8 + Iba-1:** Sections were incubated for 1 min in 80% formic acid, rinsed in TBS, then blocked in TBST plus 5% goat serum before overnight incubation at 4°C with primary antibody diluted in blocking solution (Signet Ms anti-Aβ clone 4G8 #9220-05, 1:1000;
Wako Rb anti-Iba-1, 1:500). Binding was detected with Alexa Fluor-conjugated secondary antibodies (Invitrogen Gt anti-Ms Alexa 488 #A11029, 1:400; Gt anti-Rb Alexa 568 #A11011, 1:400).

Immediately prior to use, all antibodies used for immunofluorescence were diluted 1:10 in 50 - 75 mM reduced glutathione (Sigma #G4251)/TE pH 8.0 and incubated on ice for 1 hr to decrease non-specific binding (Rogers et al., 2006).

Channel levels in all figures have been adjusted in Photoshop to equalize background signal across panels.

Quantitation of histology

Amyloid burden and microglial activation—Sections were analyzed using a macro written for AxioVision 4.7. Color thresholds were used to identify amyloid plaques or activated microglia in high-resolution digital scans of the stained slides. Background staining and shading artifacts were manually excluded from the analyses. The region of interest was specified by tracing the cortex (6.5 and 12 mo animals) or hippocampus (24 mo animals) of the corresponding section and the area of pixels above threshold computed relative to the total area for the region of interest. Five (CS-silver, 6.5 and 12 mo), four (thio-S), or three (CD68 and CS-silver 24 mo) sections spaced at 210 (CS-silver) or 420 μm (thio-S and CD68) intervals were measured for each animal.

Vascular amyloid—Amyloid-containing blood vessels were counted manually from CS-silver and thioflavine-S-stained sections. Counts were restricted to cortical layer 1 where penetrating vessels run perpendicular to the pial surface and allow for easy quantitation compared with the complex vascular involvement of deeper cortical layers. Five sections spaced at 420 μm intervals were counted for each animal.

Microhemorrhage—The number of perivascular clusters of hemosiderin-positive cells was counted manually from Perls-stained sections under DIC illumination. Eight sections spaced at 420 μm intervals were counted for each animal. Severity was estimated by grading each hemorrhage: grade 0: 0-1 cell, grade 1 – clusters of 2-5 cells; grade 2 – clusters of 6-10 cells; grade 3 – clusters of 10+ cells.

Statistics

All statistics were done using Prism 5.0. Data sets were analyzed for outliers by Grubb’s test, which resulted in the removal of no more than 1 data point per group and no more than 2 data points per 4-group comparison. Comparisons of multiple groups (6 mo experiment) were done by one-way ANOVA followed by Tukey post hoc testing. All p-values listed are for post-test comparisons. Comparisons limited to two groups (18 mo experiment) were done by Student’s t-test with Welch’s correction for unequal variances where appropriate. All graphs display group mean ± SEM.

Results

Suppression of Aβ synthesis enables plaque clearance by passive antibody treatment

We used tetoff APP transgenic mice to test whether therapeutically reducing Aβ production could enhance the efficacy of passive immunization against Aβ (Fig. 1a-c). At the outset of treatment, 6 mo old APP/TTA mice had a significant amyloid burden throughout the forebrain, with 7.8±0.48% of the cortical surface area covered by amyloid deposits (Fig. 2a,b). Left untreated, amyloid burden rises almost 10-fold between 6 and 12 mo of age in this transgenic line (63.2% at 12 mo, Fig. 2e, f). As previously shown (Jankowsky et al.,
2005), this increase can be prevented by suppression of transgenic APP. Consistent with our past work, amyloid load in APP/TTA mice treated with dox (dox-only) from 6-12 mo was only slightly greater than it had been at the start of treatment 6 mo earlier (dox-only: 12.1±0.58% vs. pre-treatment: 7.8±0.48% p<0.01). Animals treated with dox and given weekly injections of a non-specific mouse IgG (dox + IgG) harbored nearly identical amyloid levels to mice receiving dox alone (13.36±1.09%). Notably, mice treated with dox and given weekly injections of anti-Aβ IgG carried less amyloid than animals harvested before treatment (3.72±0.49%, p<0.05). Biochemical measurements of Aβ concentration confirmed that the reduction in amyloid burden came from a decrease in Aβ (Fig. 3). Mice treated with dox + Ab9 had 28.0% less Aβ42 than animals harvested prior to treatment (p<0.05; Fig. 2c and Table 1). This reduction may be largely attributable to effects on SDS-soluble Aβ, which was 37.3% lower following combination therapy than pre-treatment levels (p<0.05; Fig. 2d and Table 1). The few plaques remaining in these mice were naked cores, stripped of the expansive diffuse amyloid that surrounds plaques in all other conditions. These cores were apparent on thioflavine-S stained sections and were not significantly affected by treatment (Fig. 4a,b). Consistent with the thioflavine histology, there was no change in formic-acid-extracted Aβ between groups (Fig. 4c). Given that the majority of Aβ is contained in cored formic-acid-soluble deposits, combination treatment provided a much greater reduction in total amyloid load (measured by silver stain) than in total Aβ content (measured by ELISA).

