Blocking Tgf-β/Smad2/3 innate immune signaling mitigates Alzheimer-like pathology

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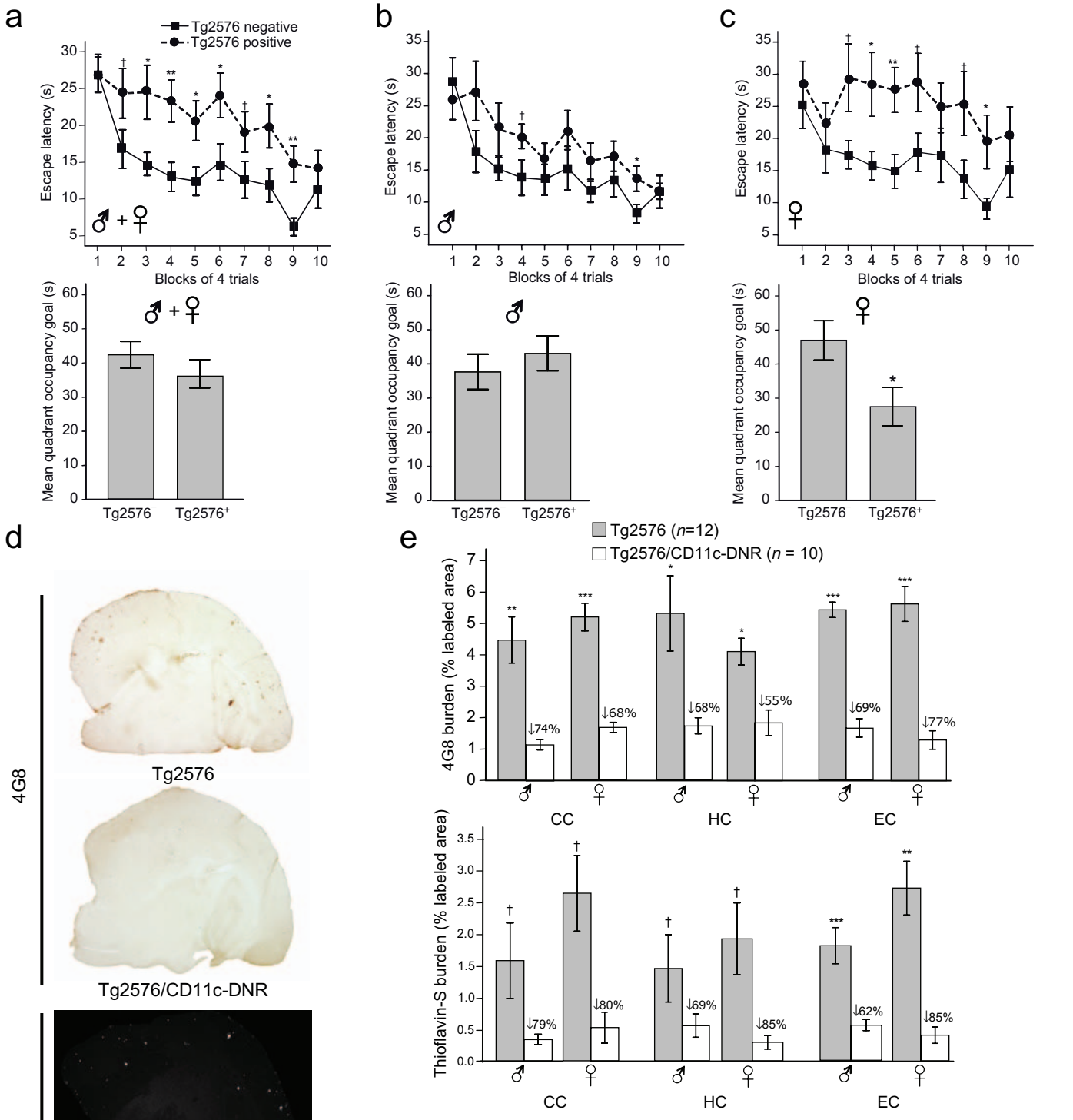
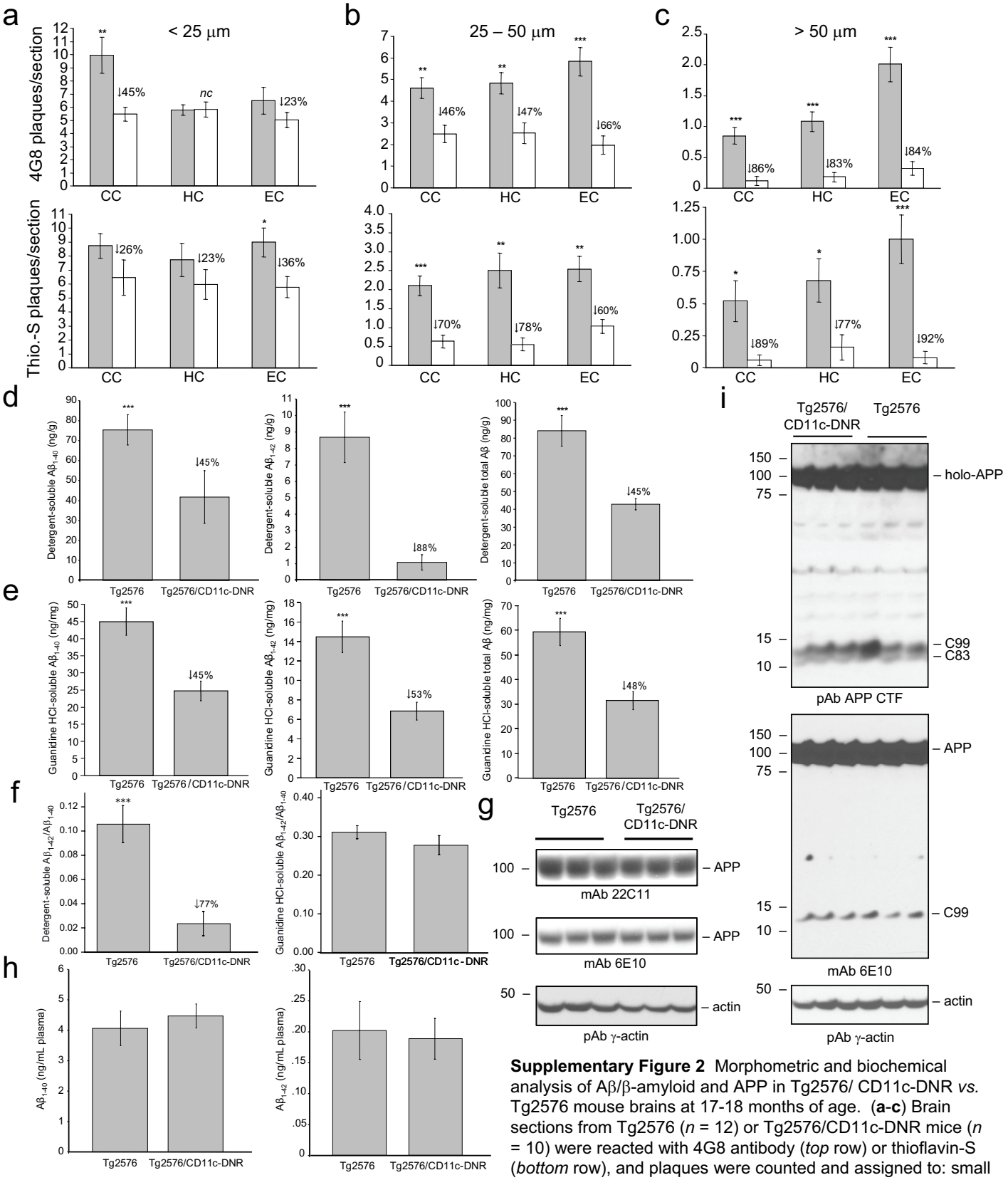
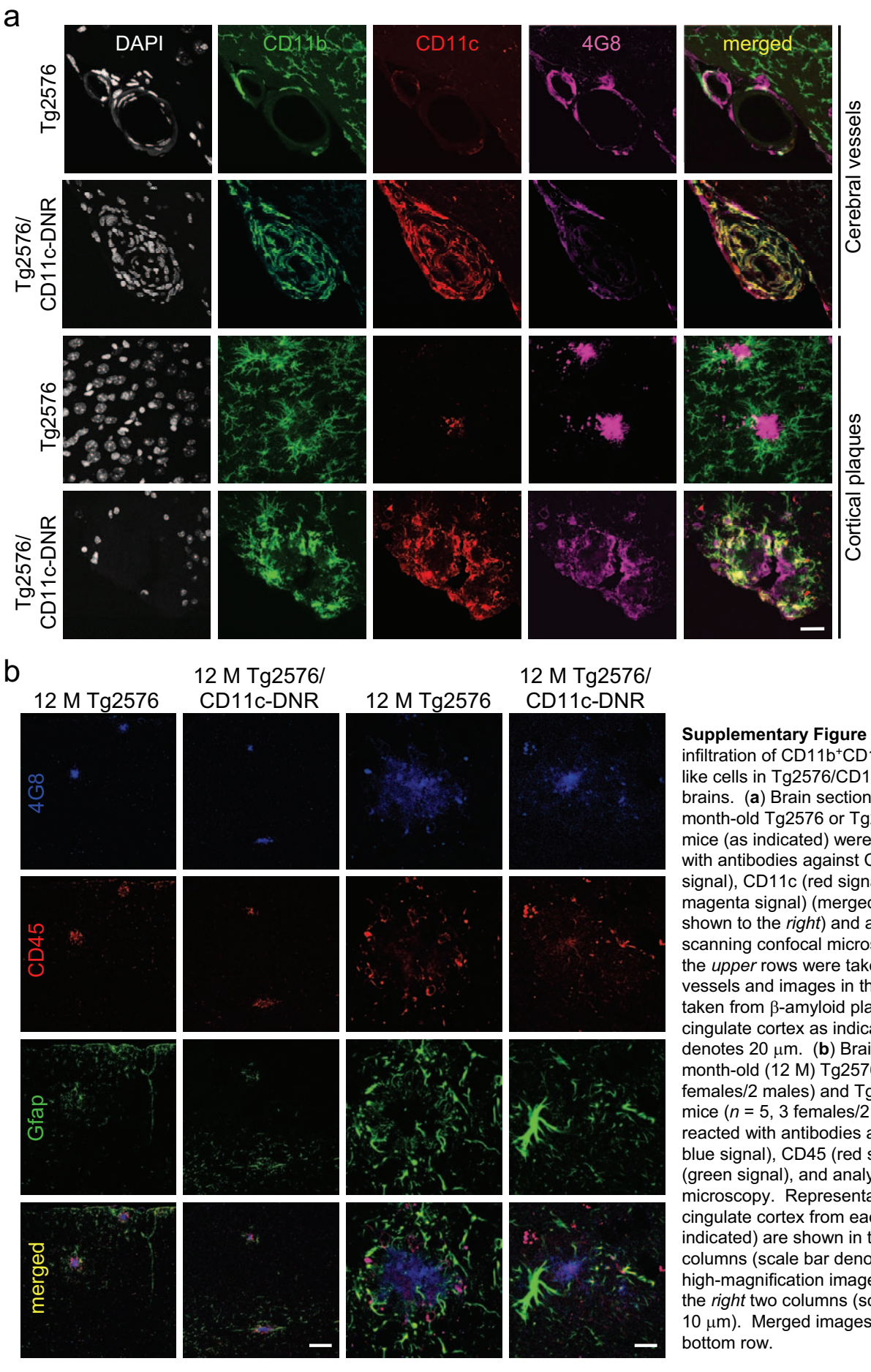
Supplementary Information



