Methods

Mice.

$ACE^{10/10}$ mice have been previously described (24). In this strain, targeted homologous recombination was used to insert a c-fms promoter to direct tissue overexpression of ACE in myelomonocytic cells. The ACE genotypes are wild type ($ACE^{WT/WT}$), heterozygous ($ACE^{10/WT}$) and homozygous for the targeted allele ($ACE^{10/10}$). These mice were backcrossed at least 10 generations to C57BL/6J mice (Jackson Laboratory).

The Alzheimer's double-transgenic mice (ADtg) strain was originally purchased from Jackson Laboratory [B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J; stock number: 005864], then bred and maintained at Cedars-Sinai Medical Center. The transgenes contain familial AD mutations and are expressed in CNS tissue under the control of the mouse prion protein (PrP) promoter. They contain cDNA sequences for the mutant human/mouse chimeric Aβ (A4) precursor protein (Swedish double mutations in $APP_{K595N, M596L}$) and the exon 9 deletion of human presenilin 1 ($PS1_{\Delta E9}$) (74). ADtg (AD^{+}; C57BL/6J background) were crossed with $ACE^{10/10}$ mice to produce the following genotypes: $AD^{+}ACE^{WT/WT}$, $AD^{+}ACE^{10/WT}$, $AD^{+}ACE^{10/10}$, $AD^{-}ACE^{WT/WT}$, and $AD^{-}ACE^{10/10}$. Mice were genotyped for ACE (24), APP, and PS1 (36) alleles using conditions previously described. Age- and gender-matched mice for each genotype were assessed at various ages from 3 to 14 months old. All mice were maintained in microisolator cages. All experiments were conducted and recorded by researchers blinded to the mouse genotypes.

Blood pressure

Systolic blood pressure was measured in conscious $AD^{+}ACE^{WT/WT}$ and $AD^{+}ACE^{10/10}$ mice using a Visitech tail cuff system (75). The individual blood pressure is the average of the blood pressure measured on four consecutive days following four days of training to accustom the animals to the procedure. Blood pressure from male and female mice was analyzed separately. The average age was similar for the two genotypes of mice.

Tissue Processing.

Following perfusion with ice-cold 0.9% saline containing 0.5 mM EDTA, harvested brains were cut in half (36). One hemisphere was used for histology. The second hemisphere was further cut into two halves and snap frozen for biochemical and molecular assays.

Immunohistochemistry.

For paraffin sections, tissue was fixed and embedded using standard techniques. Five-micron sections were cut, deparaffinized and put in 88% formic acid for 5 min. After rinsing, the tissue was incubated at pH 9 for 20 min, then rinsed and incubated with either the mouse monoclonal antibody 6F/3D (1:800; Leica Biosystems) or a rabbit polyclonal anti-human Aβ antibody Abcam Ab2539 (1:150) that targets amino acid 1-14 for 15 min in buffer pH 7.6 (Leica Biosystems, cat. AR9640). The secondary anti-mouse antibody and the DAB chromogen were dispensed using an automated Leica Bond-III slide staining machine and the Leica Bond Polymer Refine Detection reagents (Leica Biosystems, cat. DS9800). The secondary goat anti-rabbit antibody was Dako

EnVision+ System-HRP Labelled Polymer (Dako K4003). Standard peroxidase-based and hematoxylin/eosin (H&E) staining techniques were used.

For immunofluorescence, tissue was fixed in 2.5% PFA following dehydration with 30% sucrose. Coronal cryosections (30 μm) were treated with Target Retrieval solution (Dako) at 95^{o} C for 30 min. Tissue was then permeabilized and blocked as previously described (36). Sections were stained overnight at 4°C with combinations of the following primary antibodies (Abs) in PBS with blocking solution: mouse anti-human Aβ mAb clone 6E10 (1:100; Covance) recognizing the amino acid 1-16, or mouse anti-human Aβ mAb clone 4G8 (1:100; Covance) recognizing the amino acids 17-24, rabbit anti-CD31 polyclonal Ab (1:50; Abcam Ab28364), rabbit anti-GFAP polyclonal Ab (1:100; Sigma-Aldrich G9269), rabbit anti-Iba1 (1:250; Wako Chemicals USA 019-19741), rabbit anti-ACE polyclonal Ab (1:500) (76), and rat anti-CD45 mAb (1:25; BD Pharmingen 550539). Secondary polyclonal Abs donkey anti-mouse, anti-rat and anti-rabbit antibodies (1:200; Jackson ImmunoResearch Laboratories) conjugated with Cy-2, Cy-3 or DyLight^{TM} 649, were incubated for 1 hr at 37°C. Washing, DAPI nuclei staining, mounting and microscopic analysis (ApoTome-equipped Carl Zeiss Z1 fluorescence microscope) were performed as previously described (36, 62). The specificity of the labeling was established by omitting the primary antibody from parallel brain sections.

Aβ Plaque Staining by Thioflavin-S.

Following secondary antibody staining, brain sections were stained with thioflavin-S (Thio-S, 1% w/v in 70% ethanol) (Sigma-Aldrich) as previously described (36).

Quantitative Immunohistochemistry.

For each genotype, equivalent brain regions were analyzed. Three to six coronal sections per mouse were taken at 150 μm intervals. For the analysis of Aβ plaque, $GFAP^+$ immunoreactive area and manual count of cell number, and $Iba1^+CD45^{high}$ area and cell number, images were captured encompassing the hippocampal and cortical regions (including entorhinal and cingulate cortex) using the same exposure time. For perivascular Aβ deposition, areas of Thio-S and 6E10 co-labeled with CD31 (a marker for vascular endothelial cells) covering the hippocampal fissure in the outer molecular layer (OML) and vessels between the hippocampus and the lateral posterior thalamic nucleus, mediorostral part (LPMR), were assessed in five to seven serial brain sections per mouse, and images captured using the same exposure time. Images were then converted to grey scale and standardized to baseline using histogram-based thresholds by NIH ImageJ software (ver. 1.38x and 1.46r) as described (36, 77).

