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CX3CR1 Deficiency Alters Microglial Activation and Reduces Beta-Amyloid Deposition in Two Alzheimer's Disease Mouse Models

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Microglia, the primary immune effector cells in the brain, continually monitor the tissue parenchyma for pathological alterations and become activated in Alzheimer's disease. Loss of signaling between neurons and microglia via deletion of the microglial receptor, CX3CR1, worsens phenotypes in various models of neurodegenerative diseases. In contrast, CX3CR1 deficiency ameliorates pathology in murine stroke models. To examine the role of CX3CR1 in Alzheimer's disease-related β -amyloid pathology, we generated APPPS1 and R1.40 transgenic mouse models of Alzheimer's disease deficient for CX3CR1. Surprisingly, CX3CR1 deficiency resulted in a gene dose-dependent reduction in β -amyloid deposition in both the APPPS1 and R1.40 mouse models of AD. Immunohistochemical analysis revealed reduced staining for CD68, a marker of microglial activation. Furthermore, quantitative immunohistochemical analysis revealed reduced numbers of microglia surrounding β -amyloid deposits in the CX3CR1-deficient APPPS1 animals. The reduced β -amyloid pathology correlated with reduced levels of TNF α and CCL2 mRNAs, but elevated IL1 β mRNA levels, suggesting an altered neuroinflammatory milieu. Finally, to account for these seemingly disparate results, both *in vitro* and *in vivo* studies provided evidence that CX3CL1/CX3CR1 signaling alters the phagocytic capacity of microglia, including the uptake of A β fibrils. Taken together, these results demonstrate that loss of neuron-microglial fractalkine signaling leads to reduced β -amyloid deposition in mouse models of AD that is potentially mediated by altered activation and phagocytic capability of CX3CR1-

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by extracellular deposits of β -amyloid (A β) peptides in senile plaques. Accumulating evidence suggests that neuroinflammatory processes may contribute to the pathophysiology of AD. Microglia, the resident inflammatory cells of the brain, are found in a highly activated state in the AD brain, including morphological alterations, proliferation, increased expression of cell surface receptors, and secretion of inflammatory cytokines and chemokines.^{1,2} However, recent *in vivo* imaging studies demonstrated that activated microglia migrate to newly formed A β deposits in mouse models of AD and may restrict their growth by removing A β fibrils.^{3–5} Therefore, depending on the local conditions, microglia may exhibit beneficial or detrimental activation programs.⁶ Indeed, various genetically modified mouse models have convincingly demonstrated the role for various inflammatory pathways in altering microglial activation and A β deposition, including chemokines/cytokines,^{7–9} cyclooxygenase,¹⁰ and complement.¹¹ While these studies suggest a correlative link between neuroin-

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flammation and AD pathophysiology, the molecular mechanisms underlying beneficial or deleterious microglial activities remain incompletely understood.

The chemokine fractalkine (CX3CL1) and its cognate receptor, CX3CR1, play an important role in neuroinflammation via paracrine signaling between neurons and microglia.^{12–14} While CX3CR1 is expressed by many leukocyte cell types in the periphery,¹⁵ in the brain CX3CR1 is exclusively expressed by microglia¹³ whereas CX3CL1 is highly expressed within neurons.¹⁴ CX3CL1 can signal to CX3CR1 in a membrane bound form, or as a secreted ligand after constitutive or inducible cleavage by the ADAM10 and ADAM17 metalloproteases, respectively.¹⁶ Genetic variants in *CX3CR1*, which result in defective CX3CL1-CX3CR1 interactions, have been associated with age-related macular degeneration, a neuroinflammatory disorder.^{17,18} Finally, disruption of CX3CL1-CX3CR1 signaling promotes neurodegeneration in mouse models of Parkinson's disease, amyotrophic lateral sclerosis, as well as in neuroinflammation induced by systemic administration of lipopolysaccharide.¹³ Conversely, lack of CX3CR1 is protective in murine stroke models.^{19,20}

To examine the role of CX3CR1 signaling in amyloid pathology associated with AD, we mated *Cx3cr1* knockout mice to two different mouse models of AD that exhibit either rapid deposition of primarily A β 42 (the APPPS1 model)²¹ or gradual deposition of primarily A β 40 (the R1.40 model).^{22–24} Surprisingly, CX3CR1 deficiency resulted in a gene dose-dependent reduction in A β deposition as well as the numbers of microglia surrounding the A β deposits in both mouse models of AD. CX3CR1-deficient mice with amyloid pathology exhibited altered microglial activation with reduced immunohistochemical staining for the microglial activation marker CD68 and altered expression of cytokines and chemokines. Finally, we demonstrate that CX3CL1-CX3CR1 signaling reduces microglial phagocytic capabilities and that blocking CX3CL1-CX3CR1 signaling increases uptake of A β , suggesting that CX3CR1 deficiency leads to enhanced A β clearance.