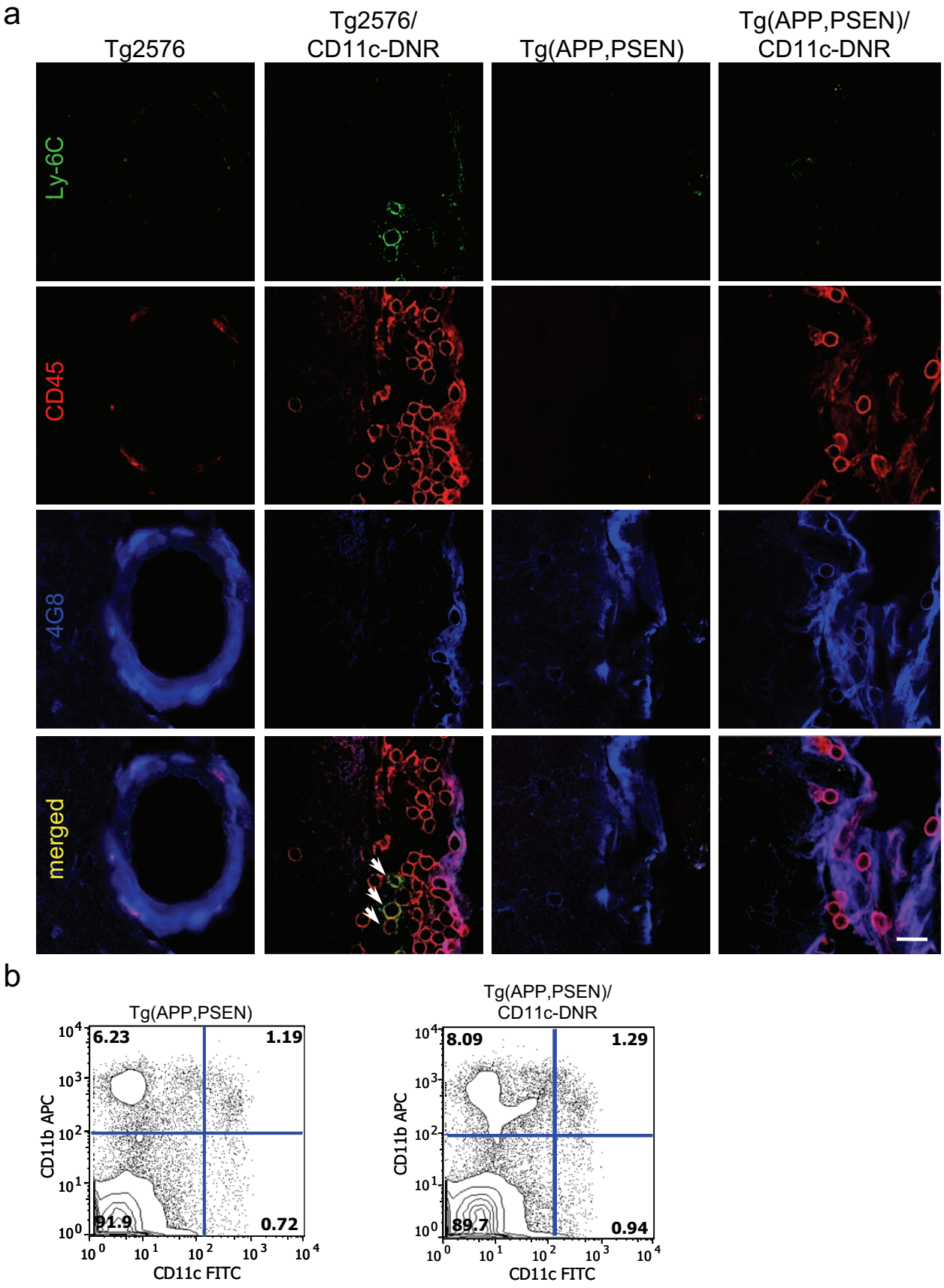
Supplementary Figure 1 Gender- and Tg2576 transgene-dependent Morris water maze impairment and β-amyloid plaque reduction in Tg2576/CD11c-DNR *vs.* Tg2576 mice. (**a-c**) Escape latency in the visible platform (s, *top*) or day 10 probe trial quadrant occupancy (s, *bottom*) group means ± SEM. (**a**) Combined males and females, (**b**) males, or (**c**) females are shown. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, † a trend of *P* < 0.10 for Tg2576^{+} *vs*. Tg2576^{-} groups. (**d**) Representative low-magnification photomicrographs from Tg2576 or Tg2576/CD11c-DNR mouse brain sections stained as indicated. (**e**) Tg2576 (*n* = 12; 5 females and 7 males) or Tg2576/CD11c-DNR (*n* = 10; 7 females and 3 males) mouse brains were analyzed for 4G8 (*top*) or Thioflavin-S "burden" (*bottom*). "Burden" values (% labeled area, group means ± SEM with percentage reduction) are shown on the *y*-axis and brain region (CC, cingulate cortex; HC, hippocampus; EC, entorhinal cortex) sorted into males and females is shown on the *x*-axis. † a trend of *P* < 0.10, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, when comparing Tg2576/ CD11c-DNR *vs.* Tg2576 mice within brain region and gender by *t*-test.