Analysis of cerebral inflammation by flow cytometry

Brains were perfused before harvest. The brains were then minced and digested for 30 min with 1.5 mg/ml collagenase IV (Worthington) and DNase I (StemcellTechnologies) at 37°C. After centrifugation, homogenization and washing, the pellet was suspended in 70% Percoll (GE Healthcare) and then layered under a 30%/50% step Percoll gradient. After centrifugation for 25 min at 700 g, the cells at the interface of the 70% and 50% layers were collected and washed. Cells were stained with FITC-conjugated anti-CD45 (1:200; Clone 30-F11), PerCP/Cy5.5-conjugated anti-CD11b (1:100; Clone M1/70) and Pacific blue-conjugated anti-Ly6C (1:200; Clone HK1.4). Antibodies were purchased from either eBioscience or BioLegend. The stained samples were analyzed on a Beckman Coulter CyAn ADP (Beckman Coulter), and data were analyzed with FlowJo software (Tree Star, Inc.).

LPS-induced cytokine expression by microglia

8 to 10-week-old $AD^{-}ACE^{WT/WT}$ and $AD^{-}ACE^{10/10}$ mice were perfused and the brains were collected. Brains were chopped into small pieces and then digested for 30 min with 1.5 mg/ml collagenase IV and DNase I (StemcellTechnologies) at 37°C. After centrifugation, homogenization and washing, the pellet was suspended in 70% Percoll and then layered under a 30%/50% step Percoll gradient. After centrifugation for 25 min at 700 g, the cells at the interface of the 70% and 50% layers were collected, washed, and incubated in vitro for 6 hr with 5 μg/ml brefeldin A (eBioscience) with or without lipopolysaccharide (LPS; *Escherichia coli* 055:B5, Sigma-Aldrich) stimulation. Cells were then stained for the surface markers CD45 (1:200) and CD11b (1:100), followed by intracellular staining with PE-conjugated anti-IL-1β (1:333) (Clone NJTEN3; eBioscience) and APC-conjugated anti-TNFα (1:100) (Clone MP6-XT22; eBioscience). The $CD11b^{+}CD45^{low\text{-}intermediate}$ microglia were evaluated for cytokine expression by flow cytometry.

Biochemical Determination of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ Levels by Sandwich ELISA

For brain soluble and insoluble ELISA analysis, brain tissues were homogenized in PBS buffer with 0.5% Triton X-100 and 1x protease inhibitor cocktail set I (Calbiochem). After removal of cell debris, the homogenate was centrifuged at 10,000g for 20 min at 4°C (36, 61). The supernatant was considered the 'soluble' fraction and was used to assess soluble Aβ. The pellet was diluted with the above homogenizing buffer. This was the 'insoluble' fraction and was used to assess insoluble Aβ. After determination of protein concentration (ThermoScientific), soluble and insoluble transgenic-derived $A\beta_{1\text{-}42}$ levels were determined using an anti-human $A\beta_{1\text{-}42}$ end-

specific sandwich ELISA kit (Invitrogen, KHB3442) according to the manufacturer's instruction. This kit does not recognize mouse Aβ, nor does it recognize human $A\beta_{1-40}$, or $A\beta_{1-43}$. The kit uses two antibodies and is specific for the N- and COOH-termini of the $A\beta_{1-42}$ sequence; bound rabbit antibody was detected through the use of a horseradish peroxidase-labeled anti-rabbit antibody and was read at 450 nm using a microplate reader (Spectra Max 384 plus, Molecular Devices). Soluble $A\beta_{1-40}$ levels were measured using a similar Invitrogen ELISA kit (KHB3482).

Western blot analysis of APP

Snap-frozen forebrain samples (left hemi brains) were homogenized with a handheld micro-grinder (Argos Technologies) in RIPA buffer with 1X protease inhibitor cocktail (Calbiochem). The homogenate was centrifuged at 10,000 g for 25 min at 4°C. Protein concentration was determined using the Bicinchoninic Acid Protein Assay kit (Pierce). Protein samples (40 µg) were separated on Bis-Tris 4-12% gel (Bio-Rad) with either NuPAGE MOPS or MES buffer and transferred to 0.45 µm nitrocellulose or PVDF membranes. Blots were probed with 6E10 mAb (Covance; anti-Aβ 1–16 aa; 1:1000). Blots were reprobed with anti-β-actin mAb (Sigma-Aldrich; 1:1000) as a loading control. Relative band intensity was quantified using ImageJ software (ver. 1.46r NIH).

Brain ACE Expression and Activity

ACE activity and Western blotting were performed as previously described (78). For the preparation of protein extracts, the brains were collected from euthanized animal, weighed and immediately frozen in liquid nitrogen. Later, they were gently homogenized in ACE buffer (HEPES, pH 7.4, 50 mM; NaCl, 150 mM; $ZnCl_2$, 25µM; and phenylmethanesulfonylfluoride, 1 mM). After centrifugation at 10,000 g, the pellets were

vigorously rehomogenized with ACE buffer containing 0.5% Triton X-100. After a second centrifugation at 10,000 g, the supernatant was collected and protein concentration was measured with the BCA protein assay reagent kit (Pierce) following the manufacturer's instructions. ACE activity was measured using the ACE-REA kit from American Laboratory Products Company, Ltd. (ALPCO). ACE activity was defined as that inhibitable by captopril (Sigma-Aldrich).

For Western blotting, 20 µg of total proteins were separated by SDS-PAGE on a 10% gel and transferred to a 0.45 µm nitrocellulose membrane. The membranes were probed with a 1:250 dilution of a rabbit polyclonal anti-mouse ACE antibody (76) and developed with a goat ant-rabbit IR dye 800CW (LI-COR Biosciences). The blot was scanned with the LI-COR Odyssey.

To inhibit ACE, $AD^{+}ACE^{10/10}$ mice were treated for 28 days or 60 days with 160 mg/liter drinking water of either the ACE inhibitor ramipril (Watson, NDC 16252-573-01) or hydralazine, (Sigma-Aldrich) which lowers blood pressure independently of ACE.

Open field test.

The open field test was performed as previously described (79). Briefly, mice were placed individually in the open field apparatus (San Diego Instruments) with ambulatory and rearing activity measured for 60 min after at least 1 hr habituation. Ambulatory activity was recorded when the mouse broke successive lower beams within a 1 sec period. Rearing behavior was counted when an upper beam was broken.