**Combination therapy is equally effective in aged mice**

Our studies clearly demonstrated that combination therapy could reduce amyloid load in young adult transgenic mice (6-12 mo of age). However, the effectiveness of our approach in geriatric animals (18-24 mo) would serve as a more realistic model of its therapeutic potential in human patients. A major limitation to conducting age-appropriate interventional studies in standard transgenic animals has been that the continued overexpression of APP results in severe plaque burden before mid-life. We took advantage of the temporal control provided by the tet-off APP system to overcome this limitation. By suppressing transgenic APP until the mice reached 12 months of age, we were able to generate 18 mo-old mice that carried only 6 mo of amyloid load. We then tested the same treatment protocol used in the younger mice (Fig. 5a).

As in the 6 mo study, animals treated at 18 mo with a combination of dox + Ab9 had visibly less amyloid than siblings treated with dox + non-specific IgG (Fig. 5b,c). Amyloid burden was 72.3% lower in dox + Ab9 treated mice than in controls (1.65±0.18% dox + IgG vs. 0.455±0.072% dox + Ab9; Fig. 5d). Biochemical measures of Aβ content were consistent with the histology (Fig. 6). The concentration of Aβ42 was 47.1% lower in the dox+Ab9 mice (p<0.005 after Welch's correction for unequal variances; Fig. 5e, and Table 2), SDS-soluble Aβ was reduced by 55.6% (p<0.01; Fig. 5f), and FA-soluble Aβ by 44.6% (p<0.05; Fig. 5g). Thus, combination therapy was equally effective in aged animals and in young adults.

**Reductions in plaque load can be maintained by monotherapy**

All of the mice tested at 6 mo and 18 mo were continued on dox for 2.5 mo after the final anti-Aβ antibody injection to test whether improvements made through combination treatment could be maintained long-term with monotherapy (Figs. 1a and 5a). In both young and old cohorts, we demonstrate that the reduction in plaque load attained by combination treatment was still present months after the final dose of antibody. Chronic suppression of APP/Aβ prevents the regrowth of amyloid deposits after acute co-administration of anti-Aβ antibody.
Mechanism of combination therapy

Given the permanence of amyloid aggregates in dox-only mice, plaque clearance by combination therapy likely involves an active process to dismantle pre-existing deposits. One possible mechanism for removal of amyloid by combination therapy is activation of microglial phagocytosis. To initiate this process, peripherally administered antibody must reach the brain and bind to aggregated Aβ. However, past work in wild-type mice has shown that less than 0.2% of a parenteral dose of anti-Aβ antibody is found in the brain 6 hours later, and the percentage decreases with time after injection (Levites et al., 2006b). Plaques may shift this equilibrium by offering a ‘central sink’ of antigen to sequester antibody in the brain. Consistent with this hypothesis, a subset of plaques in 12 out of 14 dox + Ab9 mice were intensely decorated with immunoglobulin (Fig. 7a). This was surprising given that these mice received their last dose of Ab9 several months earlier. Nonetheless, further analysis demonstrated that the plaque-bound antibody was the same isotype as Ab9 (IgG2a), supporting the idea that the originally-injected antibody remained bound to the plaques for months after injection (Fig. 7b, c).

Combination therapy also prolonged a focused microglial response around deposits (Fig. 8a, b). Relative to amyloid burden (measured by Campbell-Switzer silver stain), the area of CD68 immunostaining (a marker of phagocytic microglia (Bornemann et al., 2001)) was twice as great in combination-treated animals than in animals half their age, and more than 3.5x greater than in animals treated only with dox (Fig. 8d). Surprisingly, the absolute area of CD68 activation – approximately 1% of the cortex - remained constant across all conditions, suggesting that the number of CD68+ microglia may be limited and that combination therapy engages cells that are already primed for phagocytosis (Fig. 8c, e).

