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Supplemental Information

Alzheimer's Disease Risk Gene *CD33*

Inhibits Microglial Uptake of Amyloid Beta

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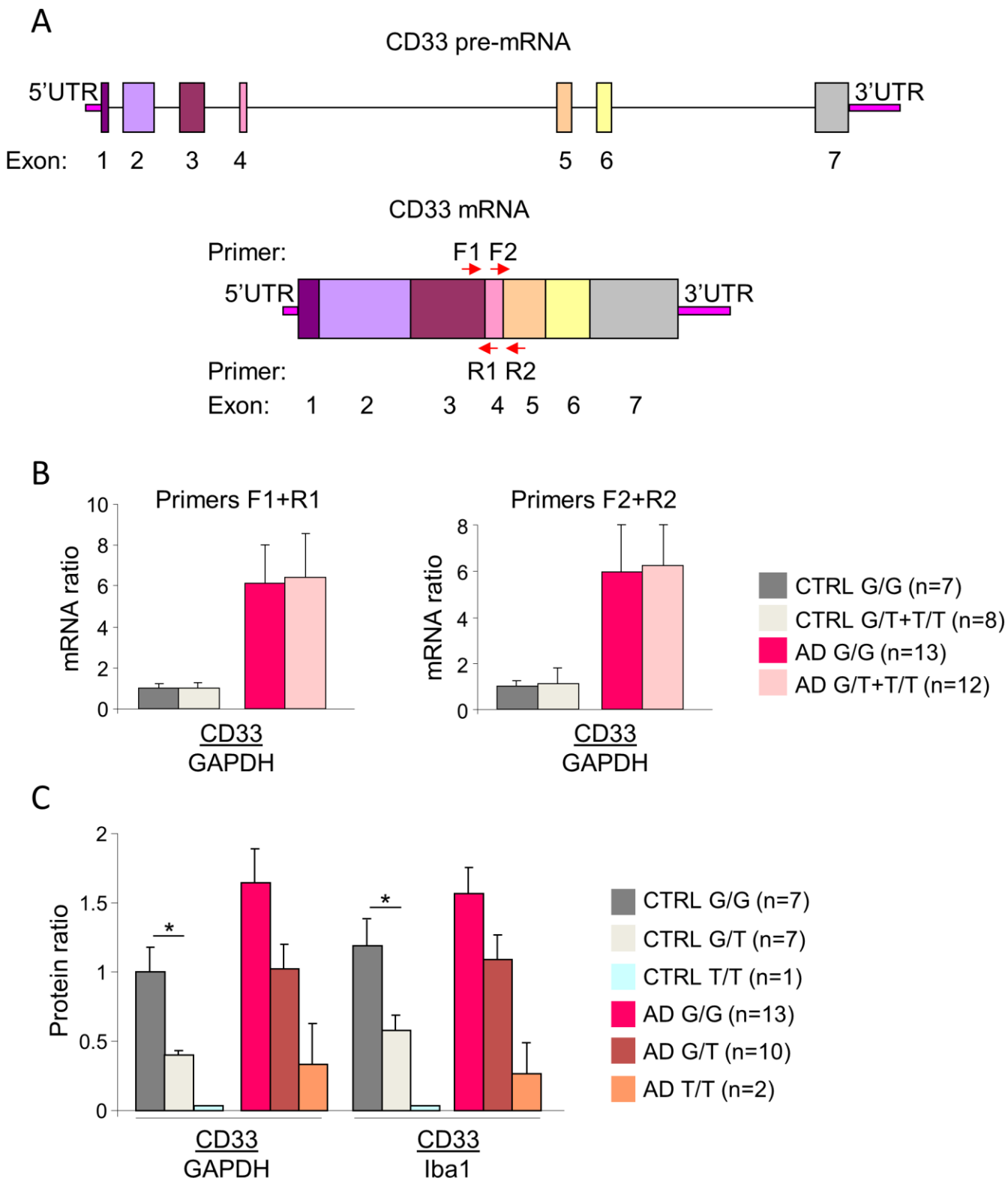

A
CD33 pre-mRNA
5'UTR
3'UTR
Exon: 1 2 3 4 5 6 7
CD33 mRNA
Primer: F1 F2
5'UTR
3'UTR
Primer: R1 R2
Exon: 1 2 3 4 5 6 7
B
Primers F1+R1
mRNA ratio
10
8
6
4
2
0
CD33
GAPDH
Primers F2+R2
mRNA ratio
8
6
4
2
0
CD33
GAPDH
CTRL G/G (n=7)
CTRL G/T+T/T (n=8)
AD G/G (n=13)
AD G/T+T/T (n=12)
C
Protein ratio
2
1.5
1
0.5
0
*
*
CD33
GAPDH
CD33
Iba1
CTRL G/G (n=7)
CTRL G/T (n=7)
CTRL T/T (n=1)
AD G/G (n=13)
AD G/T (n=10)
AD T/T (n=2)

Figure S1, related to Figure 1. Decreased levels of CD33 protein in carriers of the rs3865444 minor (T) allele. (A) Schematic diagram of the transcribed *CD33* RNA (*CD33* pre-mRNA; upper) and *CD33* mRNA (lower). The sets of primers (Forward F1 and reverse R1 targeting exons 3-4; forward F2 and reverse R2 targeting exons 4-5) used for quantitative RT-PCR are shown in red. (B) Results of qRT-PCR using the indicated sets of primers. mRNA was isolated from frozen cortical extracts obtained from AD cases (n=25) and age-matched controls (n=15) of the indicated genotypes. (C) CD33 protein levels are decreased in carriers of the rs3865444 minor (T) allele. Western blotting was used to quantify CD33 protein levels in AD cases (n=25) and age-matched controls (n=15) of the indicated genotypes. CD33 expression was normalized to GAPDH or to the microglial marker Iba1 (*$p<0.05$, student's t-test). Data are represented as mean ± SEM.