Materials and Methods

Mice

The APPPS1-21 (APPPS1) mouse line coexpresses human amyloid precursor protein harboring the K670M/N671L familial AD mutation and presenilin 1 harboring the L166P familial AD mutation, under the control of the neuron-specific Thy1 promoter.²¹ APPPS1 mice (in an isogenic C57BL/6J background; kindly provided by Mathias Jucker) were mated to *Cx3cr1*^{−/−} animals¹⁵; congenic on the C57BL/6J background) and subsequently intercrossed to generate APPPS1;*Cx3cr1*^{+/-} and APPPS1;*Cx3cr1*^{−/−} genotypes on a defined C57BL/6J genetic background. Aged-matched APPPS1;*Cx3cr1*^{+/-} animals were used as controls.

The R1.40 transgene is a full genomic copy of human *APP* (carried on a 650-kb yeast artificial chromosome clone) carrying the K670M/N671L familial AD mutation

associated with early-onset familial AD. Creation of the R1.40 transgenic mouse strain and subsequent backcrossing to inbred strains has been described previously.^{22,23,25} Homozygous R1.40 animals, maintained on the C57BL/6J genetic background, were mated to *Cx3cr1*^{−/−} animals (also maintained on the C57BL/6J genetic background) and subsequently intercrossed to generate animals homozygous for the R1.40 transgene and either homozygous or heterozygous for the *Cx3cr1* knockout allele and controls. Animals were housed at the Cleveland Clinic Biological Resources Unit, a facility fully accredited by the Association of Assessment and Accreditation of Laboratory Animal Care. All procedures were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic.

Western Blot Analysis

Mice were sacrificed by cervical dislocation, and their brains were removed, sagittally bisected, and snap frozen. Tissues were subsequently homogenized in 10 volumes of Tris-buffered saline (50 mmol/L Tris; pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% Triton-X100) or 20 volumes of T-PER reagent (Thermo Scientific, Rockford, IL) with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Total brain homogenates were subsequently sonicated to shear the DNA and centrifuged to remove nuclei and cellular debris. Total protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific). Brain protein (25–30 μ g) was run on a Novex NuPage, 4 to 12% Bis-Tris gel (Invitrogen) and transferred to a PVDF membrane. The resulting Western blots were blocked with 5% milk and subsequently incubated with CT15 antibody (rabbit polyclonal; 1:5000, kindly provided by Edward H. Koo) raised against the C terminus of APP²⁶ or 6E10 antibody (mouse monoclonal; 1:2000; Signet Labs) raised against amino acids 1–16 of human A β .

After overnight incubation at 4°C with primary antibodies, blots were washed with PBS and detected using anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (1:20,000; Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in 5% milk with 0.1% Tween 20. To confirm equal protein loading, anti- α -tubulin antibody (mouse monoclonal; 1:5000; Neomarkers, Fremont, CA) or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; mouse monoclonal; 1:20,000; Millipore, Billerica, MA) was used. For quantification, blots were incubated with secondary antibody mixtures (goat anti-mouse IRDye 700 and goat anti-rabbit IRDye 800; 1:20,000; Rockland Immunochemicals, Gilbertsville, PA) for one hour at RT. Infrared signals were measured and quantified in an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE). The integrated intensity ratio for APP/ α -tubulin or C-terminal fragment (CTF) β / α -tubulin was calculated, and the mean $\pm$ SEM for eight independent experiments were used for statistical analysis.

A β ELISA

For determination of A β 42 levels, a commercially available ELISA kit was used (Invitrogen). Mouse hemi-brains were extracted with 5 mol/L guanidine HCl and incubated at room temperature for four hours. Homogenates were centrifuged to remove insoluble debris, supernatants were diluted 1:5000 in standard diluent buffer, and ELISA was carried out according to manufacture instructions. The plate was read using a microtiter plate reader. Optical density values were fitted against the standard curve and pmol/g wet brain calculated. The results were tabulated as mean $\pm$ SEM and compared using an unpaired *t*-test.

Histology

Mice were deeply anesthetized with Avertin and transcardially perfused with ice-cold 0.1 mol/L sodium phosphate buffer, followed by 4% paraformaldehyde in PBS as described previously.²⁷ The brain was dissected, immediately removed from the cranium, and transferred to fresh 4% paraformaldehyde in PBS at 4°C overnight. The brains were subsequently cryoprotected by sinking in 30% sucrose/PBS at 4°C overnight. After bisecting across the midline, the brains were embedded in Optimal Cutting Temperature (OCT) Compound and 30 μ m free-floating cryostat sections cut and stored in PBS at 4°C until use.

Thioflavine S Staining

Sections were pretreated in 10 mmol/L sodium citrate buffer (pH 6.0, 0.05% Triton-X100) for 15 minutes at 95°C to quench eGFP expression in CX3CR1-deficient animals, which reduced background fluorescence to that observed in non-transgenic controls (modified from Ref. 28; data not shown). Sections were mounted onto Super-Plus glass slides and air-dried and immersed in 1% Thioflavine S (in dH₂O) for five minutes. Sections were subsequently hydrated in 70% ethanol and washed twice in PBS. The slides were coverslipped with 1:1 PBS:glycerol, and edges were sealed with nail polish.