The effectiveness of combination treatment compared to past studies with Ab9 alone (Levites et al., 2006a; Levites et al., 2006b) suggested that suppressing Aβ production might allow more Ab9 to reach the brain before becoming inactivated by antigen in the periphery. We tested this hypothesis by administering a brief series of Ab9 injections to two sets of mice, one in which transgenic APP and Aβ was suppressed with dox and another in which they continued to be expressed (Fig. 9a). ELISA measurements of plasma Aβ confirmed that Aβ40 was 62% lower (p<0.05) and Aβ42 71% lower (p<0.01) in the dox-treated group (Fig. 9b). Harvesting the mice several days after the final Ab9 injection – rather than several months as in our initial experiments – allowed us to compare the extent of antibody penetration into the brain in each condition. Immunostaining for mouse immunoglobulin revealed that antibody decoration of amyloid plaques was dramatically increased after Ab9-injection, but to our surprise, was independent of Aβ synthesis and was seen in mice treated with dox + Ab9 and Ab9 only (Fig. 9d). Consistent with this finding, Winkler et al. have detected mouse immunoglobulin at neocortical plaques within hours after a single i.v. dose of anti-Aβ antibody (Winkler et al., 2010). Intriguingly, we found immunoglobulin bound not only to amyloid but also to microglia surrounding the plaques (Fig. 9c). Although we cannot definitively identify the source of the IgG bound to plaques and microglia in acutely-treated mice (and the staining was too weak to allow isotype-specific detection), the relative absence of signal in untreated animals suggests that it is likely Ab9.

Animals harvested shortly after the second dose of Ab9 also allowed us to directly test for microglial internalization of Aβ during treatment. While microglia are closely associated with amyloid deposits in untreated mice, we rarely found Aβ inside the cells. In contrast, microglia containing Aβ were frequently found in mice treated with Ab9 (Fig. 9e and Supplemental Movies 1-3). As with amyloid opsonization, Ab9-induced enhancement of Aβ uptake was independent of Aβ synthesis. These data suggest that Ab9 administration spurs microglial phagocytosis of amyloid, but that only when further production of Aβ is arrested can it make an impact on amyloid burden.
Caution in the potential use of combination therapy

Recent studies in multiple lines of APP transgenic mice have shown that repeated injections with anti-Aβ antibodies can increase vascular amyloid and microhemorrhage (Thakker et al., 2009, but see Schroeter et al., 2008). We tested whether combination therapy impacted either of these conditions in our chronically-treated animals. Unlike past studies of monotherapy with anti-Aβ antibody, mice receiving dox + Ab9 showed no change in the number of cortical penetrating vessels with perivascular amyloid compared to dox + IgG controls (Fig. 10a, b). However, the frequency of microhemorrhage but not the severity of individual bleeds was significantly increased by Ab9 (Frequency: dox + Ab9 = 0.71 ± 0.16 vs. dox + IgG = 0.26 ± 0.054 bleeds/section, p < 0.05, t-test with Welch’s correction; Severity: dox + Ab9 = 1.231 ± 0.057 vs. dox + IgG = 1.107 ± 0.060, p = n.s.; Fig. 10c). These findings confirm that therapeutic use of anti-Aβ antibody comes with some risk. However, Aβ suppression to maintain improvements made during combination treatment may allow greater flexibility in the timing and dosage of antibody administration so that microhemorrhage can be minimized.

Discussion

In light of recent imaging data showing that many patients develop amyloid deposits long before diagnosis (Rabinovici and Jagust, 2009), future treatments for AD may be most effective if they can clear pre-existing plaques (Thakker et al., 2009; Rinne et al., 2010). Here we demonstrate significant clearance of deposited amyloid by combining two complementary approaches - one based on arresting further production of Aβ and another on sequestering peptide after it’s released. Both approaches have been shown to slow or stop the progression of amyloid pathology in transgenic mice, but neither alone reverses deposition (see discussion below). Here we show that used in combination, these treatments result in lower amyloid burden than was present prior to treatment. Importantly, both therapeutic approaches used in our study are currently in human clinical trials, including several anti-Aβ antibodies and secretase inhibitors (clinicaltrials.gov). Although broad-spectrum γ-secretase inhibitors may now be viewed with caution following the recent discontinuation of trials for semagacestat (LY-450139), the development of new APP-specific γ-secretase inhibitors (Mayer et al., 2008; Basi et al., 2010) offers renewed hope for treatment strategies based on limiting Aβ production and the possibility of combining these drugs with antibody-based therapies.