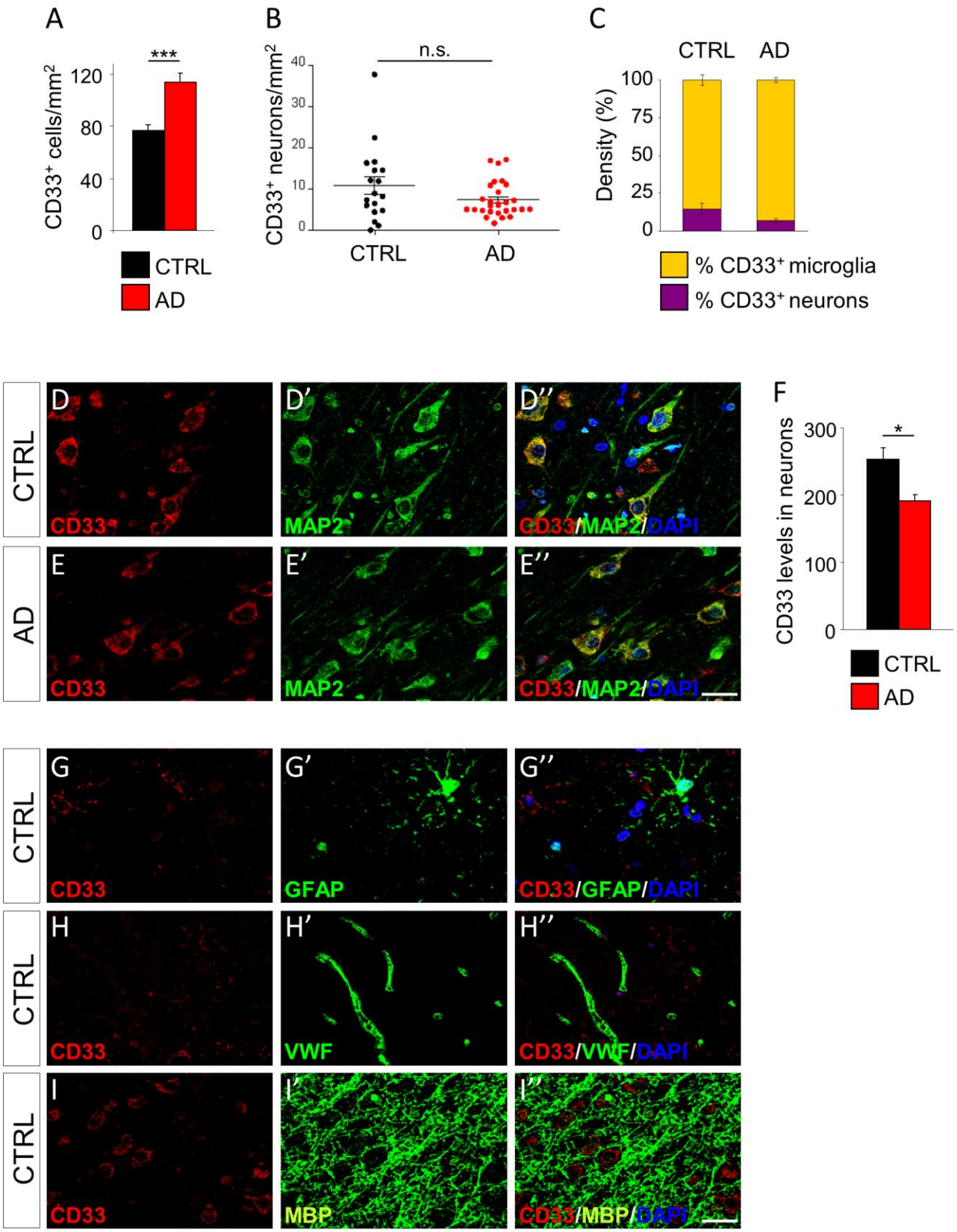

A
***
CD33⁺ cells/mm²
120
80
40
0
CTRL
AD
B
n.s.
CD33⁺ neurons/mm²
40
30
20
10
0
CTRL
AD
C
CTRL
AD
Density (%)
100
75
50
25
0
% CD33⁺ microglia
% CD33⁺ neurons
D
D'
D''
CTRL
CD33
MAP2
CD33/MAP2/DAPI
E
E'
E''
AD
CD33
MAP2
CD33/MAP2/DAPI
F
*
CD33 levels in neurons
300
200
100
0
CTRL
AD
G
G'
G''
CTRL
CD33
GFAP
CD33/GFAP/DAPI
H
H'
H''
CTRL
CD33
VWF
CD33/VWF/DAPI
I
I'
I''
CTRL
CD33
MBP
CD33/MBP/DAPI

Figure S2, related to Figure 2. CD33 expression pattern in the human brain. (A) Stereology-based quantifications of the number of CD33-positive cells in the frontal cortex of AD cases (n=28) and age-matched controls (n=18) (***p<0.001, student's t-test). (B) Stereology-based quantifications of the number of CD33-positive neurons in the frontal cortex of AD cases (n=28) and age-matched controls (n=18) (p=n.s., Mann-Whitney *U* test). (C) Distribution of CD33-positive cells in AD cases and age-matched controls. Most CD33 cells are microglial, and only a minor fraction corresponds to neurons. (D-D'', E-E'') Fluorescent immunolabeling reveals co-localization between CD33 (red) and the neuronal marker MAP2 (green) in controls (D-D'') as well as AD cases (E-E''). (F) Quantification of CD33 levels within MAP2-positive neurons (*p<0.05, student's t-test). Data are represented as mean ± SEM. (G-I) CD33 is not expressed in astrocytes, endothelial cells or oligodendrocytes in the aging human brain. Immunolabeling for CD33 (red; G-I) and astrocytic (GFAP; green; G'), endothelial (von Willebrand factor [VWF]; green; H'), or oligodendrocytic (myelin basic protein [MBP]; green; I') markers reveals no co-localization between CD33 signal and these markers. Scale bar is 25µm.