Barnes Maze test.

The Barnes Maze apparatus consists of a flat white circular platform with 20 equally spaced holes around the circumference. One of the 20 holes leads to an escape

box while the other 19 lead to false-boxes that are too small to be entered (2 cm below the surface area of the platform). The test is conducted in 3 phases. In the training/acquisition phase, the escape box is placed under 1 of 20 holes. Animals are first placed into an opaque cylinder at the center of the maze for 30 sec to promote initial spatial disorientation. After 30 sec, the cylinder is removed and the animal explores the maze until it finds and enters the escape box. The escape latency is the duration of time between removal of the cylinder and animal entry into the escape box. Two bright lights illuminate the center of the Barnes platform. If the mouse fails to enter the escape box within 240 sec, the experimenter gently leads it into the escape box. The animal remains in the escape box for an additional 30 sec before the mouse is removed and taken to the home cage. The escape box, additional boxes, and maze surface are sprayed with 70% isopropyl alcohol and wiped in a non-systematic fashion to dissipate and disseminate odor cues for subsequent trials. The location of the escape box remains the same for a mouse during every trial of the training phase but is shifted between mice to reduce the potential for unintended intra-maze cues. Training is repeated 3 times per day with a 15 min interval separating each trial. Data from each trial is retained and averaged. The training phase is 4 days. Then there is a 2-day break without any exposure to the maze. On day 7, each animal was re-tested with a 3-trial session using the same escape box location and method from the training phase. On day 8 following the retention phase, the reversal phase begins. Now, the escape box is placed in a different quadrant to the original escape box location. Using the same procedure as above, reversal trials are repeated 3 times per day over 2 consecutive days (days 8 and 9). Data was recorded both manually and by a video camera located above the maze.

Statistical Analysis.

Data were analyzed using GraphPad Prism software (ver. 5.0b). Values are presented as individual mice and/or expressed as means ± SEM. One-way analysis of variance (ANOVA) with Tukey post-hoc test was used for multiple comparisons. The behavioral data was analyzed using both repeated-measures two-way ANOVA and one-way ANOVA with Tukey post-hoc test for each day. Two-group comparisons were analyzed by 2-tailed unpaired Student's t-test. A P value less than 0.05 was considered significant.

Animal Studies Approval

All experimental protocols were approved and conducted according to the instructions of the Institutional Animal Care and Use Committee at Cedars-Sinai Medical Center.

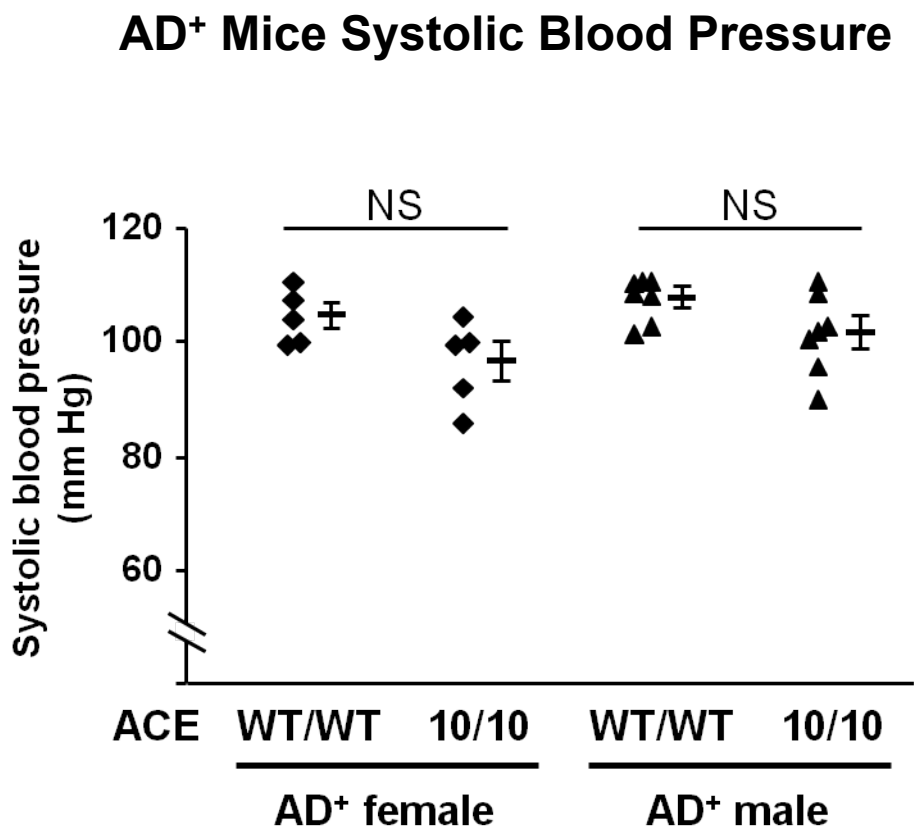

Figure S1. Blood pressure. Systolic blood pressure was measured in conscious $AD^{+}ACE^{WT/WT}$ and $AD^{+}ACE^{10/10}$ mice. The average age for each genotype was similar, 9.4 ± 1.4 and 10.1 ± 1.6 months, respectively. Both female and male mice were studied. Data from individual mice, as well as the group means and SEM are shown. For male and female mice, the blood pressures were not significantly different (NS) between $AD^{+}ACE^{WT/WT}$ and $AD^{+}ACE^{10/10}$.