As we have previously shown with the tet-off APP mice, therapeutic intervention to stop the production of APP/Aβ slows amyloid accumulation but does not remove plaques formed prior to treatment (Jankowsky et al., 2005); our current results confirm this conclusion. Two recent studies draw the same conclusion from chronic treatment with γ-secretase inhibitors (Abramowski et al., 2008; Garcia-Alloza et al., 2009). In contrast, anti-Aβ antibody treatment rapidly clears pre-existing plaques in transgenic mice, but only when administered directly into the brain (Bacskai et al., 2001; Bacskai et al., 2002; Lombardo et al., 2003; Wilcock et al., 2003; Oddo et al., 2004; Wilcock et al., 2004a; Brendza et al., 2005; Maeda et al., 2007; Thakker et al., 2009). Although the term ‘clearance’ is frequently used when describing the impact of peripherally-administered antibodies, the effect in most mouse models is likely the same as with Aβ suppression: slowing or arrest of amyloid accumulation. Few studies have compared Aβ levels or amyloid load after peripheral antibody transfer to what was present when treatment started. Instead, most compare the difference between treated and untreated animals (Bard et al., 2000; DeMattos et al., 2001; Pfeifer et al., 2002; Bussiere et al., 2004; Wilcock et al., 2004c; Buttini et al., 2005; Hartman et al., 2005; Levites et al., 2006a; Wilcock et al., 2006; Brody and Holtzman, 2008). In many cases, studies have relied on prior characterization of the transgenic line to estimate the amyloid load present at the outset of treatment. However, because many factors can
influence the onset of amyloid formation, including gender (Wang et al., 2003; Hirata-Fukae et al., 2008), strain background (Lehman et al., 2003), diet (Howland et al., 1998; Levin-Allerhand et al., 2002; Pratico et al., 2002; Bayer et al., 2003; Lim et al., 2005; Patel et al., 2005; Oksman et al., 2006), and housing conditions (Jankowsky et al., 2003; Lazarov et al., 2005; Cracchiolo et al., 2007), it is unwise to assume that a transgenic line will perform identically in all studies. Without knowledge of the pre-treatment burden, it is difficult to demonstrate true clearance as opposed to attenuation of deposition. Recognizing this distinction, recent phase 2 clinical trials with bapineuzumab incorporated 11C-PIB binding to measure amyloid load both before and during treatment to reveal a significant 8.5% reduction in the immunized patients (Rinne et al., 2010). This study demonstrated that with prolonged treatment (18 months to effect in the phase 2 trial), passive antibody treatment appears capable of modestly reducing amyloid levels, though given the relatively small number of subjects additional confirmatory studies are needed. Our current findings suggest that a combination approach could accelerate and enhance this effect.

As with passive immunization, several studies of active immunization against Aβ have also claimed to show amyloid clearance either without measuring pre-treatment levels or when the evidence better supports a more cautious interpretation. The first immunotherapy study to be published for Alzheimer’s disease stated that immunization of 11 month old mice with Aβ42 resulted in several mice with “fewer diffuse and mature amyloid-β deposits at 15 and 18 months, suggesting that the treatment had resulted in the clearance of pre-existing amyloid-β deposits,” however, no statistical comparison to the pre-treatment group was performed. When Aβ concentrations were measured by ELISA, they were identical in the immunized mice and in those harvested prior to treatment (Schenk et al., 1999), making it difficult to conclude that pre-existing Aβ deposits were cleared. Thus, this finding like many others is more consistent with treatment preventing further deposition than clearing antecedent deposits. This interpretation might also be applied to the results of the AN-1792 clinical trials, where low levels of amyloid in several treated patients were taken to suggest evidence for plaque clearance (Nicoll et al., 2003; Nicoll et al., 2006; Holmes et al., 2008). Based on decades of clinico-pathological studies, it was reasonable to assume that most of the study participants with mild-to-moderate AD have amyloid, and to be fair, when these studies began there was no way to evaluate plaque burden prior to death. But in the absence of data showing the presence and extent of pathology prior to immunization, it would be safer to conclude that treatment prevented pathology from worsening rather than reversing pathology that may not have existed. Admittedly, one conundrum in this interpretation is the appearance in several immunized patients of cortical patches lacking amyloid immediately adjacent to areas with considerable plaque accumulation (Nicoll et al., 2003; Boche et al., 2010). However, as the appearance of meningoencephalitis in several of the immunized patients dramatically revealed (Orgogozo et al., 2003; Bayer et al., 2005), active immunization invokes responses beyond antibody production and any clearance that may have occurred in these patients could have resulted from antibody-independent mechanisms.

Our impetus for marrying passive immunization with Aβ suppression was straightforward: draining a flood is easier once the water stops. We had initially hypothesized that limiting the amount of Aβ in the periphery would allow more free antibody to reach the brain. Our data suggest that anti-Aβ antibody Ab9 reaches the brain and co-localizes with both amyloid and microglia, independent of transgenic APP expression. However, even when the transgene is active the tet-off APP mice produce only moderate levels of plasma Aβ; models with higher levels of peripheral Aβ might show a greater influence on how much free antibody reaches the CNS. Past studies revealed a critical role for microglia in determining plaque burden, with the suggestion that these cells phagocytose Aβ aggregates during normal disease progression (D’Andrea et al., 2004; Simard et al., 2006; El Khoury et al., 2007; Kellner et al., 2009) and become specifically activated in response to anti-Aβ...
immunotherapy (Bard et al., 2000; Wilcock et al., 2003; Wilcock et al., 2004a; Wilcock et al., 2004c; Koenigsknecht-Talboo et al., 2008). Our findings further support a role for microglial phagocytosis in antibody-driven plaque clearance and suggest that the capacity of microglial clearance is insufficient to overcome the continued accumulation of amyloid in APP transgenic mice. The efficacy of combination therapy lies in its ability to stave off further aggregation while microglia dispose of earlier deposits. We expect that the near-complete Ab suppression attained here using a genetic switch may only be required for passive immunization to overcome the exaggerated Ab production of our mouse models. Successful treatment may be achieved in human patients with less efficient suppression. Our findings suggest that reducing Ab release by any amount will improve amyloid clearance by passive immunization.