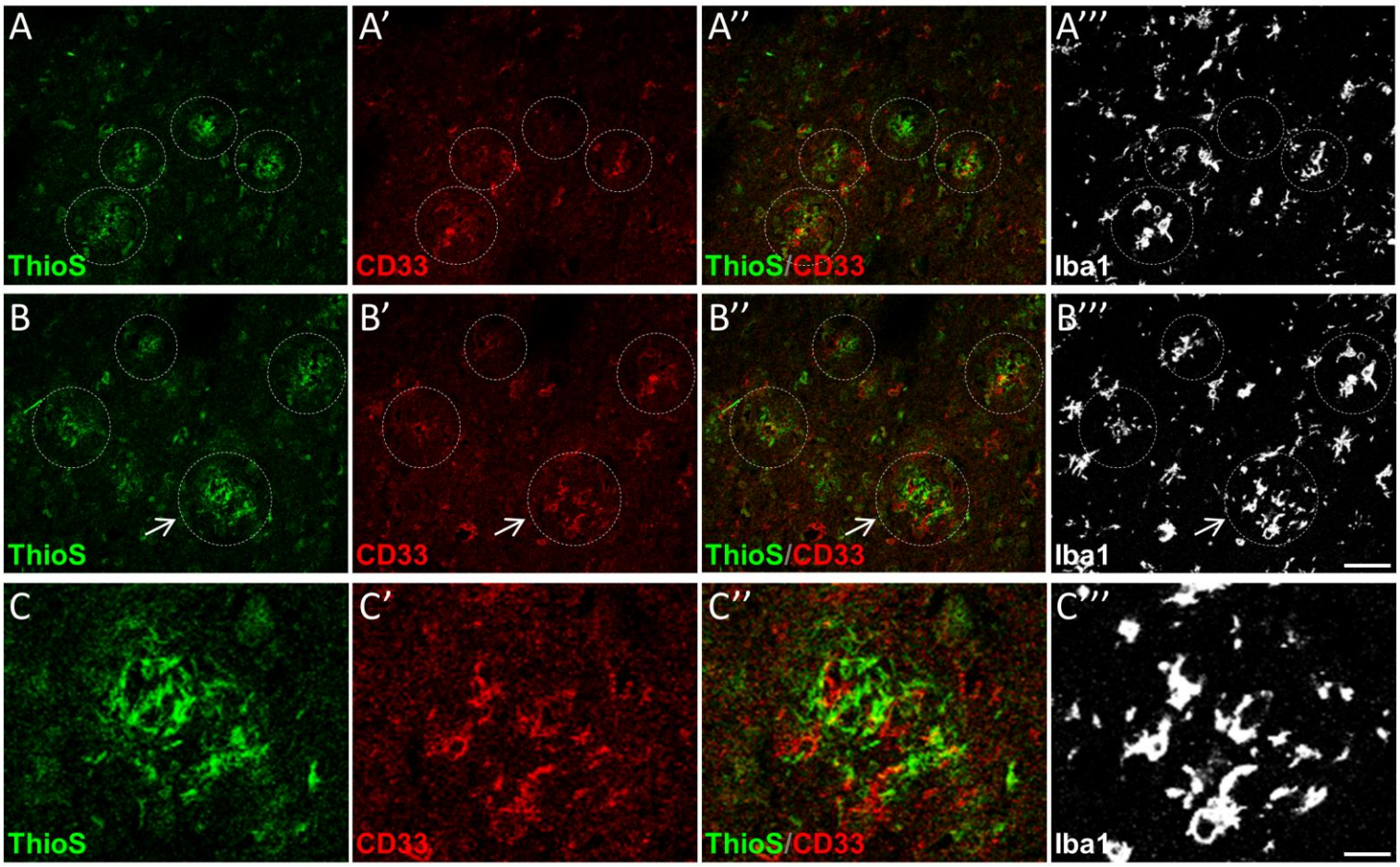
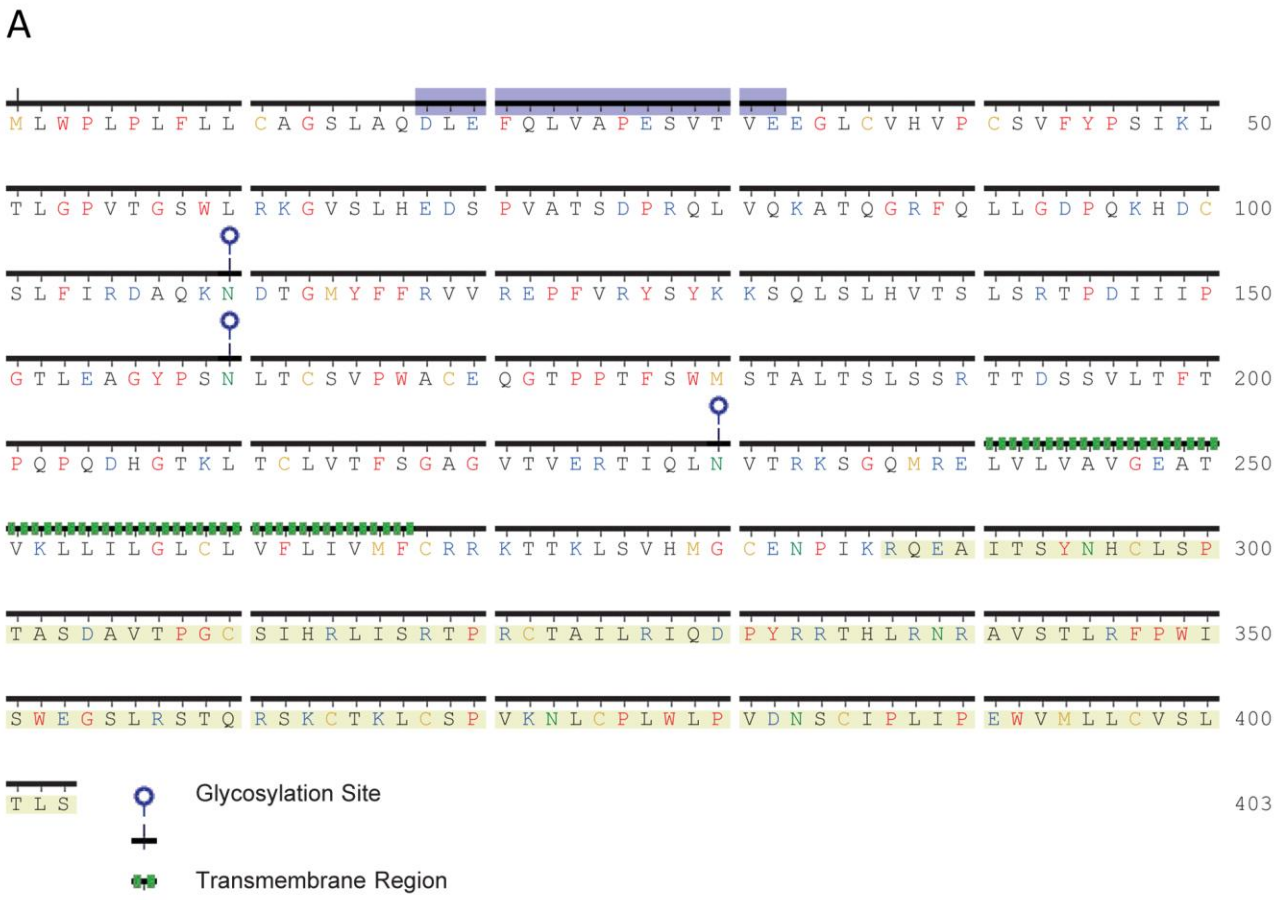