Low-power images of cortex and hippocampus were compiled to form a series of overlapping images that were used to obtain an integrated image using Photoshop. The number of Thioflavine S-positive plaques in the cortex (below the pial surface and above the corpus callosum) and the hippocampus (excluding the subiculum and the fimbriae) was quantified. For all experiments, four separate sections, 360 μ m apart, were quantified from each mouse, and all sections were taken from the same brain regions. All data were expressed as mean density or total count of Thioflavine S-positive plaques $\pm$ SEM and statistically significant alterations between the three genotypes determined by one-way analysis of variance followed by Newman-Keuls post hoc test (GraphPad Prism, La Jolla, CA).

Immunohistochemistry

For diaminobenzidine immunohistochemistry, sections were first rinsed in PBS containing 0.1% Triton-X100 (PBST), pretreated with 10 mmol/L sodium citrate buffer (pH 6.0, 0.05% Triton-X100) for 15 minutes at 95°C for epitope retrieval. Sections were subsequently cooled for 30 minutes at room temperature, immersed in 0.3% hydrogen peroxide in PBS for 30 minutes to remove endogenous peroxidase activity, and blocked in 5% normal goat serum in PBS containing 0.3% Triton X-100 for one hour at room temperature. After incubation overnight at 4°C in blocking buffer containing either rat monoclonal anti-CD45 (diluted 1:500; Serotec, Oxford, UK), rat monoclonal anti-CD68 (diluted 1:500; Serotec, Oxford, UK), mouse monoclonal anti-human A β (clone 6E10; diluted 1:500; Signet Labs), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; diluted 1:500; Sigma-Aldrich), mouse monoclonal anti-phosphorylated tau (clone AT8; diluted 1:500; Thermo Scientific), or anti-APP (clone CT15; diluted 1:500) antibodies, slides were washed three times in PBST and incubated for one hour at room temperature in blocking buffer containing biotinylated anti-rat, anti-mouse, or anti-rabbit IgG antibody (diluted 1:200; Vector, Burlingame, CA). After three additional PBST washes, sections were incubated in Vectastain ABC Elite reagent (Vector) for one hour at room temperature and developed using diaminobenzidine with nickel chloride enhancement, according to the manufacturer's instructions. Sections stained for CD45 or CD68 were additionally counterstained with Congo Red to visualize dense core A β deposits. Finally, free-floating sections were mounted onto SuperPlus glass slides and coverslipped with Permount.

For double fluorescent immunohistochemistry, sections were rinsed in PBST, pretreated with sodium citrate buffer to quench microglial eGFP, cooled for 30 minutes at room temperature, blocked for one hour at room temperature, and incubated overnight at 4°C in blocking buffer containing anti-Iba1 rabbit polyclonal antibody (diluted 1:1000; Wako, Richmond, VA) and the anti-A β mouse monoclonal antibody 4G8 (diluted 1:500; Signet Labs). After three PBST washes, sections were incubated for one hour at room temperature in blocking buffer containing secondary antibodies conjugated to fluorescent Alexa dyes (diluted 1:1000; Invitrogen). The sections were finally washed three times in PBST, incubated with TO-PRO-3 (diluted 1:1000 in PBS; Invitrogen) for 10 minutes at room temperature, mounted onto SuperPlus glass slides, and coverslipped with hard-set Vectashield mounting media (Vector). Controls included sections in which the fluorescent dyes were swapped as well as no primary antibody (data not shown).

Quantification of Microglia

Twenty- to thirty- μ m thick Z-stacks were collected at $\times 40$ magnification with 1 μ m between each slice using a Leica SP5 confocal microscope, and images were post-

processed using ImageJ. To minimize any potential experimental bias, high-resolution confocal Z-stacks that spanned the entire thickness of the section were obtained and quantified via an unambiguous parameter (ie, number of cells rather than percent area or optical density). In addition, all sections were immunostained and imaged together using the same parameters. To determine the number of microglia surrounding each A β deposit, Iba1-positive cells whose nuclei or primary processes directly overlapped 4G8 immunoreactivity in a given plane of focus were counted. Only those microglia whose nuclei were present in the Z-stack were included in the analysis. The size of the A β deposits were calculated from arbitrarily thresholded maximum projections of 4G8 immunoreactivity and subsequently used to determine the number of microglia surrounding A β deposits of different sizes. Three random fields within the cortex were analyzed from three independent non-adjacent sections per mouse, with four animals analyzed per genotype. Data from 35 plaques of each size category were included for statistical analyses using one-way analysis of variance followed by Newman-Keuls post hoc test (GraphPad Prism) and presented as mean $\pm$ SEM.