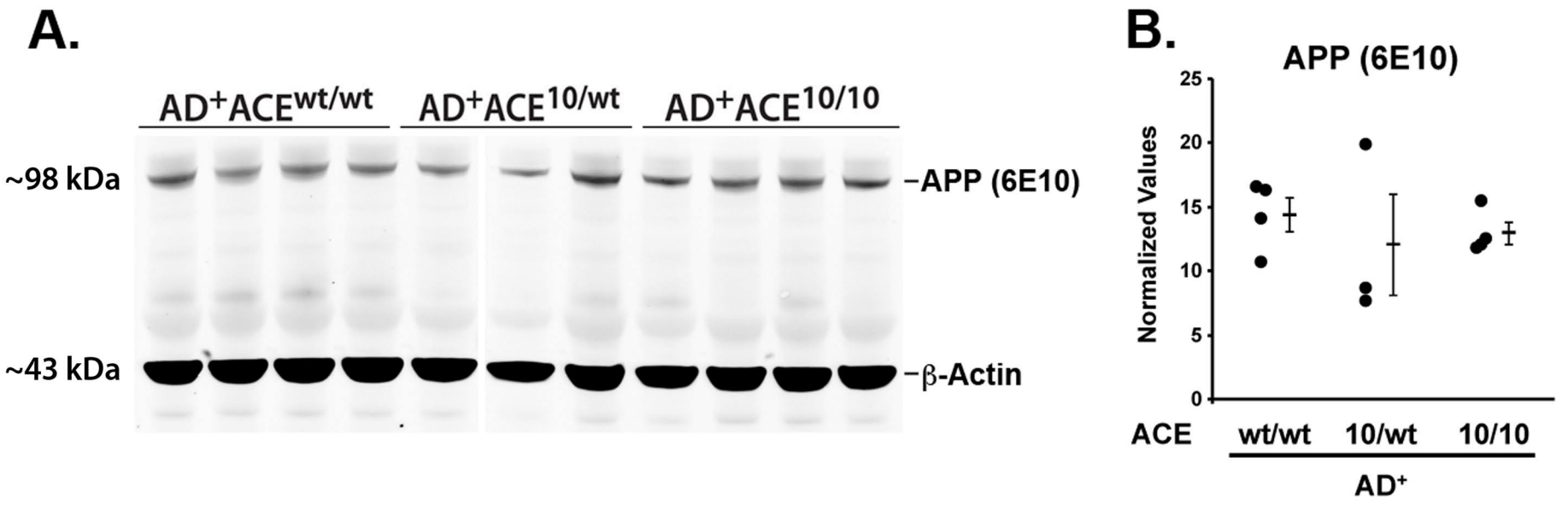

Figure S2. Amyloid precursor protein (APP) expression is not significantly different in $AD^{+}ACE^{10/WT}$ and $AD^{+}ACE^{10/10}$ mice. A Western blot of brain homogenates from 5-month-old $AD^{+}ACE^{10/WT}$, $AD^{+}ACE^{10/10}$, and age-matched control $AD^{+}ACE^{WT/WT}$ mice were probed for transgenic-derived APP using the monoclonal antibody 6E10. (**A**) A representative blot probed with 6E10 and anti-β-actin. The lanes were run on the same gel but were noncontiguous. (**B**) Densitometry of APP normalized to β-actin revealed no significant changes in APP levels. Data from individual mice and the group means and SEM are shown.

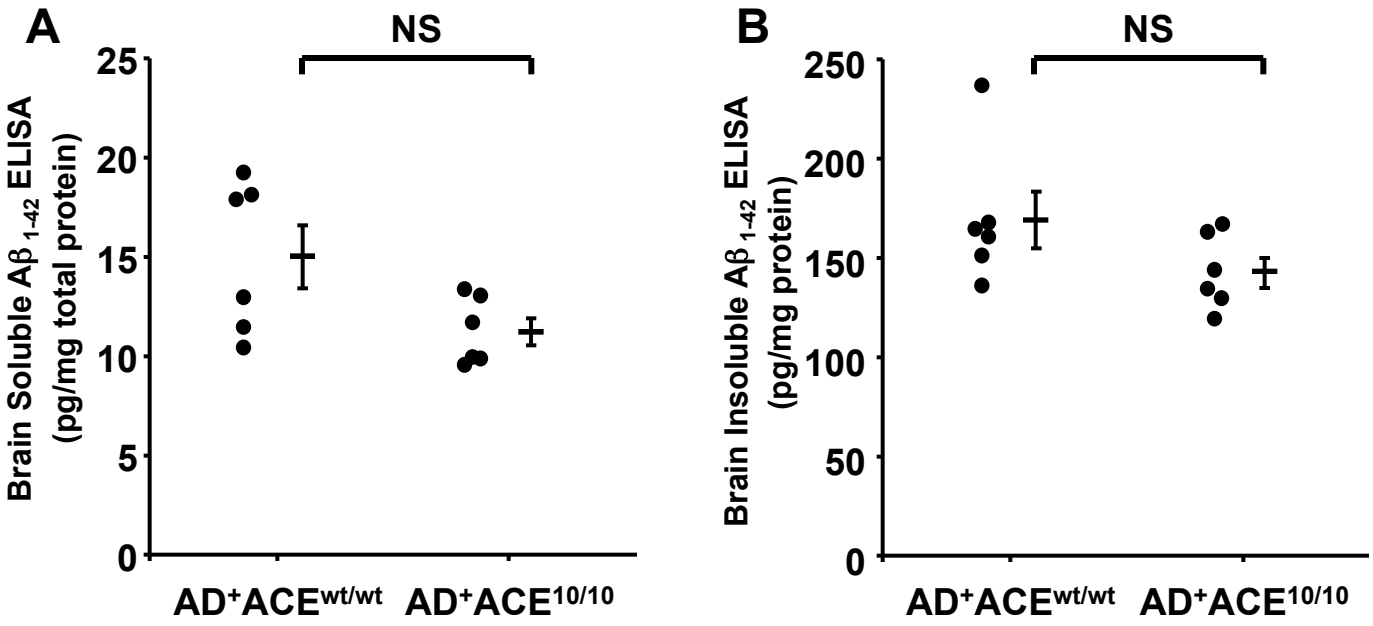

Figure S3. Brain $A\beta_{1-42}$ levels are equivalent in 3 month old mice. ELISA measurements of soluble (**A**) and insoluble (**B**) human $A\beta_{1-42}$ in the brains of 3 month old $AD^{+}ACE^{wt/wt}$ or $AD^{+}ACE^{10/10}$ mice. Data from individual mice, as well as the group means and SEM, are shown. At this age, there is no significant (NS) difference in both soluble and insoluble $A\beta_{1-42}$ between the two groups of mice.