Figure S3, related to Figure 3. Localization of CD33 and relationship to amyloid plaques. (A-C) Frontal cortices from AD cases were immunolabeled for CD33 (red; A'-C'), the microglial marker Iba1 (white; A'''-C''') and stained with Thioflavin S (green; A-C) to detect amyloid plaques. CD33 exhibited a prominent microglial localization that was broadly distributed throughout the frontal cortex. In addition, an increased density of CD33-positive microglia was noted around amyloid plaques (white circles; A'', B''). (C-C''') Higher-magnification views of the selected Aβ plaque (white arrow in B''). Scale bar is 25μm in A''' and B''' and 5μm in C'''.


A
M L W P L P L F L L C A G S L A Q D L E F Q L V A P E S V T V E E G L C V H V P C S V F Y P S I K L 50
T L G P V T G S W L R K G V S L H E D S P V A T S D P R Q L V Q K A T Q G R F Q L L G D P Q K H D C 100
S L F I R D A Q K N D T G M Y F F R V V R E P F V R Y S Y K K S Q L S L H V T S L S R T P D I I I P 150
G T L E A G Y P S N L T C S V P W A C E Q G T P P T F S W M S T A L T S L S S R T T D S S V L T F T 200
P Q P Q D H G T K L T C L V T F S G A G V T V E R T I Q L N V T R K S G Q M R E L V L V A V G E A T 250
V K L L I L G L C L V F L I V M F C R R K T T K L S V H M G C E N P I K R Q E A I T S Y N H C L S P 300
T A S D A V T P G C S I H R L I S R T P R C T A I L R I Q D P Y R R T H L R N R A V S T L R F P W I 350
S W E G S L R S T Q R S K C T K L C S P V K N L C P L W L P V D N S C I P L I P E W V M L L C V S L 400
T L S
Glycosylation Site
Transmembrane Region
403

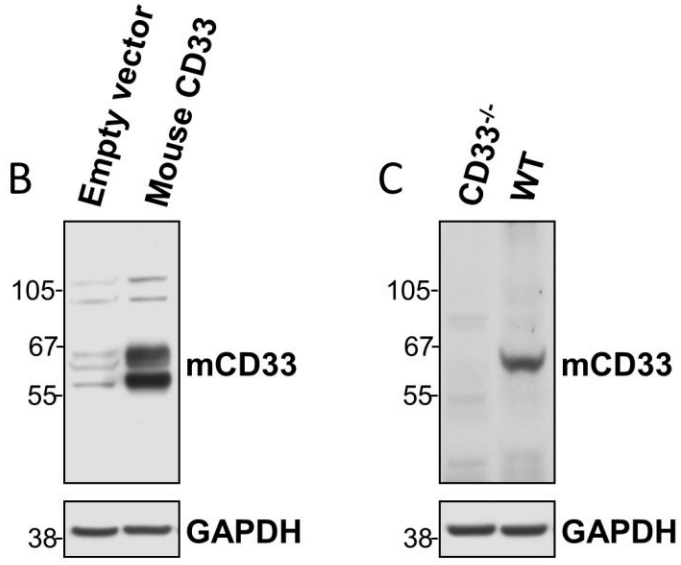

B
Empty vector
Mouse CD33
105
67
55
mCD33
38
GAPDH
C
CD33-/-
WT
105
67
55
mCD33
38
GAPDH