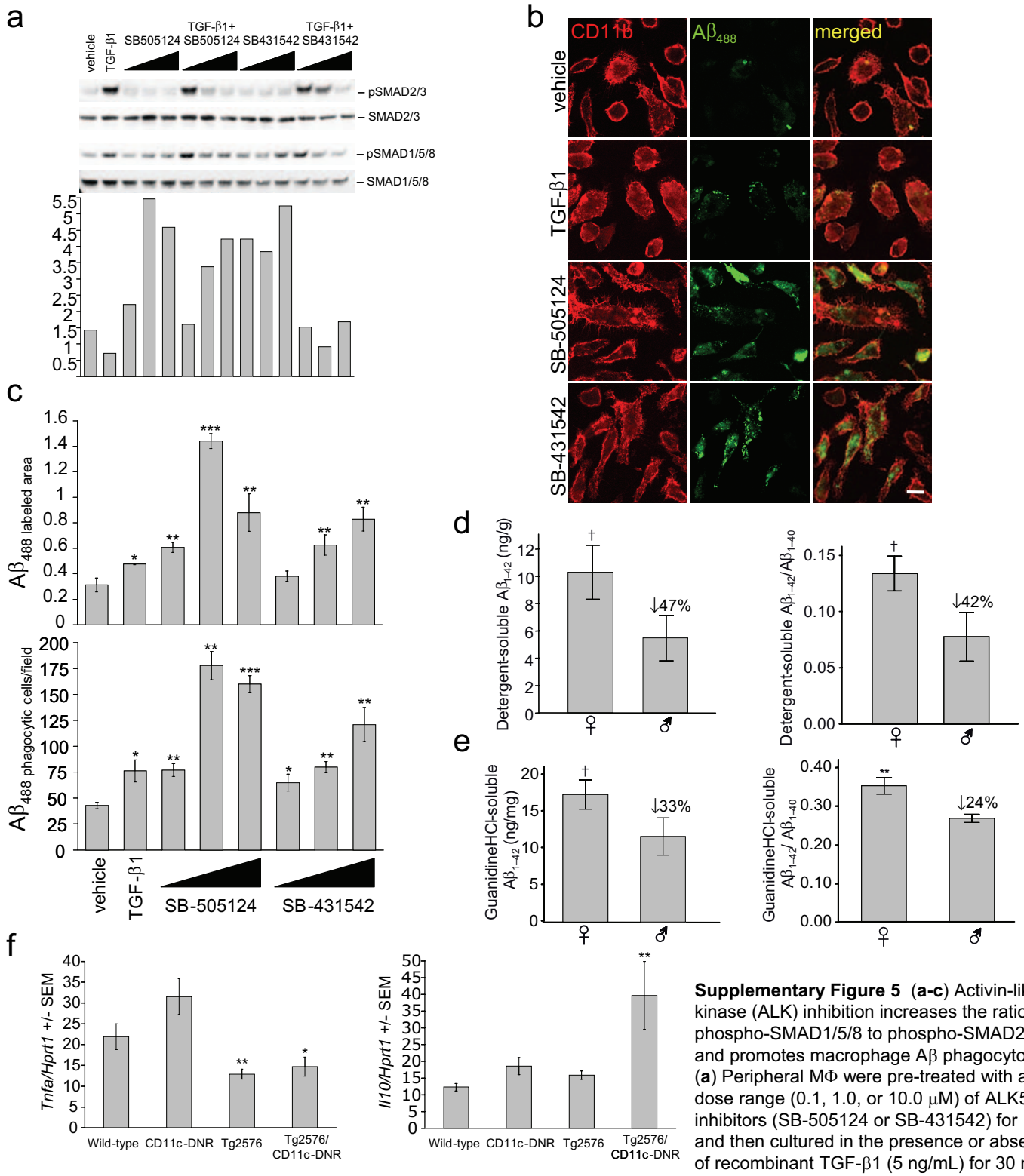
Supplementary Figure 2 Morphometric and biochemical analysis of Aβ/β-amyloid and APP in Tg2576/ CD11c-DNR *vs.* Tg2576 mouse brains at 17-18 months of age. (**a**-**c**) Brain sections from Tg2576 (*n* = 12) or Tg2576/CD11c-DNR mice (*n* = 10) were reacted with 4G8 antibody (*top* row) or thioflavin-S (*bottom* row), and plaques were counted and assigned to: small (< 25 μm; *left*), medium (from 25 to 50 μm; *middle*), or large (> 50 μm; *right*) (group means ± SEM with percentage reduction). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, when comparing Tg2576/CD11c-DNR *vs*. Tg2576 mice within brain region by *t*-test. (**d**-**f**) Two-step extracted brain homogenates were assayed for detergent-soluble (**d**) or 5M guanidine HCl-soluble (**e**) human $A\beta_{1-40}$ (*left*), $A\beta_{1-42}$ (*middle*), or total Aβ (*right*). $A\beta_{1-42}/A\beta_{1-40}$ ratios are shown in (**f**), ****P* < 0.001. (**g**) Western blots for brain APP and actin (control), and (**h**) blood plasma steady-state $A\beta_{1-40}$ (*left*) or $A\beta_{1-42}$ (*right*) levels are shown. (**i**) Western blots of detergent-soluble brain homogenates using an APP carboxyl-terminal fragment antibody (Ab) (*top*; full-length (holo)-APP and APP carboxyl-terminal fragments generated by amyloidogenic (C99) or non-amyloidogenic (C83) cleavage are shown) or antibody 6E10 against the N-terminus of human Aβ (*bottom*; holo-APP, soluble APP species, and C99 fragments are shown, and actin is a loading control).