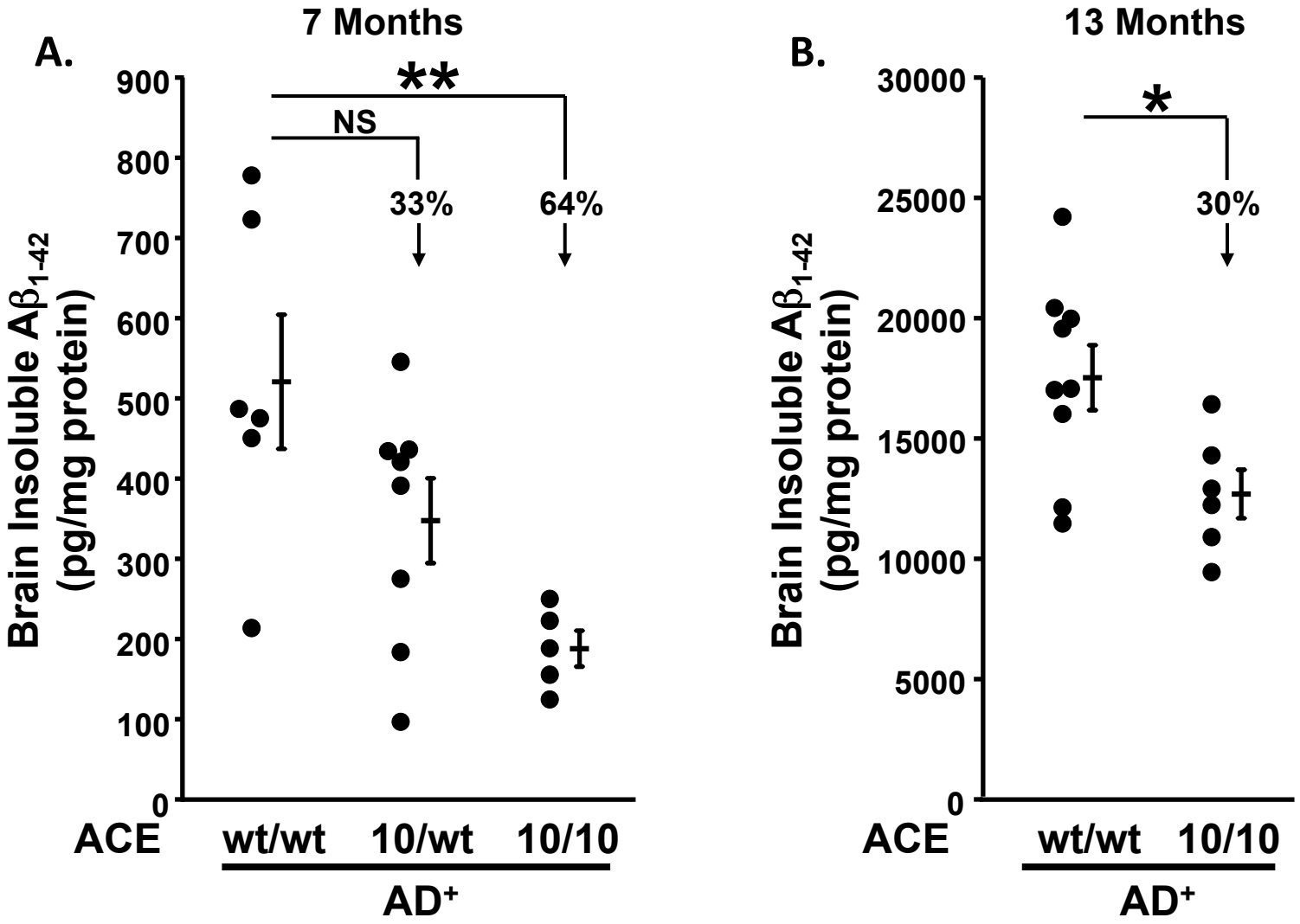

Figure S4. Reduced insoluble $A\beta_{1-42}$ levels in $AD^{+}ACE^{10/10}$ mice. Quantitative ELISA was used to measure insoluble human $A\beta_{1-42}$ in the brains of 7 month old $AD^{+}ACE^{WT/WT}$, $AD^{+}ACE^{10/WT}$, and $AD^{+}ACE^{10/10}$ mice and of 13 month old $AD^{+}ACE^{WT/WT}$ and $AD^{+}ACE^{10/10}$ mice. Data from individual mice, as well as the group means and SEM, are shown. Arrows indicate the percentage reduction in group means, as compared to $AD^{+}ACE^{WT/WT}$ mice. NS is non significant. * $P<0.05$, ** $P<0.001$