Figure S4, related to Figure 4. mCD33-specific polyclonal antibody. (A) Shown is a schematic representation of the mouse CD33 (mCD33) protein, highlighting the location of the immunizing peptide (blue shaded area; residues 18-32). (B) The rabbit polyclonal antibody raised against this CD33 peptide was tested on protein extracts derived from HEK293 cells transfected with either an empty vector or a vector encoding full-length mouse *CD33*. GAPDH served as loading control. (C) Western blotting using the anti-CD33 rabbit polyclonal antibody and cortical lysates reveals CD33 expression in WT mice and absence of CD33 expression in *CD33*$^{-/-}$ mice. GAPDH served as loading control.

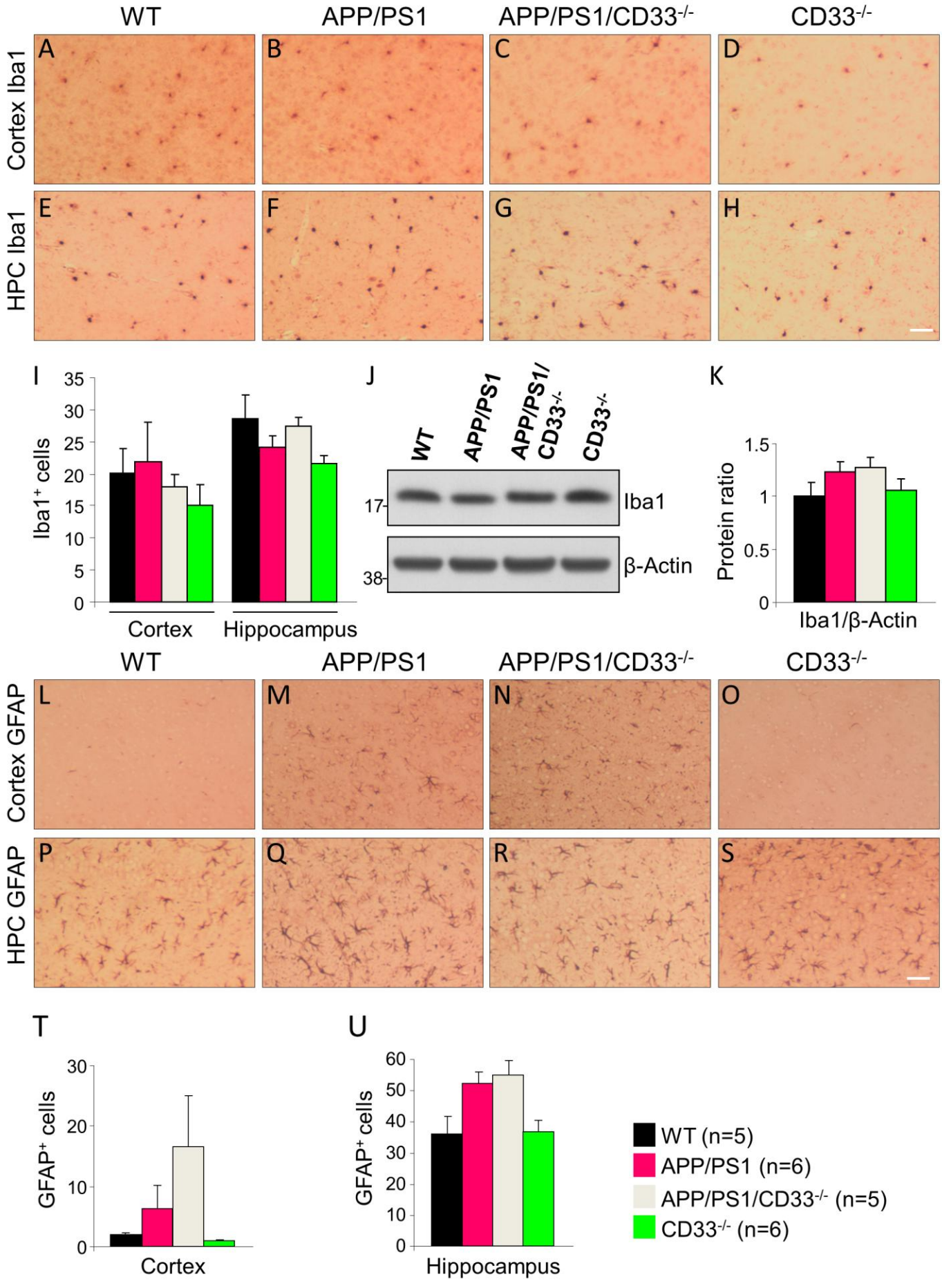

WT
APP/PS1
APP/PS1/CD33-/-
CD33-/-
Cortex Iba1
HPC Iba1
A
B
C
D
E
F
G
H
I
Iba1+ cells
35
30
25
20
15
10
5
0
Cortex
Hippocampus
J
WT
APP/PS1
APP/PS1/ CD33-/-
CD33-/-
17
38
Iba1
β-Actin
K
Protein ratio
1.5
1
0.5
0
Iba1/β-Actin
WT
APP/PS1
APP/PS1/CD33-/-
CD33-/-
Cortex GFAP
HPC GFAP
L
M
N
O
P
Q
R
S
T
GFAP+ cells
30
20
10
0
Cortex
U
GFAP+ cells
60
50
40
30
20
10
0
Hippocampus
WT (n=5)
APP/PS1 (n=6)
APP/PS1/CD33-/- (n=5)
CD33-/- (n=6)