RNA Extraction and Reverse Transcription-PCR

Hemi-brains of APPPS1;Cx3cr1^{+/+}, APPPS1;Cx3cr1^{+/-}, APPPS1;Cx3cr1^{-/-}, and nontransgenic C57BL/6J animals were snap frozen and stored at -80°C until RNA extractions were performed. RNA was extracted using the Tri Reagent as described by the manufacturer (Invitrogen). Total RNA (50 ng/ μL) was converted to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems Inc. [ABI], Foster City, CA) and amplified using specific TaqMan probes on the ABI 7300 Real-Time PCR System. TaqMan probes used in the current studies include: chemokine (C-C motif) ligand 2 (CCL2; Mm00441241_m1), CX3CL1 (Mm01243036_m1), interferon γ (IFN γ ; Mm00801778_m1), interleukin 1 β (IL1 β ; Mm00434228_m1), IL4 (Mm00445259_m1), IL10 (Mm00439615_g1), IL6 (Mm00446191_m1), and tumor necrosis factor α (TNF α ; Mm00443258_m1). The relative amount of each transcript was determined via normalization across all samples to the endogenous control, GAPDH (part #4308313) as recommended by ABI. In addition, before initiation of the studies, cDNA was serially diluted and amplified using the respective TaqMan probes to ensure that the amount of cDNA used was in the linear range. RNA samples from each individual animal were run in triplicate.

To quantify the relative expression levels of the different transcripts for each genotype, we calculated the difference (ΔCt) between the cycle threshold of the genes of interest (GOI) and the housekeeping gene *Gapdh*. From this data, the $-\Delta\Delta\text{Ct}$ ($-\Delta\text{Ct}_{\text{GOI}} - \Delta\text{Ct}_{\text{B6}}$) was computed and converted to a value relative to the entire brain using the formula $2^{-\Delta\Delta\text{Ct}}$. The results were tabulated as mean $\pm$ SEM and compared between genotypes via one-way analysis of variance followed by Newman-Keuls post hoc test.

Cell Culture

Primary microglia were cultured from postnatal day 0–3 Cx3cr1^{+/+} and Cx3cr1^{-/-} mice as described previously.²⁹ Briefly, dissected cerebral cortices were sequentially rinsed with PBS containing 1 g/L glucose, minced, and digested with 0.05% trypsin-EDTA for 20 minutes at 37°C . Cells were triturated in DMEM/F12 containing 20% heat-inactivated fetal bovine serum (HI-FBS) and 1% penicillin-streptomycin and plated in cell culture flasks. Media was changed the next day to DMEM/F12 with 10% HI-FBS.

After 14 days of incubation, microglia were isolated as described previously³⁰ by removal of astrocytes by incubation in 0.25% trypsin/HBSS diluted 1:4 in serum-free DMEM for 45 minutes at 37°C . 0.25% trypsin/PBS was then added to remove adherent microglia, and cells were replated in DMEM containing 2% HI-FBS at a density of 250,000 per well in 6-well plates.

Phagocytosis Assay

The phagocytosis assay was modified from previous studies.^{31–33} After overnight incubation in serum-free DMEM in the absence or presence of 100 nmol/L CX3CL1 (chemokine domain; R&D Systems, Minneapolis, MN), Nile Red fluorescent microspheres (Invitrogen) washed in PBS containing 1 mg/ml BSA were added to the cells for 90 minutes. Cells were then extensively washed with PBS and fixed in 2% paraformaldehyde. The numbers of phagocytosed microspheres per cell were counted from random fields totaling 60–100 cells on an inverted microscope. The results were tabulated as mean $\pm$ SEM and compared between genotypes using an unpaired *t*-test. Concentration of CX3CL1 used in the study has been shown to promote microglial survival³⁴ and cell adhesion *in vitro*.³⁵

To confirm uptake, cells plated on coverslips were immunostained with antibody against Iba1. After permeabilization with 0.1% PBST for 10 minutes at room temperature, cells were blocked in 5% normal goat serum in PBS containing 0.3% Triton X-100 for one hour at room temperature. Cells were then incubated with rabbit polyclonal Iba1 antibody (Wako) diluted 1:1000 in blocking buffer for 1 hour at room temperature, washed with PBS, incubated with Alexa 648 conjugated anti-rabbit IgG secondary antibody (Invitrogen) for one hour at room temperature, washed with PBS, and coverslipped with Vectashield mounting media (Vector). Single plane images were taken using a Leica SP5 confocal microscope.

Preparation of Fibrillar A β

Unlabeled A β_{1-42} (American Peptide Company, Sunnyvale, CA) or HiLyte 555-conjugated A β_{1-42} (AnaSpec, Fremont, CA) was diluted 1 mg/ml in ddH₂O and fibrillarized for 24 hours at 37°C as previously described.²⁹ Control A β_{42-1} peptide (Invitrogen), composed of same amino acids as A β_{1-42} but in reverse sequence, was prepared in the same manner.