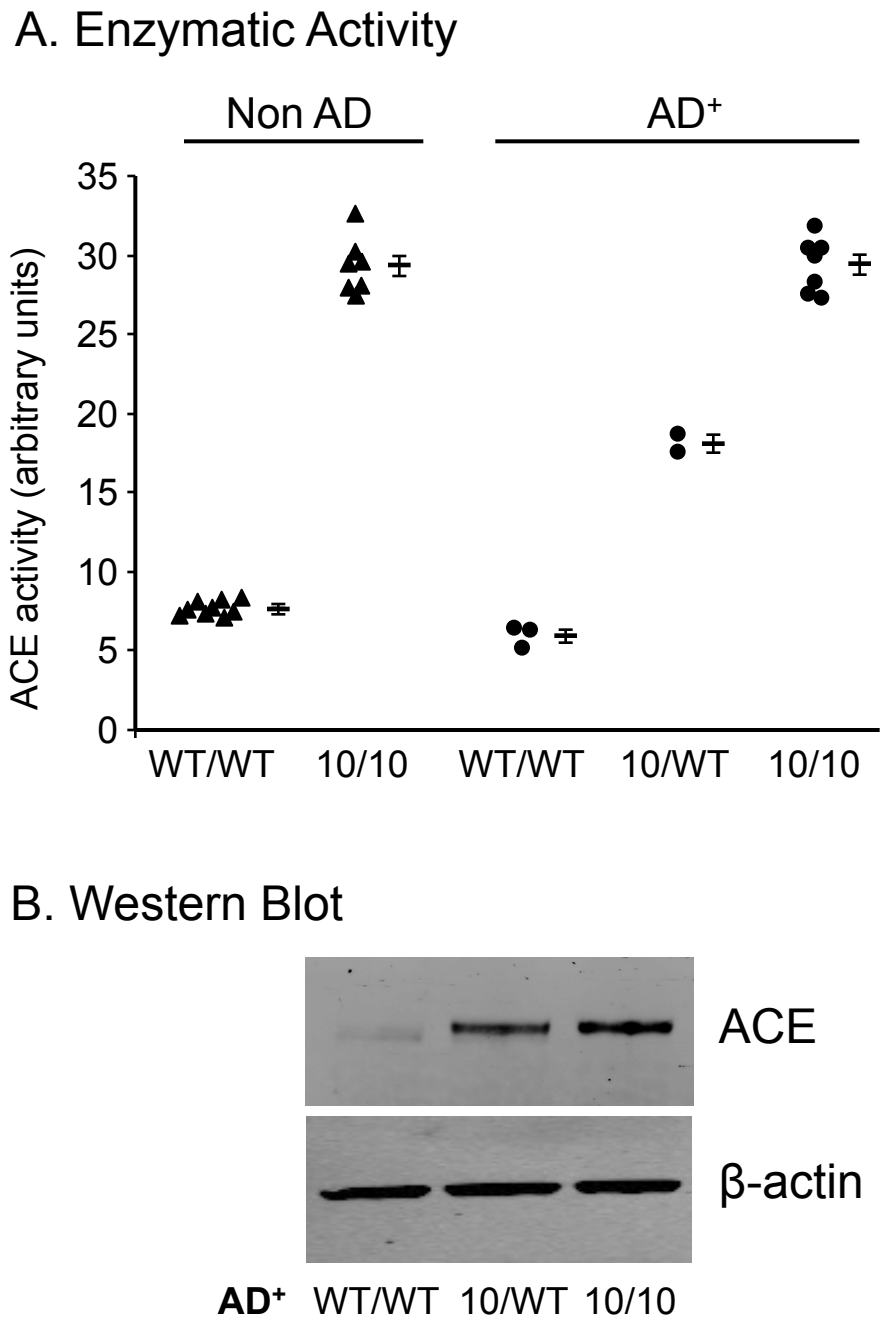

Figure S5. Increased brain ACE levels in $ACE^{10/WT}$ **and** $ACE^{10/10}$ Mice. Brains from $ACE^{WT/WT}$, $ACE^{10/WT}$ and $ACE^{10/10}$ mice were analyzed for both ACE activity and expression. AD^+ mice were 9 months old, while non-AD mice were 8 months old. (**A**) ACE activity was measured using the ACE-REA kit and was defined as that inhibitable by captopril. ACE activity is shown for individual mice, as well as group means and SEM. (**B**) ACE expression levels in the brain were assessed by Western blots probed with a rabbit polyclonal anti-mouse ACE antibody. β-actin staining showed equal loading. A substantial, and gene dosage dependent, increase in brain ACE activity and expression was found in $AD^+ACE^{10/WT}$ and in $AD^+ACE^{10/10}$ mice.