Figure S5, related to Figure 6. *APP/PS1/CD33*$^{-/-}$ mice do not display enhanced microglial recruitment or accelerated astrogliosis in comparison to *APP/PS1* mice. Shown are representative microphotographs of cortical (A-D, L-O) and hippocampal (E-H, P-S) coronal sections stained for Iba1 (A-H) and GFAP (L-S). (I) Quantification of Iba1-positive microglial cell numbers reveals similar microglia numbers in the hippocampus and cortex in mice of the indicated genotypes (n=5-6 male mice/genotype, 4 month-old). (J) Western blotting using a Iba1 antibody also reveals no changes in Iba1 protein levels in the cortex of mice of the indicated genotypes. (K) Quantifications of Iba1 protein levels, using normalization to β-Actin (n=6 male mice/genotype, 4 month-old). (T) Quantification of GFAP-positive astrocyte numbers reveals similar astrogliosis in the cortex of WT and *CD33*$^{-/-}$ mice and more astrogliosis in *APP/PS1* and *APP/PS1/CD33*$^{-/-}$ mice; however, the extent of astrogliosis was similar in the cortex of *APP/PS1* and *APP/PS1/CD33*$^{-/-}$ mice (n=5-6 male mice/genotype, 4 month-old). (U) Quantification of the GFAP-immunoreactive cell numbers in the hippocampus reveals a similar degree of astrogliosis in the hippocampus in all groups (n=5-6 male mice/genotype, one-way Kruskal-Wallis ANOVA, Dunn's test). Data are represented as mean ± SEM. Scale bar is 25μm.

Table S1, related to Figure 1. Characteristics of AD cases and controls used in the study.

Characteristics	**Controls (n = 15)**	**AD (n = 25)**
Age at death (years)	79.9 ± 11.2	79.2 ± 8.3
Disease duration (years)	NA	10.96
Males/Females	40%/60%	28%/72%
APOE ε4 carriers	5 (33.33 %)	18 (72%)
APOE ε4 homozygous carriers	0	8 (32%)
Post-mortem interval (hours)	29 ± 9	17 ± 12

Supplemental Experimental Procedures

SNP Genotyping

Genomic DNA was extracted from AD and control frozen brain tissue using the QIAamp DNA Mini Kit (Qiagen). DNA Samples were genotyped for SNPs using the TaqMan SNP genotyping assays (Shen et al., 2009). The genotyping was performed using a custom-designed TaqMan® SNP genotyping assay for the rs3865444 SNP (Life Technologies) on a CFX384 Real-Time PCR System (BioRad) in accordance with the supplier's recommendations. To determine the *APOE* genotype (alleles: *ε2, ε3*, *ε4*), DNA samples were genotyped at two SNPs, rs429358 and rs7412, using a pre-designed Taqman SNP genotyping assay (catalogue no. 4351379) from Life Technologies. The reactions were carried out in 384-well microtiter plates (BioRad) in a total reaction volume of 5ul, containing 1µl genomic DNA (5ng/µl), 2.5µl of 2x Gene expression master mix (Life Technologies), 0.063ul of 80x Taqman probes, and 1ul water. Thermal cycling was performed with 40 cycles of 92° for 15 seconds and 60° for 30 seconds. Data was analyzed using the CFX Manager Software and the allelic discrimination tool.

RNA Extraction and Real Time-PCR

RNA was extracted from brain tissue with Trizol (Life Technologies) following manufacturer's instructions. The extracted mRNA was dissolved in water and cleaned using the RNAeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Extracted mRNA (2µg) was reverse-transcribed using the SuperScript III first strand synthesis system (Life Technologies). Gene expression was assessed by performing Taqman real-time PCR assays. Probes targeting the *CD33* gene were labeled with FAM (Hs01076280_g1 specific to exons 3-4 and Hs01076281_m1 specific to exons 4-5, Life Technologies). The housekeeping genes *GAPDH* and *β-Actin* were used as controls and were labeled with a VIC/MGB probe (Life Technologies, 4326315E for *β-Actin* and 4326317E for *GAPDH*). 1:10 diluted cDNAs were mixed with the probes and 2x Gene expression master mix (Life Technologies) and amplified using a CFX384 Real-Time PCR System (BioRad). Results were analyzed by the comparative C_T method. Average C_T values for each sample were normalized to the average C_T values of the housekeeping genes. *GAPDH* expression was highly correlated with *β-Actin* expression.

Immunohistochemistry and Stereology

For immunohistochemistry, mice were deeply anesthetized with isoflurane, then perfused with 0.9% sodium chloride and ice-cold 4% paraformaldehyde (PFA). Subsequently, brains were removed from the skull and post-fixed overnight in 4% PFA. The left hemisphere was dehydrated with ethanol and embedded in paraffin, whereas the right hemisphere was cryoprotected by incubation in 15% and 30% sucrose solutions and embedded in OCT.