In contrast to our findings of improved amyloid clearance by combination therapy, an earlier study by Wilcock and colleagues found no improvement by combining immunization with a γ-secretase modulator over monotherapy alone (Wilcock et al., 2007). While both active immunization against Ab42 and non-steroidal anti-inflammatory treatment using the modified flurbiprofen derivative NCX-2216 diminished amyloid accumulation compared to untreated controls, their combination provided no benefit over either alone. However, toxicity of NCX-2216 killed many of their combination-treated mice, leaving only 3 animals for analysis. In addition, the use of active as opposed to passive immunization resulted in highly variable anti-Ab antibody titers, which may have increased variability in treatment outcomes. Additional study is needed to determine whether larger treatment groups with multivariate analysis of amyloid load against antibody titer might yield a different conclusion.

One caveat in interpreting our findings is the fact that we did not directly test the effect of Ab9 alone in our tet-off APP mice. In retrospect, we would have done well to include this control, however when designing the study we believed the question had already been answered by our past work in mice with even lower starting levels of Ab accumulation. Our original study of Ab9 passive immunization found only modest attenuation of amyloid loads in Tg2576 and CRND8 mice that started Ab9 treatment with much less amyloid than the tet-off APP mice used here (Levites et al., 2006a). In these studies, the effect was consistent with prevention of accumulation rather than clearance of pre-existing plaques. In mice with amyloid loads comparable to those studied here, direct intracranial injection of Ab9 was required to demonstrate any effect on Ab, and then only diffuse plaques were affected. Indeed, both the epitope specificity of Ab9 (Ab1-16) and its isotype (IgG2a) may have contributed to this outcome, and the possibility exists that other antibodies may function through distinct mechanisms or have different capabilities for removing pre-existing amyloid. However, based on these previous studies showing only attenuation of deposition after peripheral Ab9 treatment versus the unambiguous evidence of amyloid reduction provided here, we think it is reasonable to infer that combination therapy is needed for clearance with this antibody.

We further show that plaque reductions attained through combination treatment can be maintained long afterwards with monotherapy. All of the mice receiving long-term treatment with dox + Ab9 were harvested 2.5 mo after their final antibody injection. During that time, the animals continued on dox to limit transgenic APP/Ab synthesis. As shown in prior studies with the tet-off APP mice, γ-secretase inhibitors, and passive anti-Ab immunization, reducing Ab concentration can maintain amyloid levels indefinitely at the level present when treatment started (Jankowsky et al., 2005; Abramowski et al., 2008; Garcia-Alloza et al., 2009; Karlnoeski et al., 2009). Here we test long-term treatment with dox as a ‘maintenance dose’ to preserve improvements made during a short-term ‘loading dose’ of combination treatment. Given the potential side effects associated with use of...
passive antibody transfer in mice (Pfeifer et al., 2002; Wilcock et al., 2004b; Lee et al., 2005; Racke et al., 2005), and more recently in humans (first reported by S. Gilman et al., ICAD 2008), it may be important to limit the duration of combination therapy and preserve recovery without continued risk. Our results demonstrate that chronic Aβ suppression meets this need.

Further study is needed to determine whether our outcome could be improved by optimizing our dosing strategy. Alternatively, several groups are testing ways to increase the brain-penetration of peripherally-delivered antibody, including polyamine modification or ultrasonic disruption of the blood brain barrier (Poduslo et al., 2007; Choi et al., 2008; Raymond et al., 2008). We could also simply intervene earlier. Dox + Ab9 intervention at both 6 and 18 mo yielded an equivalent 72% reduction in amyloid load compared to dox + IgG, but because the 18 mo mice started treatment with less amyloid, they ended with almost none. The ending levels of amyloid in the two cohorts likely also reflect the greater abundance of cored plaques in the 6 mo old mice at the outset of treatment. The compact structure of fibrillar deposits may leave them less vulnerable to attack, alternatively, as the innermost core beneath a layer of diffuse amyloid that must be cleared first, they may simply require longer to clear than was tested here. Although cored amyloid represents only a small fraction of total burden (<15% of amyloid surface area), it holds the vast majority of Aβ in the brain. We felt strongly about testing combination therapy in mice that mimicked the level of pathology present in patients at the time of diagnosis. We expect that future improvements in diagnosis will allow patients to be identified at earlier stages of disease.