Stereotaxic Intracerebral A β Microinjection

Five- to seven-month-old *Cx3cr1*^{+/+} and *Cx3cr1*^{-/-} were deeply anesthetized with ketamine/xylazine cocktail (100/10 mg/kg) and immobilized in a Kopf stereotaxic apparatus. A 0.5-mm burr hole was drilled in the skull, and 2 μ l of fibrillar A β ₁₋₄₂ containing solution was injected at coordinates anteroposterior: -1.0 mm, lateral: 2.0 mm, and dorsoventral: 1.0 mm with respect to bregma in the right cortex using a 10- μ l Hamilton syringe. Some animals were injected with 2 μ l of A β ₄₂₋₁-containing solution at mirroring coordinates on the left cortex, providing an internal control. Mice were sacrificed 48 hours after injection and prepared for histology as described above. Coronal sections containing the needle track were immunostained for Iba1 (and for A β using the 6E10 antibody in animals in which unlabeled A β ₁₋₄₂ or A β ₄₂₋₁ was injected) and visualized using appropriate Alexa dye-conjugated secondary antibodies. Iba1-positive microglia within 50 μ m of the needle track were counted and examined for the presence of intracellular A β in confocal Z-stacks spanning 20–30 μ m in depth to calculate percent phagocytic microglia. Four A β ₁₋₄₂ injected brains and three A β ₄₂₋₁ injected brains were analyzed per genotype. The results were tabulated as mean $\pm$ SEM and compared using an unpaired *t*-test. Unconjugated and HiLyte 555-conjugated A β ₁₋₄₂ injections triggered identical microglial reactions (data not shown), and thus the results were combined into a single group. Furthermore, A β ₄₂₋₁-injected *Cx3cr1*^{+/+} and *Cx3cr1*^{-/-} controls showed no significant differences in microglial counts (data not shown). Finally, control sections incubated with fluorescent anti-mouse IgG secondary antibodies revealed no specific labeling (data not shown), thereby ruling out nonspecific mouse IgG binding at the injection site.

Results

CX3CR1 Deficiency Results in Reduced A β Deposition in APPPS1 Mice

APPPS1 mouse line coexpresses APP K670M/N671L and PS1 L166P mutations under the control of the neuron-specific Thy1 promoter and exhibits early and robust A β deposition beginning at 6–8 weeks of age in the cortex and 3–4 months of age in the hippocampus.²¹ As reported previously, nearly all plaques are fibrillar. There is also marked microglial activation around the A β deposits from the earliest time-points.

Cx3cr1 knockout animals were bred to the rapid-onset APPPS1 mouse model of AD to generate the following genotypes: APPPS1;*Cx3cr1*^{+/+}, APPPS1;*Cx3cr1*^{+/-}, and APPPS1;*Cx3cr1*^{-/-}. Brain sections from four-month-old mice were stained with Thioflavine S, a fluorescent dye specific for fibrillar A β . Consistent with published reports, APPPS1;*Cx3cr1*^{+/+} controls exhibited numerous fibrillar A β deposits in the cortex and hippocampus (Figure 1A). Surprisingly, however, APPPS1;*Cx3cr1*^{+/-} (Figure 1B) and APPPS1;*Cx3cr1*^{-/-} (Figure 1C) animals demon-

Figure 1. Gene dose-dependent reduction in fibrillar A β deposition in the APPPS1 mouse model of AD with CX3CR1 deficiency. Brain sections (30 μ m) from APPPS1;*Cx3cr1*^{+/+} (A, *n* = 7), APPPS1;*Cx3cr1*^{+/-} (B, *n* = 6), and APPPS1;*Cx3cr1*^{-/-} (C, *n* = 7) mice at 4 months of age were stained with Thioflavine S. A series of low-power images (Scale bar = 1 mm) were used to reconstruct the cortex and hippocampus from four sections from each animal. As expected based on the published literature,²¹ APPPS1;*Cx3cr1*^{+/+} animals exhibit abundant fibrillar A β deposition throughout the cortex with reduced A β deposition in the hippocampus (A). By contrast, age-matched APPPS1;*Cx3cr1*^{+/-} (B) and APPPS1;*Cx3cr1*^{-/-} (C) animals exhibited a gene dose-dependent reduction in fibrillar A β deposition throughout the cortex and hippocampus. Quantification of Thioflavine S staining in the cortex (D) and hippocampus (E) across all animals revealed a statistically significant decrease in APPPS1 mice with either one or two copies of *Cx3cr1* loss of function alleles when compared with age-matched APPPS1;*Cx3cr1*^{+/+} controls (**P* < 0.001). Notably, there was also a significant difference in A β deposition in the cortex between APPPS1;*Cx3cr1*^{+/-} and APPPS1;*Cx3cr1*^{-/-} genotypes (*P* < 0.05). To confirm results obtained using Thioflavine S staining, Western blots of brain extracts from *Cx3cr1*^{+/+}, APPPS1;*Cx3cr1*^{+/+}, and APPPS1;*Cx3cr1*^{-/-} animals were probed with antibodies to human A β and GAPDH as a loading control. Consistent with Thioflavine S staining, APPPS1;*Cx3cr1*^{-/-} brains exhibited lower steady-state A β levels when compared with age-matched APPPS1;*Cx3cr1*^{+/+} controls (F). Furthermore, ELISAs performed on APPPS1;*Cx3cr1*^{+/+} (*n* = 6) and APPPS1;*Cx3cr1*^{-/-} brain homogenates (*n* = 5) revealed reduced A β 42 levels in CX3CR1-deficient animals (G; **P* < 0.05).