Sections (8-µm thick) were deparaffinized and incubated with 3% H_2O_2 to quench endogenous peroxidases for DAB staining. Antigen retrieval was performed using the Diva Decloaker (Biocare Medical) or citrate buffer (0.01M, pH 6.0, 0.05% Tween-20) in a microwave oven (95°C, 20 minutes). Sections were subsequently blocked using 2% BSA, 0.1% Triton X-100 in phosphate buffered saline (PBS), or alternatively with Antibody diluent (Cell signaling). Primary antibodies were directed against human CD33 (mouse monoclonal, 1:100, clone PWS44, Vector Laboratories) (Hoyer et al., 2008; Rollins-Raval and Roth, 2012); MAP2 (rabbit polyclonal, 1:500, Millipore); Iba1 (rabbit polyclonal, 1:500, Wako); human GFAP (rabbit polyclonal, 1:1,000, Sigma); mouse GFAP (mouse monoclonal, 1:500, clone GA5, Millipore); human von Willebrand factor (rabbit polyclonal, 1:500, Millipore); human myelin basic protein (rabbit polyclonal, 1:300, Millipore). To detect amyloid plaques, sections were labeled with 1% Thioflavin S in water for 8 minutes at room temperature, then incubated in 80% ethanol for 2 minutes and mounted. For the immunostaining with the antibodies targeting human Aβ (mouse monoclonal, clone 10D5 [1:50] or clone 3D6 [1:2000], Elan Pharmaceuticals), antigen retrieval was performed using citrate buffer, in a microwave oven (95°C, 20 minutes) followed by incubation in 90% formic acid for 5 minutes. Biotinylated secondary antibodies were from Vector Laboratories.

For immunofluorescence experiments, Alexa-488/564/647-coupled secondary antibodies and Alexa 568-coupled streptavidin were acquired from Life Technologies. Sections were mounted with aqueous mounting medium containing DAPI and anti-fading reagent (Life Technologies).

For stereology-based quantitative studies, primary antibodies were detected with DAB, using different Vectastain ABC kits (Vector Laboratories) according to the provider's instructions.

Sections were dehydrated with increasing concentrations of ethanol, cleared with xylene, and cover-slipped with Cytoseal-XYL xylene-based mounting medium (Richard-Allan Scientific).

An unbiased stereology-based quantification method was used to determine the number of CD33-positive microglia and neurons on single immunostained sections from human frontal cortex (Brodmann areas 8, 9) (Serrano-Pozo et al., 2011). Briefly, sections were placed on the motorized stage of an upright Olympus BX51 microscope that is equipped with a DP70 video-camera and controlled by a computer with the image analysis software CAST (Olympus, Tokyo, Japan). The region of interest (full-width) cortex was outlined under the 4x objective and CD33-positive cells were counted under the 40x objective using a 1% meander sampling and a 10% counting frame (3565.2 μm^2).

Assessment of Aβ plaque burden was performed in an upright Leica DMRB microscope (Leica, Germany) equipped with a motorized stage and a CCD camera and coupled with the software BIOQUANT NOVA PRIME, version 6.90.10 (MBSR). Aβ plaque burden was measured as the percentage of total surface stained by the anti-Aβ antibody (clone 10D5) in a full-width strip of cortex (approximately 1-cm long) using the optical threshold application of the software (Serrano-Pozo et al., 2011).

Assessment of Aβ Plaque Burden in Mice

Coronal sections stained with the anti-Aβ antibody 3D6 (1:2000, Elan Pharmaceuticals) were imaged using an upright Olympus BX51 microscope. Six coronal sections spanning the cortex and four coronal sections spanning the hippocampus (at different depths on the rostro-caudal axis) were imaged for each animal. The amyloid plaque burden (area occupied by all plaques divided by the total area) was estimated in the cortex or hippocampus for each section using ImageJ software. Values from each section were then averaged to derive a mean plaque burden for each animal. We analyzed 9-11 male mice in each group (age 6-7 months).

ELISA

For ELISA, mice were deeply anesthetized with isoflurane, then perfused with 0.9% sodium chloride. The brains were extracted and cortices and hippocampi were dissected. To assess Aβ levels, mouse cortices or human frozen brain tissue (frontal cortex) were homogenized in 5

volumes of TBS containing 5mM EDTA, phosphatase inhibitor (ThermoFisher), EDTA-free protease inhibitor cocktail (Roche) and 2 mM 1,10-phenantroline (Sigma), using a Polytron benchtop lab homogenizer (Wheaton) at 4°C. The homogenate was centrifuged at 100,000 *g* for 1 hour at 4°C using an Optima TL ultracentrifuge and a TLA 120.2 rotor (Beckman Coulter). Supernatants were collected and used to measure TBS-soluble Aβ. The resulting pellet was homogenized in 70% formic acid (the volume of used formic acid was equal to the volume of TBS homogenate used for centrifugation). Samples were centrifuged at 100,000 *g* for 1 hour at 4°C and supernatants were collected. Formic acid-containing supernatants were neutralized with 1M Tris-base, pH 11 (1:20 v:v) and samples were used to measure formic acid-soluble Aβ. Aβ40 and Aβ42 ELISAs were performed using Aβ ELISA kits from Wako.

Western Blot Analysis

Brain tissue was homogenized in 5 volumes of TBS containing 5mM EDTA, phosphatase inhibitor (ThermoFisher), EDTA-free protease inhibitor cocktail (Roche) and 2 mM 1,10-phenantroline (Sigma). The homogenate was mixed with an equal volume of 2x radio-immunoprecipitation assay (RIPA) buffer (Millipore) supplemented with all the above-described inhibitors. Samples were incubated on a rotating wheel for 30 minutes at 4°C and centrifuged at 12,000 *g* for 15 minutes at 4°C. The supernatant was collected and used for western blot analysis. Lysates were assessed for protein concentration using the BCA kit (Pierce). Samples were boiled in sample buffer containing lithium dodecyl sulfate and β-mercaptoethanol as reducing agent (Life Technologies), and resolved on 4%–12% Bis-Tris polyacrylamide precast gels (NuPAGE system, Life Technologies). Gels were transferred onto PVDF membranes (BioRad) using wet transfer system (BioRad). The primary antibodies were directed against: human CD33 (mouse monoclonal, 1:100, clone PWS44, Vector Laboratories); Iba1 (rabbit polyclonal, 1:500, Wako); GAPDH (mouse monoclonal, 1:10,000, Millipore); β-Actin (mouse monoclonal, 1:5,000, Sigma), APP (rabbit polyclonal, 1:2,000, clone C7 targeting the amino acid residues 732-751 in APP, custom-designed by Open Biosystems) (Podlisny et al., 1991) and mouse CD33 (mCD33, rabbit polyclonal, 1:200, custom-designed by Open Biosystems). Densitometric analyses were performed using Quantity One software (BioRad). Band density values were normalized to GAPDH or β-Actin levels.