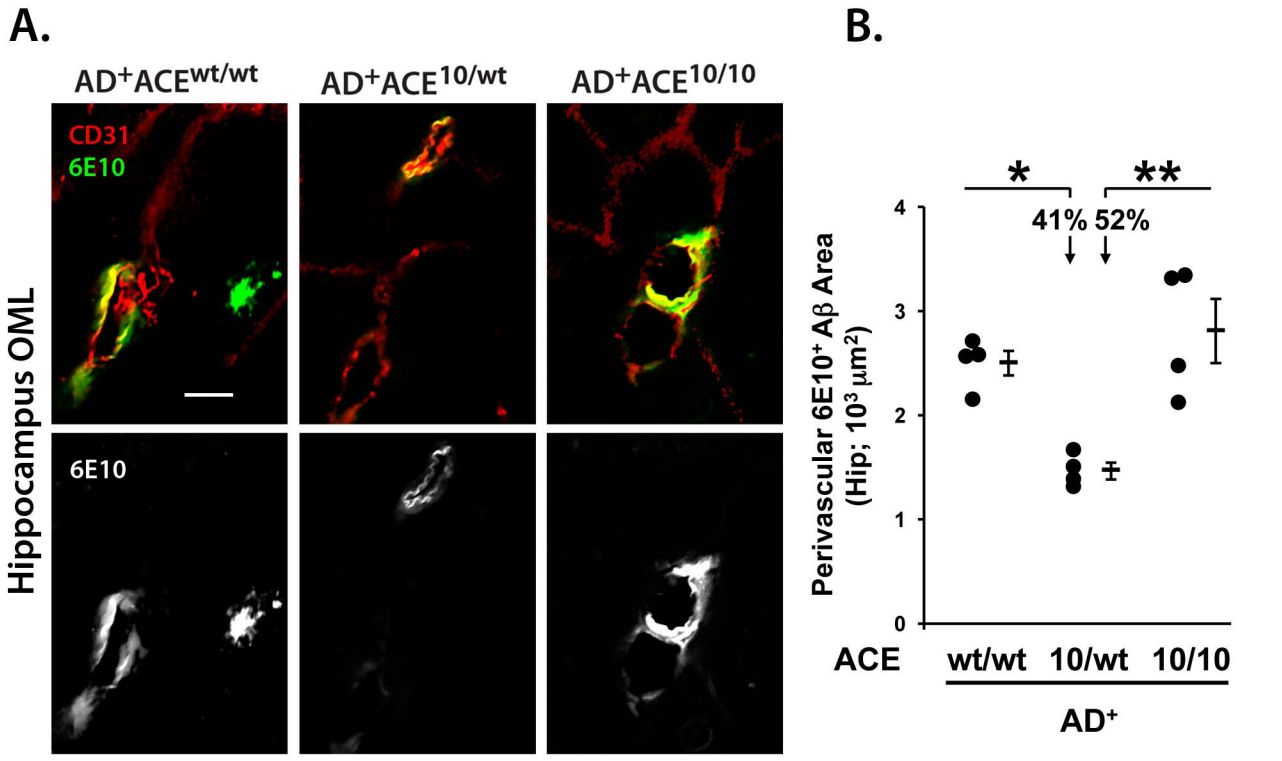

Figure S6. Perivascular Aβ deposition in AD$^+$ACE$^{10/WT}$ and AD$^+$ACE$^{10/10}$ mice. (**A**) Representative images of fluorescent-based immunohistochemistry. Paraformaldehyde-fixed hippocampal sections of mice 7-8 months of age were co-labeled with anti-CD31 (red), an endothelial cell marker, and with anti-human Aβ (6E10; green). Serial sections were quantitatively assessed for perivascular Aβ in blood vessels from the outer molecular layer (OML) of the hippocampal fissure. Scale bar: 20 μm. (**B**) Data from individual mice, as well as group means and SEM, are indicated, with the percentage reductions in mean plaque area. Perivascular Aβ deposition is significantly reduced in AD$^+$ACE$^{10/WT}$ mice compared to AD$^+$ACE$^{WT/WT}$ mice. There is no statistical difference between AD$^+$ACE$^{10/10}$ and AD$^+$ACE$^{WT/WT}$ mice. * P<0.05, ** P<0.001

Exp. Group	Sample size	Entorhinal Cortex	Cingulate Cortex	Hippocampus	Total Brain
$AD^{+}ACE^{wt/wt}$	N=6	**29.5 ± 4.8**	**19.1 ± 3.0**	**53.4 ± 8.3**	**102.1 ± 12.6**
$AD^{+}ACE^{10/10}$	N=6	**10.2 ± 1.1**	**16.6 ± 9.6**	**31.1 ± 4.7**	**57.9 ± 6.2**
$AD^{+}ACE^{10/10}$ + Hydralazine	N=4	**11.8 ± 2.2**	**12.2 ± 1.5**	**27.6 ± 4.6**	**51.5 ± 6.6**
$AD^{+}ACE^{10/10}$ + Ramipril (ACE-inh)	N=4	**31.1 ± 5.4**	**18.0 ± 3.6**	**50.1 ± 5.5**	**99.3 ± 3.5**

Figure S7. Effects of ACE inhibitor on Aβ plaque area in several brain regions. 13 month old AD^{+} mice of the indicated ACE genotypes were sacrificed and the overall cortical and hippocampal Aβ plaque area was determined by quantitative immunohistochemistry after staining 3-6 serial corresponding histologic sections with 6E10 mAb. Mice treated with either hydralazine or the ACE inhibitor ramipril received the drug for 60 days before sacrifice. The Total Brain plaque area is plotted in Figure 5b. Values are in means ± SEM of $6E10^{+}$-immunoreactive area ($\mu m^2 \times 10^3$).