A critical question to be addressed in future is whether plaque clearance is necessary for maximum functional recovery. Our current experiments could not assess behavior because of the animals’ mixed hybrid background and persistent hyperactivity (Jankowsky et al., 2005); these mice were bred for pathology rather than performance. What role plaques play in cognitive decline remains a topic of debate. Certainly, plaques have been shown to cause both physical damage such as neuritic swelling, swerving, and breakage (Le et al., 2001; D’Amore et al., 2003; Lombardo et al., 2003; Tsai et al., 2004; Meyer-Luehmann et al., 2008) and functional deficits such as desynchronized transmission, neuronal hyperactivity, and calcium dysregulation (Stern et al., 2004; Busche et al., 2008; Kuchibhotla et al., 2008). Thus, it will be critical to understand whether their clearance is required for optimum functional recovery and whether it should be a target for future treatment. Our study suggests that combination therapy may be a valuable tool in making these assessments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References


Fig 1. Using the tet-off system to suppress transgenic APP and Aβ synthesis during combination therapy

A. Experimental design: Mice expressed transgenic APP for 6 mo to generate amyloid throughout the forebrain before being placed on dox to stop further Aβ production. Mice harvested after 2 wk on dox served as the baseline against which we gauged three interventions: 1) continued monotherapy with dox (dox only); 2) combination therapy with dox plus a non-specific mouse IgG (dox + IgG); and 3) combination therapy with dox plus anti-Aβ Ab9 (dox + Ab9). After completing 12 weekly antibody injections, all mice remained on dox for an additional 2.5 mo and were harvested at 12 mo of age. B. Western blot showing APP suppression in APP/TTA mice following 2 wk or 6 mo of dox treatment. The upper part of the membrane was probed with 6E10 to detect transgene-derived full-length APP; the lower part was probed for superoxide dismutase 1 as an internal control for loading. C. Quantitation of the immunoblot shown in B demonstrates that transgenic APP levels are reduced by >90% in dox-treated mice (p<0.001).
Fig 2. Combination therapy clears amyloid and lowers Aβ levels in transgenic mice

A. Silver histology reveals significant amyloid burden throughout the forebrain of APP/TTA transgenic mice harvested at 6.5 mo of age (line 107, n=7). Plaque burden is maintained at this level by treatment with dox (n=22) or a combination of dox + IgG (n=14), but is substantially reduced by dox + Ab9 (n=14). Individual plaques in the dox + Ab9 mice look like naked cores stripped of their diffuse halo (top: 1x, middle: 5x, bottom: 63x). B. Quantitation of plaque load from silver-stained sections confirms that amyloid burden is significantly lower in mice treated with dox + Ab9 than in age-matched controls or animals harvested prior to treatment. Other significant comparisons (not shown) were: pre-treatment vs. dox only (p<0.05), pre-treatment vs. dox + IgG (p<0.01). C, D. Aβ ELISAs confirm the histology. Both Aβ42 (sum of SDS + FA-extracted 42; (C)) and SDS-soluble Aβ (sum of SDS-extracted 40 + 42 (D)) were lower in mice given dox + Ab9 than they were prior to treatment or following monotherapy. E. Untreated mice (6 mo to 12 mo) show the rise in amyloid that would occur without intervention (line 102). F. Graph illustrating the relative efficacy of combination therapy. All mice in our study started treatment with a moderate amyloid burden, similar, if slightly more pronounced, to the levels expected in Alzheimer's patients at the time of diagnosis. Left untreated, plaque burden increases more than 8-fold over the next 6 mo in this mouse model. This progression can be slowed considerably by suppressing further production of Aβ, but only a combined approach clears amyloid formed prior to treatment.
Figure 3. Graph of total Aβ concentration vs. amyloid load for animals receiving long-term combination treatment starting at 6 mo of age

Graph of Aβ levels measured by ELISA from hemi-forebrain extracts plotted against amyloid % surface area measured in the cortex of Campbell-Switzer silver-stained sections confirms a strong correlation between these values.
Figure 4. Fibrillar deposits are less amenable to clearance by combination therapy