Supplementary Figure 3 Age-dependent infiltration of $CD11b^{+}CD11c^{+}$ macrophage-like cells in Tg2576/CD11c-DNR mouse brains. (**a**) Brain sections from 17-18 month-old Tg2576 or Tg2576/CD11c-DNR mice (as indicated) were immunolabeled with antibodies against CD11b (green signal), CD11c (red signal), or Aβ (4G8, magenta signal) (merged images are shown to the *right*) and analyzed by laser scanning confocal microscopy. Images in the *upper* rows were taken from cerebral vessels and images in the *lower* rows were taken from β-amyloid plaques in the cingulate cortex as indicated. Scale bar denotes 20 μm. (**b**) Brain sections from 12 month-old (12 M) Tg2576 (*n* = 5, 3 females/2 males) and Tg2576/CD11c-DNR mice (*n* = 5, 3 females/2 males) were reacted with antibodies against Aβ (4G8, blue signal), CD45 (red signal), or Gfap (green signal), and analyzed by confocal microscopy. Representative images of cingulate cortex from each genotype (as indicated) are shown in the *left* two columns (scale bar denotes 50 μm), and high-magnification images are shown in the *right* two columns (scale bar denotes 10 μm). Merged images are shown in the bottom row.



Supplementary Figure 4 Levels of peripheral and CNS macrophages in AD and crossed mice. (**a**) Brain sections from Tg2576 and Tg2576/CD11c-DNR mice at 17-18 months of age, or Tg(APP,PSEN) and Tg(APP,PSEN)/CD11c-DNR mice at 15 months of age were reacted with antibodies against Ly-6C (green signal), CD45 (red signal), or Aβ (4G8, blue signal), and confocal images are shown of cerebral vessels in the entorhinal cortex. Merged images are shown in the bottom row, and arrowheads indicate cells double-positive for Ly-6C and CD45. Scale bar denotes 10 μm. (**b**) FACS dot-plots showing similar levels of peripheral $CD11b^{+}CD11c^{+}$ cells isolated from spleens of Tg(APP,PSEN) and Tg(APP,PSEN)/CD11c-DNR mice at 15 months of age.



Supplementary Figure 5 (**a-c**) Activin-like kinase (ALK) inhibition increases the ratio of phospho-SMAD1/5/8 to phospho-SMAD2/3 and promotes macrophage Aβ phagocytosis. (**a**) Peripheral MΦ were pre-treated with a dose range (0.1, 1.0, or 10.0 μM) of ALK5 inhibitors (SB-505124 or SB-431542) for 1 h and then cultured in the presence or absence of recombinant TGF-β1 (5 ng/mL) for 30 min as indicated. Upper panels show Western blots for phospho (p) or total forms of SMAD2/3 or SMAD1/5/8. Lower panel is a histogram showing the ratio of pSMAD1/5/8 to pSMAD2/3. (**b**) Primary MΦ were cultured in the presence or absence of TGF-β1 or ALK5 inhibitors for 1 h, pulsed for 4 h with 2 μg/mL "pre-aggregated" HiLyte Fluor™ 488-labeled $A\beta_{1–42}$ ($A\beta_{488}$), and chased for 15 min prior to analysis by laser scanning confocal microscopy in conjunction with CD11b (red signal) antibody (merged images are shown in the right panels, images are from a 1.0 μM ALK5 inhibitor dose). (**c**) Quantification of confocal images (n = 3 randomly-selected 10X magnification fields per group, group means ± SD) was performed and $A\beta_{488}$-labeled area is shown in the *upper* graph, and numbers of $A\beta_{488}$ phagocytic cells/field are shown in the *lower* graph. (**d-f**) Gender-dependent reductions in Aβ levels in $Tg2576^{+}$ animals and anti-inflammatory responses in brains of crossed mice. Biochemical analyses of (**d**) detergent-soluble and (**e**) guanidine HCl-soluble $A\beta_{1–42}$ and $A\beta_{1–42}/A\beta_{1–40}$ in male *vs*. female Tg2576 positive mice (irrespective of CD11c-DNR transgene status). (**f**) Q-PCR results for *Tnfa* (*left*) or *Il10* (*right*) expression (relative to *Hprt1*) are shown for four groups of mice, and statistical comparisons are *vs*. wild-type mice. For (**c-f**), † a trend of $P < 0.10$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