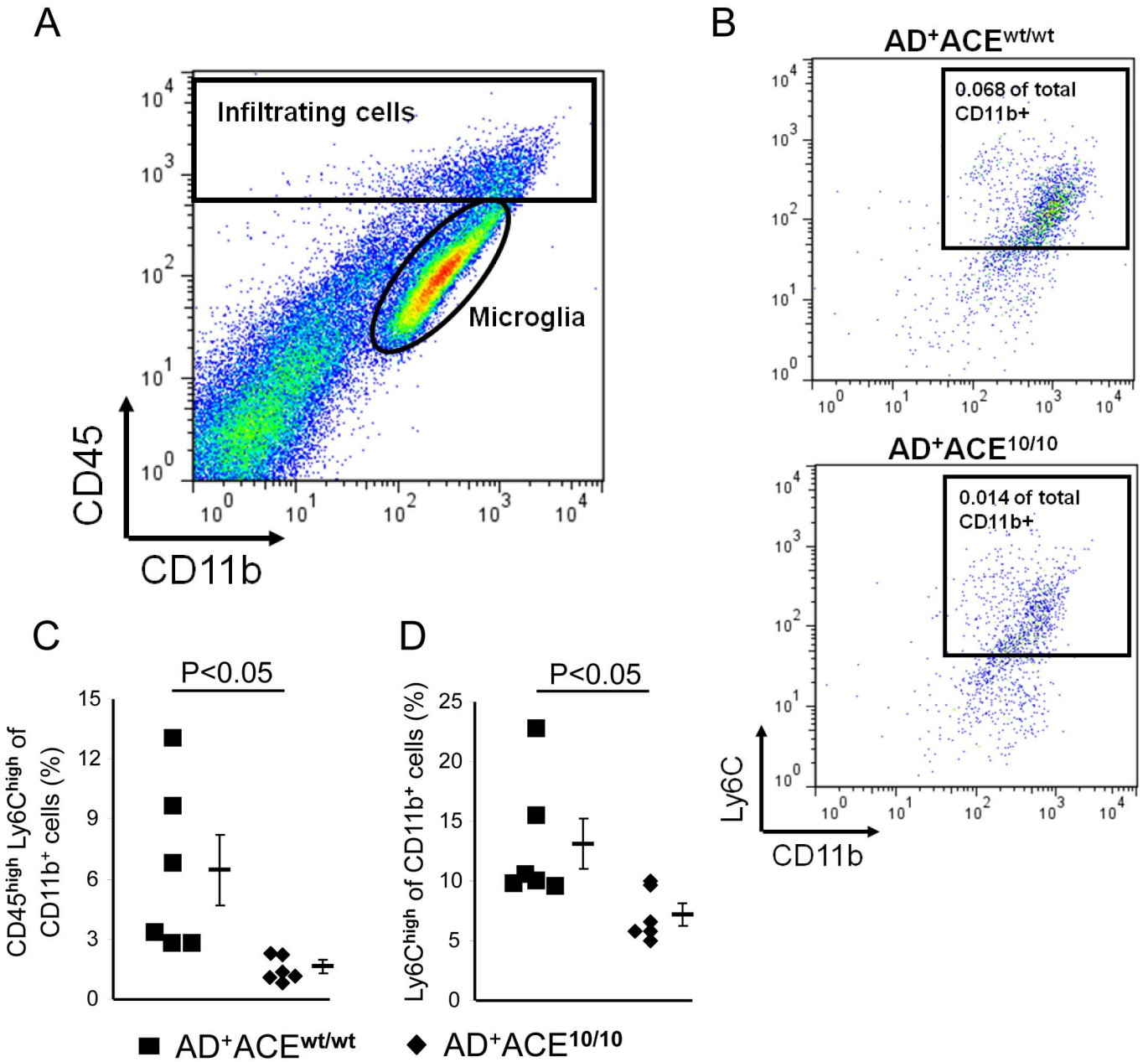

Figure S8. **Reduced inflammation in the brains of AD^{+}ACE$^{10/10}$ mice**. Brains were perfused before harvest. Infiltrating cells and microglia were purified using a Percoll gradient. (**A**) For flow cytometric analysis, infiltrating cells and microglia were defined as CD45^{high} and CD45$^{low\text{-}intermediate}$CD11b^{+}, respectively. (**B**) The CD45^{high} cells were further gated for Ly6C^{high} and CD11b^{+} expression. (**C**) Flow data showing the percent of total CD11b^{+} cells that are also CD45^{high}Ly6C^{high} for individual mice, as well as group means and SEM. (**D**) The percent of total CD11b^{+} cells that are also Ly6C^{high}. These data indicate that fewer blood-borne monocytic cells (CD45^{high}Ly6C^{high}CD11b^{+}) are present in the brains of AD^{+}ACE$^{10/10}$ mice as compared to AD^{+}ACE$^{WT/WT}$ mice. Mice were 9.9 months to 14.5 months old; there was no statistical difference in age between the two groups (P=0.097).

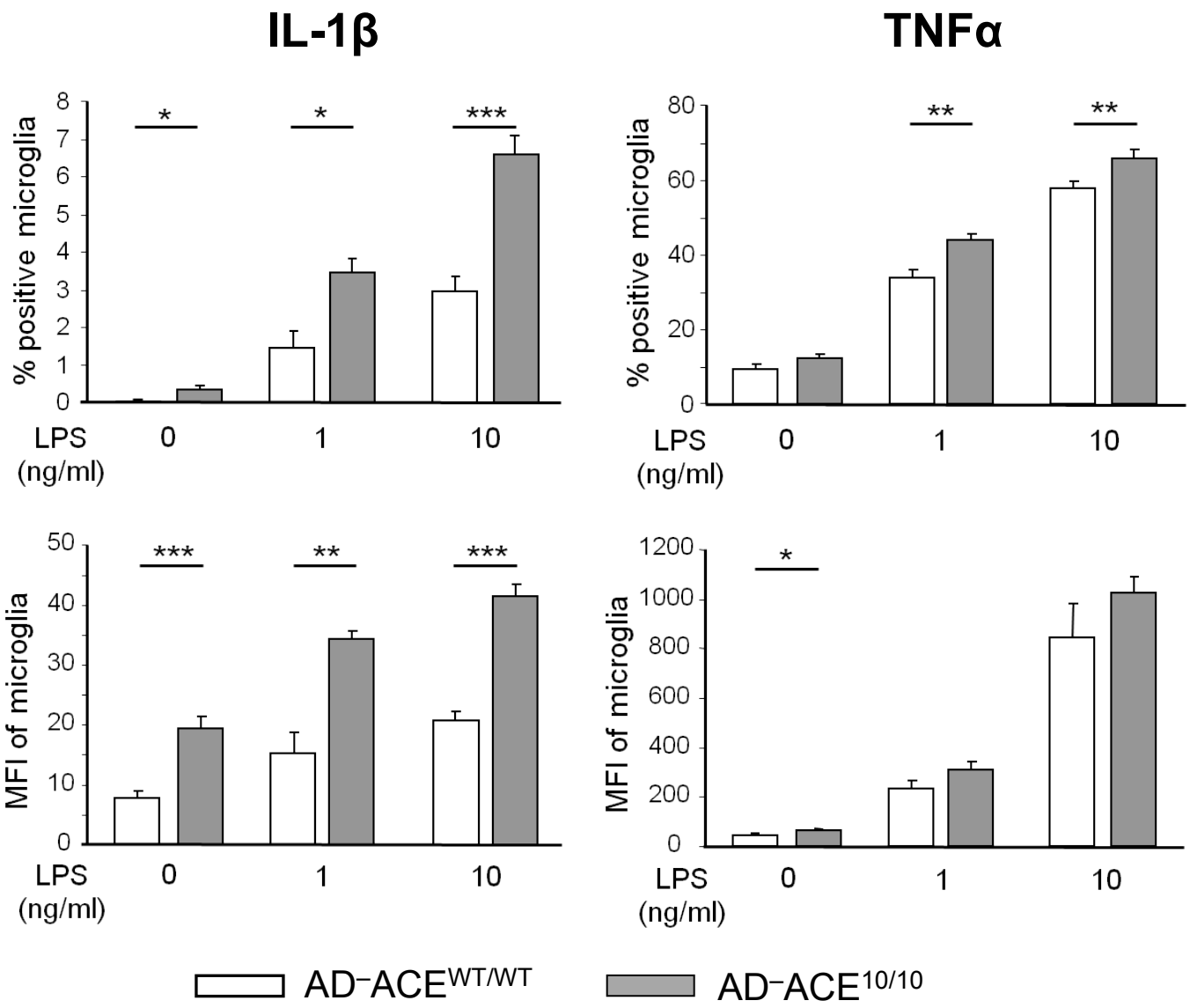

Figure S9. Altered immune reactivity of microglia isolated from brains of ACE$^{10/10}$ mice. The brains of adult AD⁻ACE$^{10/10}$ and AD⁻ACE$^{WT/WT}$ mice were collected, perfused and digested with collagenase IV. Microglia and any non-resident inflammatory cells were then collected after separation on a Percoll gradient, washed and incubated with Brefeldin A for 6 hr with or without LPS stimulation. Cells were stained for surface CD45 and CD11b, as well as intracellular IL-1β and TNFα. Microglia, defined as CD45$^{low\text{-}intermediate}$CD11b^{+}, were evaluated for cytokine expression by flow cytometry. The top two panels show the percent positive cells for IL-1β and TNFα; the bottom two panels show the mean florescent intensity (MFI) for IL-1β and TNFα. In response to LPS, there is a mild increased expression of the pro-inflammatory cytokines IL-1β and TNFα by ACE$^{10/10}$ microglia. *P<0.05, **P<0.02, ***P<0.01; n=6 per group.