A. The proliferation of thioflavine-S-positive fibrillar plaques was stabilized by suppression of APP/Aβ but there was no evidence of clearance following addition of Ab9 (upper row, 5x, lower row, 63x). B. Quantitation of thioflavine-stained sections confirmed that there was no significant change in fibrillar amyloid burden with treatment. C. Biochemical measurements of formic acid-soluble Aβ are generally consistent with the histological measurement of fibrillar amyloid. Differences in formic acid-soluble Aβ (sum of FA-extracted 40 + 42) approached significance when tested by ANOVA (p=0.062), driven largely by the difference between dox-only and dox + Ab9; direct t-test of the two groups yielded p<0.05. D. Similarly, Aβ40 (sum of SDS + FA-extracted 40) was significantly lower in the dox + Ab9 group than in dox-only controls.
Fig 5. Combination therapy works equally well in aged animals and young adults
A. Experimental design: The tet-off system was used to delay transgenic APP expression until 12 mo of age. Animals were then taken off dox to start APP overexpression and the experiment proceeded as in the younger cohort: following 6 mo of transgenic APP expression animals were treated with dox for 2 wk before starting the first of 12 weekly antibody injections with either non-specific IgG (n=7) or anti-Aβ Ab9 (n=9). Mice remained on dox for 2.5 mo after the final injection and were harvested at 24 mo of age. B. Campbell-Switzer silver staining reveals significantly less amyloid in mice treated with dox + Ab9 than in age-matched animals given control IgG (10x hippocampal sections). C. Individual plaques from dox + Ab9-treated mice appear as ‘naked cores’ missing the halo of diffuse amyloid that surrounds larger plaques in control mice (63x). D. Quantitation of silver-stained sections demonstrates that dox + Ab9-treated mice have significantly less amyloid than dox + IgG controls. E-G. ELISA measurement of Aβ levels confirm the histological findings. Significant reductions were found in Aβ42 (sum of SDS + FA-extracted 42 (E)), SDS-soluble Aβ (sum of SDS-extracted 40 + 42 (F)), and FA-soluble Aβ (sum of FA-extracted 40 + 42 (G)).
Fig. 6. Graph of total Aβ concentration vs. amyloid load for animals receiving long-term combination treatment starting at 18 mo of age

Graph of Aβ levels measured by ELISA from hemi-forebrain extracts plotted against amyloid % surface area measured from the hippocampus of Campbell-Switzer silver-stained sections confirms a significant correlation between these values.
Fig 7. Mouse immunoglobulin co-localizes to plaques long after the final injection of Ab9
A. Immunostaining for mouse IgG reveals antibody-decorated plaques nearly 3 mo after the final dose of Ab9 (top and bottom rows show different anterior-posterior levels of the brain at 1x magnification). Amyloid-associated antibody is found only in mice treated with Ab9, and decorates only a subset of plaques. Boxes highlight similar areas in dox+IgG and dox +Ab9 sections to facilitate comparison. B. Plaques in dox+Ab9 treated mice were isotyped by immunostaining with subtype-specific secondaries. Plaques were strongly positive for IgG2a – the same isotype as Ab9 (red, upper row, 5x) - but negative for IgG1 (green, upper and lower rows, 5x). Instead, IgG1 signal can be found associated with blood vessels in the thalamus and meninges ventral to the hippocampus (arrows, lower row), indicating that the absence of signal at plaques is not due to failure of the immunostain. C. No staining above background for either isotype is found at deposits in mice administered dox + control IgG (as in B, arrow indicates IgG1-positive blood vessel, 5x).
Fig 8. Combination therapy focuses the microglial response to amyloid

A. Immunostaining with Iba-1, a generic microglial marker, shows a dense gathering of microglia around amyloid cores in untreated mice. In the absence of continued amyloid deposition (dox-only and dox + IgG), the concentration of microglia becomes less pronounced. By comparison, co-administration of Ab9 maintains the presence of microglia around amyloid foci (top row, 5x; bottom row, 63x). Non-transgenic animals show basal Iba-1 staining in the absence of pathology. B. Immunostaining with CD68, a marker of microglial lysosomes, confirmed that the cells concentrated around plaques are activated and potentially phagocytic (top row, 5x). Counterstaining with Congo red highlights the disparity between the size of plaques in each condition and the number of CD68-positive microglia surrounding them (bottom row, 63x). C. Quantitation of CD68-immunostaining indicates there is no difference in the area occupied by CD68+ microglia between dox + Ab9-treated mice and age-matched controls. D. When considered in relation to the plaque burden carried by each treatment group, the dox + Ab9-treated mice have significantly more CD68+ microglia per unit amyloid than any of the control groups (measured as CD68 area vs. amyloid burden from Campbell-Switzer silver staining). Significant comparisons are shown for dox + Ab9; other significant comparisons were: microglial activation: pre-treatment vs. dox + IgG (p<0.001), dox only vs. dox + IgG (p<0.01). F. The area occupied by phagocytic CD68+ microglia is relatively constant across amyloid burden and treatment condition, consistent with the idea that combination therapy focuses a fixed microglial response around less remaining amyloid. There is no correlation between % CD68 area and amyloid load measured by Campbell-Switzer silver stain (slope of the linear regression is not significantly different from 0 (p=0.94, R²=0.0001); dashed lines indicate the 95% confidence interval).
Fig 9. Passive immunization promotes microglial internalization of Aβ aggregates