strated a gene dose-dependent reduction in Thioflavine S-positive A β deposits in both the cortex as well as the hippocampus. In a quantitative analysis of fibrillar A β burden, cortical Thioflavine S positive A β deposition was reduced by 35% in APPPS1;*Cx3cr1*^{+/-} and by 57% in APPPS1;*Cx3cr1*^{-/-} mice when compared to APPPS1;*Cx3cr1*^{+/+} controls (*P* < 0.001; Figure 1D). The APPPS1;*Cx3cr1*^{-/-} mice demonstrated significantly reduced cor-

tical deposition when compared to APPPS1;*Cx3cr1*^{+/-} mice, indicating a gene dose-dependent effect ($P < 0.05$). A β deposition was also reduced in the hippocampus by 70% and 85% in the APPPS1;*Cx3cr1*^{+/-} and APPPS1;*Cx3cr1*^{-/-} animals ($P < 0.001$), when compared to control mice (Figure 1E). To determine whether the A β deposits recognized by Thioflavine S were representative of the total A β within the brain, Western blots of *Cx3cr1*^{+/+}, APPPS1;*Cx3cr1*^{+/+}, and APPPS1;*Cx3cr1*^{-/-} brain extracts were probed with the human-specific anti-A β monoclonal antibody 6E10 (Figure 1F). Confirming results obtained with Thioflavine S, APPPS1;*Cx3cr1*^{-/-} brains exhibited drastically reduced levels of biochemically extracted A β when compared to APPPS1;*Cx3cr1*^{+/+} brains. In support of the Thioflavine S staining, ELISA analysis revealed reduced A β 42 levels in APPPS1;*Cx3cr1*^{-/-} brains (1375 ± 803 pmol/g wet brain; Figure 1G) when compared to APPPS1;*Cx3cr1*^{+/+} brains (4608 ± 861 pmol/g wet brain; Figure 1G). Finally, 6E10 immunohistochemistry also confirmed a gene dose-dependent reduction A β deposits in the cortex (see Supplemental Figure S1 at <http://ajp.amjpathol.org>) and hippocampus (data not shown). Taken together, these data indicate that CX3CR1 deficiency results in a gene dose-dependent reduction in A β deposition in the APPPS1 mouse model of AD.

CX3CR1 Deficiency Results in Reduced A β Deposition in R1.40 Mice

To address the effect of CX3CR1 deficiency in another AD mouse model, we bred *Cx3cr1* knockout animals to the R1.40 mouse model of AD. The R1.40 transgene is a full genomic copy of the human APP gene expressing the K670M/N671L mutation carried on a yeast artificial chromosome. The R1.40 transgenic mouse line develops gradual AD-like A β pathology, exhibiting both diffuse and fibrillar A β deposition beginning at 14–16 months of age in the cortex and 18–20 months of age in the hippocampus.³⁶ Notably, fibrillar A β deposits in R1.40 mice are associated with reactive microglia.

Brain sections from R1.40;*Cx3cr1*^{+/+}, R1.40;*Cx3cr1*^{+/-}, and R1.40;*Cx3cr1*^{-/-} animals at 20–24 months of age were stained with Thioflavine S to visualize fibrillar A β . R1.40;*Cx3cr1*^{+/+} controls exhibited abundant Thioflavine S positive fibrillar A β deposits in the cortex (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). Similar to the results in CX3CR1-deficient APPPS1 mice, R1.40;*Cx3cr1*^{+/-} and R1.40;*Cx3cr1*^{-/-} animals also demonstrated a gene dose-dependent reduction in Thioflavine S-positive A β deposits in the cortex. Quantification of the total number of Thioflavine S-positive A β deposits in the cortex revealed that R1.40;*Cx3cr1*^{+/-} and R1.40;*Cx3cr1*^{-/-} exhibited markedly fewer cortical Thioflavine S-positive plaques when compared to site-matched brain regions in R1.40;*Cx3cr1*^{+/+} mice ($P < 0.05$). Taken together, these data indicate that CX3CR1 deficiency results in a gene dose-dependent reduction in A β deposition in mouse models of AD, regardless of the kinetics of deposition.

CX3CR1 Deficiency Reduces A β -Associated Dystrophic Neurites in APPPS1 and R1.40 Mice

To assess neuronal integrity in the current studies, several additional experiments were performed. First, TUNEL and cleaved caspase 3 staining of brain sections from CX3CR1-deficient APPPS1 and R1.40 animals did not reveal any gross alterations in neurodegeneration (data not shown), consistent with previous studies demonstrating no overt neurodegeneration in either the APPPS1 or R1.40 animals.^{21,36} Second, additional staining with antibodies for dystrophic neurites, including APP (antibody CT15) and tau phosphorylation (antibody AT8) revealed a reduction in staining in both the APPPS1;*Cx3cr1*^{-/-} (See Supplemental Figure S3 at <http://ajp.amjpathol.org>) and R1.40;*Cx3cr1*^{-/-} animals (data not shown) that likely reflects the reduction in A β deposition observed in these mice.