	Behavioral Assay									
Test	Activity	Y-maze		Morris water maze						
					Probe trial day 5			Probe trial day 10		
Measure	Distance traveled	Arm entries	% alternation	Latency	goal quadrant occupancy	goal platform crossings	% goal platform crossings	goal quadrant occupancy	goal platform crossings	% goal platform crossings
Genotype groups	3	3	3	2	2	2	2	2	2	2
Variables										
gender	$F_{(1,34)}$=0.01 p=0.93	$F_{(1,33)}$=2.43 p=0.13	$F_{(1,33)}$=1.09 p=0.31	$F_{(1,35)}$=3.88 p=0.06	$F_{(1,35)}$=0.10 p=0.76	$F_{(1,35)}$=1.89 p=0.18	$F_{(1,35)}$=0.19 p=0.67	$F_{(1,35)}$=0.34 p=0.57	$F_{(1,35)}$=0.53 p=0.47	$F_{(1,35)}$=0.44 p=0.51
time	$F_{(3.8,129.8)}$=7.96 p<0.001	~ ~	~ ~	$F_{(9,315)}$=8.69 p<0.001	~ ~	~ ~	~ ~	~ ~	~ ~	~ ~
genotype	$F_{(2,34)}$=4.61 p<0.05	$F_{(2,33)}$=7.61 p<0.01	$F_{(2,33)}$=4.05 p<0.05	$F_{(1,35)}$=10.81 p<0.01	$F_{(1,35)}$=1.46 p=0.24	$F_{(1,35)}$=1.83 p=0.19	$F_{(1,35)}$=0.51 p=0.48	$F_{(1,35)}$=1.65 p=0.21	$F_{(1,35)}$=0.09 p=0.77	$F_{(1,35)}$=0.0001 p=0.99
gender X genotype	$F_{(2,34)}$=0.27 p=0.77	$F_{(2,33)}$=0.22 p=0.81	$F_{(2,33)}$=0.31 p=0.74	$F_{(1,35)}$=1.49 p=0.23	$F_{(1,35)}$=2.81 p=0.10	$F_{(1,35)}$=2.13 p=0.15	$F_{(1,35)}$=0.90 p=0.35	$F_{(1,35)}$=5.24 p=0.03	$F_{(1,35)}$=2.09 p=0.16	$F_{(1,35)}$=2.34 p=0.14
genotype X time	$F_{(7.6,129.8)}$=1.22 p=0.30	~ ~	~ ~	$F_{(9,315)}$=1.22 p=0.28	~ ~	~ ~	~ ~	~ ~	~ ~	~ ~
gender X time	$F_{(3.8,129.8)}$=1.37 p=0.25	~ ~	~ ~	$F_{(9,315)}$=1.33 p=0.22	~ ~	~ ~	~ ~	~ ~	~ ~	~ ~
gender X genotype X time	$F_{(7.6,129.8)}$=0.89 p=0.53	~ ~	~ ~	$F_{(9,315)}$=0.62 p=0.78	~ ~	~ ~	~ ~	~ ~	~ ~	~ ~

Supplementary Table 1 ANOVA results for behavioral testing. Multiple ANOVA models were designed with gender, genotype, and (where appropriate) time as covariates. Genotype was coded as three groups (including combined wild-type and CD11c-DNR mice as controls, Tg2576 mice, or Tg2576/CD11c-DNR mice) or two groups (Tg2576 positive or Tg2576 negative mice). Main effects and interactive terms are shown for each behavioral measure. Stratification by gender produced similar trends in both males and females (data not shown).

Mouse ID	Genotype	Score
02	+/+	++
03	+/+	++
05	+/–	–
09	+/–	+/–
10	+/–	+/–
13	+/+	++
28	+/–	–
41	+/+	+++
49	+/+	+++
50	+/–	–

Supplementary Table 2 Semi-quantitative histology results for $CD45^{+}CD11b^{+}$ infiltrating MΦ in individual Tg (APP,PSEN) or Tg(APP,PSEN)/CD11c-DNR mice at 15 months of age. Brain sections (4 per mouse, spaced 50 μm apart) from individual Tg(APP,PSEN) or Tg(APP,PSEN)/CD11c-DNR mice were immunostained with antibodies against CD45 and CD11b and cortical areas and hippocampus were scored on a five-point semi-quantitative scale for presence of infiltrating MΦ. Mouse identification (ID) numbers are shown in the *left* column, mouse genotypes (formatted as Tg(APP,PSEN)/CD11c-DNR) are shown in the *middle* column, and average scores from each set of sections per mouse are shown in the *right* column. Scoring was as follows: (–), absence of cells; (+/–), very few cells present; (+) cells mildly present; (++), cells moderately present; (+++), cells present in large quantity; $P < 0.01$ when comparing +/+ to +/- mouse groups.

Supplementary methods

Behavioral analyses. We evaluated exploratory activity by individually placing mice into a novel environment with fresh bedding, and monitoring their activity for 20 min by an overhead CCD camera and computerized tracking system (Ethovision®; Noldus). Data are reported as distance traveled (cm) per 2.5-min time bin. We assayed spontaneous alternation and total arm entries essentially as described elsewhere [1-3]. Briefly, we individually placed mice in one arm of a radially symmetric Y-maze made of opaque black acrylic (arms: 40 cm long, 4 cm wide; walls: 30 cm tall), and recorded the sequence of arm entries and total number of entries over a period of 8 min, beginning when the animal first entered the central area. Percentage of alternation was defined as the number of sequential triplets of arm visits during the session as a proportion of total triplets of arm visits (*i.e.*, visiting arms A-B-C or C-B-A constituted a sequential triplet, while A-B-A or A-C-A did not).

We performed testing in the Morris water maze essentially as previously described [1,4]. The water maze consisted of a circular pool (diameter of 1 m) filled with water made opaque with non-toxic white paint maintained at 23-26 ^{o}C. The 12.5 cm square plexiglass platform was located 1 cm below the water surface. After a minimum of 20 min habituation to the room, we placed mice in the pool and allowed them to search for the platform for 60 s. We guided animals to the platform that did not locate it within 60 s, and allowed them to remain there for 15 s before returning them to their cages. We trained mice four times per day with a 20 min inter-trial interval. On the first two days, we placed a visible cue on the platform and randomly varied its location among four possible locations. Then, we trained animals for an additional 10 days with the platform

invisible below the surface of the water and in an invariant location (counterbalanced across mice). We performed probe trials, in which the animal was placed in the pool in the absence of the escape platform and its search was monitored for 30 s, on days 5 and 10 of hidden platform training. We performed all behavioral tests in a room (6'x 8') with indirect lighting and multiple visible cues on the walls. We recorded trials using an overhead CCD camera and analyzed them using Noldus:Ethovision®. We performed all trials at the same time of day (± 1 h), during the animals' light phase. An examiner (C.P.) who was blind to mouse genotype performed behavioral analyses.