Generation of an anti-Mouse CD33 Antibody

mCD33 is a rabbit polyclonal antibody that was generated for our laboratory by Open Biosystems. It was raised against a mouse CD33 epitope that is absent in other mouse CD33-related Siglecs and corresponds to amino acid residues 18–32 (DLEFQLVAPESVTVE). The antibody was characterized by western blot analysis and immunocytochemistry (Figures 4 and S4).

Primary Microglia Isolation

Microglial cells were prepared from WT or *CD33*$^{-/-}$ brains at postnatal day 1 as previously described (Choi et al., 2008; Gorlovoy et al., 2009). Briefly, meninges and leptomeningeal blood vessels were removed from the cortex. Cells were dissociated by trituration and cultured in DMEM containing 10% heat-inactivated fetal bovine serum, 2mM L-glutamine and 1% penicillin/streptomycin (Life Technologies) for 14-21 days in poly-D-lysine-coated 75 cm^2 flasks (Biocat) to form a confluent glial monolayer. Half of the medium was replaced with fresh cell culture medium every three days. To collect microglial cells, the cultures were shaken on a rotary shaker (placed in a cell culture incubator, 37°C and 5% CO_2) at 250 rpm for 3 hours. The detached microglial cells were collected by centrifugation and the enriched microglial cell suspension was plated onto poly-D-lysine-coated 6-well plates (Biocat) or onto 24-well plates containing poly-D-lysine-coated glass coverslips. After the cells attached, the medium was replaced with fresh cell culture medium. The purity of the isolated microglia was determined by immunostaining with antibodies directed against Iba1. In average, 93% of cultured cells were immunostained with Iba1.

Cell Culture and Transfection

Two pcDNA3.1 plasmids encoding human wild-type *CD33* (*CD33*^{WT}) and mutant *CD33*$^{K283/288/309/312/313/315/352R}$ (*CD33*^{K7R}) were generously provided by Roland B. Walter and were previously described (Walter et al., 2008a). pCMV6-XL5 plasmid encoding *CD33* that lacks the sialic acid-binding V-type immunoglobulin-like domain (*CD33*$^{\Delta V\text{-}Ig}$) was acquired from OriGene. *CD33*$^{\Delta V\text{-}Ig}$ cDNA was amplified and subcloned into a pcDNA3.1 vector (Life Technologies). pcDNA3.1 plasmid encoding *GFP* was acquired from Life Technologies. All constructs were verified by sequencing. BV2 microglial cell line was kindly provided by Linda

Van Eldik (Bachstetter et al., 2011) and was maintained in DMEM containing 5% heat-inactivated fetal bovine serum, 2mM L-Glutamine and 1% penicillin/streptomycin (Life Technologies). Cells were transiently transfected with plasmids using Lipofectamine-Plus (Life Technologies) according to the manufacturer's instructions.

Aβ Uptake and Degradation Assays

Primary mouse microglia or BV2 cells transfected with *CD33* plasmids were treated with 2 μg/ml Aβ42 (AnaSpec) in serum-free DMEM medium for 3 hours. Cells were washed with DMEM and maintained for additional 3 hours in serum- and Aβ42-free DMEM. Afterwards, cells were extensively washed with PBS and were lysed in cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5mM EDTA, 1% SDS) supplemented with EDTA-free protease inhibitors (Roche) and 2 mM 1,10 phenantroline (Sigma). Lysates were centrifuged at 12,000 *g* at 4°C for 15 minutes. Supernatants were collected and further used for ELISAs. Aβ42 levels were measured using Aβ42 ELISA kit (Wako) and normalized to total protein concentration that was assessed by BCA method (Pierce).

For immunofluorescence experiments, primary mouse microglial or BV2 cells were incubated with 2 μg/ml Alexa 555-labeled Aβ42 (AnaSpec) in serum-free DMEM for 3 hours, washed extensively with PBS and fixed with 4% PFA for 20 minutes. Cells were washed with PBS, blocked with 4% BSA in PBS and incubated with the primary antibodies targeting: mouse CD33 (rabbit polyclonal, 1:200, custom-designed by Open Biosystems), human CD33 (mouse monoclonal, 1:200, clone PWS44, Vector Laboratories) and Iba1 (rabbit polyclonal, 1:500, Wako, or goat polyclonal, 1:500, Abcam). Alexa 488/647 conjugated secondary antibodies were purchased from Life Technologies. Coverslips were mounted with aqueous mounting medium containing DAPI and anti-fading reagent (Life Technologies).

To quantify intracellular Alexa555-labeled Aβ42, pictures displaying the intracellular Aβ42 in transfected BV2 or mouse primary microglial cells were used. The area corresponding to intracellular Aβ42 was carefully delineated and the average intensity of the signal was determined within this area. To correct for differences in background intensity, an area devoid of Aβ42 was used and the average intensity in this area was subtracted from the average intensity of

Aβ42 signal. The quantifications were performed using the ImageJ software and the values are represented in arbitrary units. At least 30 cells were analyzed per condition.

Supplemental References

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