CX3CR1 Deficiency Does Not Alter APP Levels or APP Processing

One potential explanation for the alterations in A β deposition observed in the APPPS1;*Cx3cr1*^{+/-} and APPPS1;*Cx3cr1*^{-/-} animals was that either APP transgene expression or APP processing was altered. To examine this possibility, brain extracts from two-month-old APPPS1;*Cx3cr1*^{+/+}, APPPS1;*Cx3cr1*^{+/-}, and APPPS1;*Cx3cr1*^{-/-} animals were analyzed by Western blot for the levels of holo-APP and APP CTFs. No significant differences in the steady state levels of holo-APP were observed in the samples from three different genotypes (Figure 2, A [top] and B). The steady-state levels of APP CTFs, including CTF β , the direct precursor of A β , were unaltered (Figure 2, A [middle] and C). Similar results were also obtained in the R1.40 mouse model of AD with CX3CR1 deficiency (see Supplemental Figure S4 at <http://ajp.amjpathol.org>). In addition, there were no alterations in the steady-state

Figure 2. Lack of effect of CX3CR1 genotype on APP processing. **A:** Western blots of brain extracts from the APPPS1;*Cx3cr1*^{+/+} (lanes 1–3), APPPS1;*Cx3cr1*^{+/-} (lanes 4–6), and APPPS1;*Cx3cr1*^{-/-} (lanes 7–9) animals were probed with an antibody to the C terminus of APP (top two panels) and subsequently stripped and reprobed with an antibody against α -tubulin as a loading control. Shown on the right is the approximate size in kDa. **B:** Relative levels of holo-APP were quantified ($n = 8$) from each genotype by normalizing the intensity values of APP to α -tubulin. **C:** Relative values of CTF β were quantified similarly averaging the values of APP CTF β to α -tubulin. No significant differences were observed in the relative levels of either holo-APP or CTF β between genotypes.

levels of brain A β in pre-depositing APPPS1 and R1.40 mice (data not shown). Taken together, these results indicate that the differences in A β deposition observed in this study are not due to altered expression or processing of APP by neurons and instead suggest involvement of brain microglia, where CX3CR1 is exclusively expressed.

CX3CR1 Deficiency Leads to an Altered Neuroinflammatory Milieu

One potential explanation for the alterations in A β deposition observed in the APPPS1;Cx3cr1^{+/-} and APPPS1;Cx3cr1^{-/-} animals was that loss of CX3CL1-CX3CR1 signaling leads to altered microglial activation. To examine this possibility, brain sections from four-month-old wild-type C57BL/6J mice (Figure 3, A and B), APPPS1;Cx3cr1^{+/-} (Figure 3, C and D), APPPS1;Cx3cr1^{+/-} (Figure 3, E and F), and APPPS1;Cx3cr1^{-/-} (Figure 3, G and H) were immunostained with either an antibody to CD68 (Figure 3, A, C, E, and G), a marker for macrophages (including microglia-derived macrophages), or CD45 (Figure 3, B, D, F, and H), a marker for cells of the hematopoietic lineage which is moderately up-regulated in activated microglia. Sections were subsequently counterstained with the amyloid dye Congo Red to visualize dense-core A β deposits. Wild-type non-transgenic sections demonstrated no Congo Red-positive deposits and sparse CD68 (Figure 3A) and CD45 immunoreactivity (Figure 3B). CD68- and CD45-positive microglia are closely associated with Congo Red-positive deposits in all APPPS1 genotypes (Figure 3, insets). CD68 immunoreactivity was qualitatively reduced in APPPS1;Cx3cr1^{+/-} (Figure 3E) and APPPS1;Cx3cr1^{-/-} animals (Figure 3G) compared to APPPS1;Cx3cr1^{+/-} controls (Figure 3C). By contrast, CD45 immunoreactivity between the APPPS1;Cx3cr1^{+/-} (Figure 3D), APPPS1;Cx3cr1^{+/-} (Figure 3F) and APPPS1;Cx3cr1^{-/-} (Figure 3H) genotypes appeared relatively unchanged. These data suggest that CX3CR1 deficiency alters the microglial activation program in reaction to dense-core A β deposits.

To determine whether microglial CX3CR1 signaling mediates non-cell-autonomous effects in other inflammatory cells in the brain, sections from four-month-old wild-type, APPPS1;Cx3cr1^{+/-}, and APPPS1;Cx3cr1^{-/-} animals were stained with an antibody to GFAP, an astrocytic marker and Congo Red (see Supplemental Figure S5 at <http://ajp.amjpathol.org>). As expected, wild-type controls exhibited no Congo Red-positive A β deposits and GFAP immunoreactivity was limited to the meninges and perivascular regions. By contrast, APPPS1;Cx3cr1^{+/-} mice exhibited abundant Congo Red-positive A β deposits that were ringed by numerous GFAP-positive astrocytes. In addition to a substantial reduction in the amount of A β deposition in the APPPS1;Cx3cr1^{-/-} animals (Figure 1), there was also significantly reduced GFAP immunoreactivity. Taken together, these results indicate that, in addition to alterations in microglial activation, there is also reduced astrocytosis in CX3CR1-deficient APPPS1 mice, although both of these alterations could be due to the reduced A β deposition observed.