Aβ ELISA and Bio-Plex cytokine assays. We analyzed brain homogenates (detergent- or 5 M guanidine HCl-soluble protein fractions [5]) or EDTA-treated plasma samples from Tg2576 or Tg2576/CD11c-DNR mice at 17-18 months of age (taken at time of sacrifice) by sandwich ELISA for human $A\beta_{1-40}$ or $A\beta_{1-42}$, or total human Aβ (estimated by summing $A\beta_{1-40}$ and $A\beta_{1-42}$ values) using commercially available kits strictly according to the manufacturer's instruction (Invitrogen-Biosource). We used dilution factors of 1:10, 1:5,000, and 1:4 for detergent-soluble brain homogenates, guanidine HCl-soluble brain homogenates, and plasma samples, respectively, and all samples fell within the linear range of standard curves. ELISA values are reported as ng of $A\beta_{1-x}$/wet g (or mg) of brain. We assayed cytokines in detergent-soluble brain homogenates at a 1:1 dilution using the Beadlyte® mouse multi-cytokine detection system 2 [allows simultaneous detection of interleukin (Il)-1β, Il-2, Il-4, Il-5, Il-6, Il-10, Il-12(p70), tumor necrosis factor-α, interferon-γ, and granulocyte-macrophage colony stimulating factor; Millipore]

in conjunction with the Bio-Plex™ multiplex cytokine bead reader (Bio-Rad Laboratories) according to the manufacturer's instruction.

Fluorescent-activated cell sorter (FACS) analysis. We performed FACS analysis on brain mononuclear cells of hematopoetic origin according to previously published methods with minor modifications [6]. Briefly, we rapidly isolated brains from Tg(APP,PSEN) and Tg(APP,PSEN)/CD11c-DNR mice under deep isofluorane anesthesia (n = 5 per group; 3 males, 2 females 15 months of age) and quartered them as described above. We placed cerebral quarters on ice in RPMI 1640 media (Invitrogen-Gibco), and homogenized them in an Eppendorf hand homogenizer (eight strokes) to obtain single cell suspensions. We pooled samples of the same genotype and isolated mononuclear cells by discontinuous Percoll gradient (GE Healthcare-Pharmacia). We rinsed these cells in FACS buffer (1% FCS, 0.1% w/v sodium azide), incubated them with Fc Block™ (BD Biosciences-Pharmingen) for 20 min on ice, and stained them with FITC-conjugated CD45 (1:100), APC-conjugated CD11b (1:200), and PE-conjugated CD11c (1:50) antibodies for 20 min on ice (all FACS antibodies were from BD Biosciences-Pharmingen). We then rinsed cells three times in FACS buffer, and analyzed them using a FACSCaliber™ instrument (BD Biosciences). As previously described [6], mononuclear cells that were CD11b positive and intermediate CD45 expressers ($CD11b^{+}CD45^{int}$) were taken as resident microglia whereas $CD11b^{+}CD45^{high}$ cells were taken as infiltrating peripheral macrophages (MΦ).

Cell isolation and culture. We isolated cortical microglia from neonatal (1-2 day-old) CD11c-DNR or wild-type C57BL/6 mice according to previously published methods [7,8]. Briefly, we isolated brains under sterile conditions and incubated cerebral cortices in tripsin-EDTA (Invitrogen-Gibco) for 15 min at 37 ^{o}C. We then added complete RPMI 1640 medium (supplemented with 10% FCS and 1 mM penicillin-streptomycin) and dissociated brains by trituration. Subsequently, we plated cerebral cortex material in 25 cm^2 flasks (Fisher Scientific), and changed media every 2-3 days. When the appearance of microglia was noted (typically 14 days after plating), we exchanged culture media with RPMI 1640 medium supplemented with 5% FCS, and isolated microglia by shaking in an incubator-shaker at 200 rpm for 2 h at 37 ^{o}C. We isolated peripheral MΦ from adult CD11c-DNR or wild-type C57BL/6 mice according to standard immunological methods by intraperitoneally (i.p.) injecting mice with 900 μL of 3% (w/v) sterile thioglycollate solution diluted in PBS. Four days later, we i.p. injected mice with 10 mL of ice-cold PBS for peritoneal lavage. We then plated peripheral MΦ with complete medium (DMEM supplemented with 10% FCS and 1 mM penicillin-streptomycin) and allowed them to rest overnight. The following morning, we rinsed MΦ four times in ambient-temperature PBS and added fresh medium. Cultures of microglia and peripheral MΦ were both > 95% pure as determined by immunofluorescent staining with CD11b and CD45 antibodies (data not shown).