A. Short-term treatment was used to investigate the mechanism of plaque clearance in combination therapy. At 7 mo of age, animals were divided into two groups, one placed on dox chow (n=6) and the other left un-medicated (n=3). Two weeks later, both groups received the first of two Ab9 injections. Mice were harvested 3 days after the second injection. Untreated mice served as controls (n=2). B. Plasma samples taken immediately prior to the first Ab9 injection confirm that the concentration of transgene-derived Aβ was significantly reduced by dox. Graph includes mice receiving unlabeled (n=9) or biotinylated Ab9 (n=9, not used further). C. Triple labeling with thioflavine for fibrillar amyloid (green) and immunofluorescence for microglia (Iba1, blue) and mouse pan-IgG (red) demonstrates that Ab9 treatment led to IgG binding of both plaques and microglia (63x). D. Plaque decoration with mouse IgG was dramatically enhanced after Ab9 injection independent of circulating Aβ levels (5x). E. Double-immunofluorescence for microglia (Iba-1, red) and Aβ (4G8, green) reveals that Ab9 treatment stimulated microglial engulfment of Aβ aggregates (upper row, multiple image projection; bottom row, single optical section; 63x). Microglia were often found in close apposition to Aβ in untreated mice, but often contained Aβ in mice treated with Ab9. As with the increase in plaque-bound IgG, the stimulation of microglial Aβ internalization was independent of APP/Aβ expression. Three-dimensional rotations of these cells are provided as movie files in Supplemental Information.
Fig 10. Vascular amyloid is unchanged but microhemorrhage frequency is increased by combination therapy

A, B. Campbell-Switzer silver (A, 10x DIC) and thioflavine-S staining (B, 5x fluorescence) were used to assess the frequency of blood vessels harboring amyloid deposits in cortical layer 1. No significant difference was seen in the number of amyloid-positive vessels per section between animals receiving control IgG + dox and those given Ab9 + dox. C. Perls Prussian blue histology revealed an increased frequency but no change in severity of cerebral microhemorrhage (20x DIC).
Table 1

**Aβ ELISA data for animals receiving long-term combination treatment starting at 6 mo of age**

Values are expressed as pmol/g brain weight ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>12 mo</th>
<th>Pre-treatment</th>
<th>Dox only</th>
<th>IgG + Dox</th>
<th>Ab9 + Dox</th>
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<tbody>
<tr>
<td>Aβx-40</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>5.943 ± 673</td>
<td>6.794 ± 352</td>
<td>6.490 ± 332</td>
<td>5.278 ± 325†</td>
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<tr>
<td>Aβx-42</td>
<td>14.416 ± 1,773</td>
<td>13.645 ± 672</td>
<td>13,241 ± 696</td>
<td>10,381 ± 693*†</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>2.646 ± 298</td>
<td>2.778 ± 193</td>
<td>2.516 ± 142</td>
<td>1,658 ± 102*††#</td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>17,058 ± 2,023</td>
<td>17,615 ± 909</td>
<td>17,615 ± 909</td>
<td>14,000 ± 895</td>
<td></td>
</tr>
<tr>
<td>Total Aβ</td>
<td>20.359 ± 2,375</td>
<td>20,401 ± 925</td>
<td>19,731 ± 932</td>
<td>15,658 ± 956†</td>
<td></td>
</tr>
</tbody>
</table>

* p<0.05 vs. pre-treatment
† p<0.05
††† p<0.001 vs. dox only
# p<0.05 vs. IgG + dox.
**Table 2**

Aβ ELISA data for animals receiving long-term combination treatment starting at 18 mo of age

Values are expressed as pmol/g brain weight ± SEM.

<table>
<thead>
<tr>
<th>24 mo</th>
<th>IgG + Dox</th>
<th>Ab9 + DOX</th>
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</thead>
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<tr>
<td>Aβx-40</td>
<td>475.8 ± 36.1</td>
<td>365.9 ± 92.4*</td>
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<tr>
<td>Aβx-42</td>
<td>1,956 ± 95.3</td>
<td>1,034 ± 237.9**</td>
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<tr>
<td>SDS</td>
<td>198.7 ± 23.79</td>
<td>88.3 ± 24.1**</td>
</tr>
<tr>
<td>FA</td>
<td>2,366 ± 210.7</td>
<td>1,311 ± 303.6</td>
</tr>
<tr>
<td>Total Aβ</td>
<td>2,418 ± 134.8</td>
<td>1,541 ± 397.3*</td>
</tr>
</tbody>
</table>

* p<0.05

** p<0.01 vs. IgG + dox.