Aβ phagocytosis assay. We plated peripheral MΦ from CD11c-DNR or wild-type C57BL/6 mice on glass coverslips in 24-well culture plates (Fisher Scientific) at 5 x 10^5 cells/well in complete DMEM as described above. We re-suspended human synthetic

$A\beta_{1\text{-}42}$ conjugated with Hilyte Fluor™ 488 ($A\beta_{488}$; AnaSpec) in dH_2O at 1 mg/mL and pre-aggregated it for 24 h at 37 ^oC. We added $A\beta_{488}$ at 1 μg/mL to MΦ cultures and pulsed cells for 4 h at 37 ^oC. We then rinsed MΦ three times in ambient-temperature PBS, and chased them for 15 min to allow $A\beta_{488}$ to concentrate into phagolysosomes. After an additional two rinses in complete DMEM and then two final rinses in PBS, we mounted coverslips in ProLong Gold™ fluorescent mounting medium containing DAPI for confocal microscopy. We acquired three random 10X magnification fields and reported data two ways: as $A\beta_{488}$ labeled area (similar to "burden" analysis) or number of MΦ/field containing $A\beta_{488}$. In parallel experiments, we plated peripheral MΦ from CD11c-DNR or wild-type mice at 1.5 x 10^6 cells/well in 6-well culture plates (Fisher Scientific) in complete DMEM. We resuspended and pre-aggregated human synthetic $A\beta_{1\text{-}42}$ (unlabeled; Invitrogen-Biosource) as described above, and cultured MΦ as mentioned above, except that we lysed cells in cell lysis buffer and subjected them to Western blot for Aβ as described below.

Western immunoblot. We plated microglia or peripheral MΦ from wild-type or CD11c-DNR mice at 1 x 10^6 cells/well in 6-well tissue culture plates (BD Biosciences-Falcon) containing complete RPMI 1640 media or complete DMEM, respectively. We then treated these cells with a dose-range of recombinant TGF-β1 (R&D Systems; 1, 5, or 10 ng/mL) in the presence or absence of lipopolysachharide (LPS, 50 ng/mL) for 30 min. In a separate set of experiments, we pre-treated these cells for 1 h with ALK5 inhibitors SB-505124 or SB-431542 (Sigma-Aldrich; used at 0.1, 1.0, or 10.0 μM) and then added 5 ng/mL of recombinant TGF-β1. For Aβ phagocytosis assay, we pulse-chased MΦ with

Aβ as described above. We rinsed cells in ice-cold PBS three times, and lysed them in ice-cold lysis buffer (containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% v/v Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM -glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin, and 1 mM PMSF). After lysis for 30 min on ice, we centrifuged cell lysates at 15,000 x g for 30 min, and aliquoted supernatants for Western blot analysis. We determined protein concentration using the Bradford method, and ran out an aliquot corresponding to 50 µg of protein on 12% Nu-PAGE™ polyacrylamide gels (Invitrogen) and transferred proteins electrophoretically to Immobilon-P polyvinylidene difluoride membranes (Millipore). We blocked membranes in blocking buffer (5% w/v nonfat dry milk in Tris-buffered saline containing 1% v/v Tween-20) for 3 h at ambient temperature and incubated them overnight at 4°C with primary polyclonal antibodies directed against total or phosphorylated SMAD2/3, SMAD1/5/8, PAK2, or ERK1/2 (Cell Signaling Technology). We then rinsed membranes three times for 5 min each in dH_2O, and incubated them with anti-rabbit secondary antibody conjugated with horseradish peroxidase (diluted at 1/2000 in blocking buffer; GE Healthcare-Amersham Biosciences). After an additional three rinses for 5 min each in dH_2O, we incubated membranes for 5 min at ambient temperature with the enhanced chemiluminescence substrate (Thermo Fisher Scientific-Pierce Biotechnology), exposed to film, and developed.

We also Western blotted detergent-soluble brain homogenates from Tg2576/CD11c-DNR *vs.* littermate Tg2576 mice using the same protocol as above, except that the following primary antibodies were used: monoclonal antibody (mAb) 22C11 against the amino-terminus of APP (Chemicon; 1/2,000, recognizes mouse and

human transgene-derived APP), mAb 6E10 against the amino-terminus of human Aβ (Covance; 1/500, which reveals transgene-derived APP only), or polyclonal (p) antibody against γ-actin (Santa Cruz Biotechnology; 1/200, for a loading control)

Q-PCR. We extracted RNA from anterior cerebral pieces using the TRIzol reagent (Invitrogen). We synthesized complementary DNA using Superscript III reverse transcriptase (Invitrogen). We used a Taqman™ strategy to specifically amplify *Tnfa* or *Il10* from brain cDNA prepared from aged progeny from Tg2576 x CD11c-DNR matings using previously described probes and primers [9]. Probes contained a 5' reporter, FAM, and a 3' quencher, BHQ (Biosearch Technologies). We performed the assay on an ABI 7500Fast instrument (Applied Biosystems), and thermal cycling consisted of 95°C for 4 min and 45 cycles of 95 °C for 30 s and 60 °C for 1 min. To normalize the samples, we used the same amount of input cDNA in an *Hprt1* Q-PCR. The ratio of the amount of amplified target gene compared with the amount of *Hprt1* cDNA represented the relative levels in each sample.

Statistical analyses. In instances of single comparisons of the means, we used Levene's test for equality of the variance followed by *t*-test for independent samples to assess significance, except for semi-quantitative histology (*i.e.*, "CAA Score" and "$CD45^{+}CD11b^{+}$ infiltrating macrophages" data), where we used the Mann-Whitney U test. In instances of multiple means comparisons, we used analysis of variance (ANOVA), followed by post-hoc comparison by Fisher's LSD (for behavioral data) or Bonferroni's method (for all other analyses). For all analyses, we set alpha levels at 0.05

(SPSS for Windows, release 15.0, SPSS Inc.). An examiner blinded to sample identities performed all analyses, and code was not broken until analyses were completed.

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