Figure 3. Altered expression of microglial markers in the APPPS1 mouse model of AD with CX3CR1 deficiency. Brain sections (30 μ m) from wild-type C57BL/6J (A and B), APPPS1;Cx3cr1^{+/-} (C and D), APPPS1;Cx3cr1^{+/-} (E and F), and APPPS1;Cx3cr1^{-/-} (G and H) mice at 4 months of age were immunostained with antibodies to either CD68, a marker for phagocytic microglia/macrophages (A, C, E, and G), or CD45, a marker for cells of hematopoietic lineage which is up-regulated in activated microglia (B, D, F, and H). Sections were also counterstained with Congo Red, a dye specific for dense-core A β deposits. Wild-type sections contained no Congo Red-positive deposits and rare CD68 (A) and CD45 (B) immunoreactivity. Consistent with the published literature, APPPS1;Cx3cr1^{+/-} animals show abundant CD68 (C) and CD45 immunoreactivity (D), especially in the immediate vicinity of Congo Red-positive A β deposits (insets). While age-matched APPPS1;Cx3cr1^{+/-} and APPPS1;Cx3cr1^{-/-} animals exhibit gene dose-dependent reduction in CD68 immunoreactivity compared to APPPS1;Cx3cr1^{+/-} controls (E and G, respectively), CD45 immunoreactivity is relatively unchanged in APPPS1;Cx3cr1^{+/-} and APPPS1;Cx3cr1^{-/-} animals compared with APPPS1;Cx3cr1^{+/-} controls (F and H, respectively). As expected, higher magnification revealed that CD68- and CD45-positive microglia are mostly clustered around Congo Red-positive A β deposits (insets). Scale bar = 250 μ m.

CX3CR1 Deficiency Leads to Reductions in Plaque-Associated Microglia

Our immunohistochemical studies suggested that CX3CR1 deficiency leads to reductions in CD68-positive microglia. This finding could be due to the reduced A β deposition observed in CX3CR1-deficient mice. To address this possibility, we quantified the number of microglia on a per-plaque basis. Brain sections from four-month-old APPPS1;Cx3cr1^{+/-} (Figure 4, A–C), APPPS1;Cx3cr1^{+/-} (Figure 4, D–F), and APPPS1;Cx3cr1^{-/-} mice (Figure 4, G–I) were coimmunostained with monoclonal anti-A β antibody, 4G8 (red; Figure 4, A, D, and G) and an

Figure 4. Reduced plaque-associated microglia in the APPPS1 mouse model of AD with CX3CR1 deficiency. Brain sections (30 μm) from APPPS1;Cx3cr1^{+/+} (A–C), APPPS1;Cx3cr1^{+/-} (D–F), and APPPS1;Cx3cr1^{-/-} (G–I) mice at 4 months of age were immunostained with monoclonal Aβ antibody 4G8 (red; A, D, and G), with an antibody against the pan-microglial marker Iba1 (green; B, E, and H), and counterstained with the nuclear TO-PRO-3 dye (blue; C, F, and I). Confocal microscopy was used to obtain maximum projections reconstructed from Z-stacks spanning 20–30 μm in depth. As expected, APPPS1;Cx3cr1^{+/+} controls exhibit extensive accumulation of Iba1-positive microglia around senile plaques (A–C). However, age-matched APPPS1;Cx3cr1^{+/-} (D–F) and APPPS1;Cx3cr1^{-/-} animals (G–I) exhibit gene dose-dependent reduction in the number of Iba1-positive microglia surrounding the senile plaques. The number of Iba1-positive microglia associated with senile plaques in the three genotypes was quantified in three nonadjacent sections from each of the four animals per genotype. APPPS1 mice with either one or two copies of *Cx3cr1* loss-of-function alleles exhibited a statistically significant reduction in microglia surrounding both large (>1000 μm²; J), medium (500–1000 μm²; K), and small (<500 μm²; L) Aβ deposits when compared to age-matched APPPS1;Cx3cr1^{+/+} controls (**P* < 0.05; ***P* < 0.001, respectively). The APPPS1;Cx3cr1^{+/-} and APPPS1;Cx3cr1^{-/-} genotypes exhibited a statistically significant difference in microglial accumulation around medium Aβ deposits (*P* < 0.05), suggesting a gene dose-dependent effect. *n* = 35 for all analyses. Scale bar = 25 μm.

antibody against the pan-microglial marker Iba1 (green; Figure 4, B, E, and H). Sections were subsequently counterstained with a TO-PRO-3 nuclear dye (blue; Figure 4C, F, and I) and imaged via confocal microscopy to obtain Z-stacks. We observed a gene dose-dependent reduction in the number of plaque-associated microglia in the cortex of APPPS1;Cx3cr1^{+/-} (Figure 4, D–F) and APPPS1;Cx3cr1^{-/-} (Figure 4, G–I) animals when compared to APPPS1;Cx3cr1^{+/+} controls (Figure 4, A–C).

To evaluate the possibility that CX3CR1 signaling to microglia might mediate a differential response to larger plaques, Aβ deposits were grouped based on area and numbers of Iba1-positive microglia whose nuclei or primary processes directly overlapped Aβ deposits in a given tissue section were quantified. Large plaques

(>1000 μm²) in APPPS1;Cx3cr1^{+/+} controls exhibited an average of ~13 plaque associated microglia, whereas those in APPPS1;Cx3cr1^{+/-} and APPPS1;Cx3cr1^{-/-} animals exhibited only ~11 and ~9 plaque-associated microglia, respectively (Figure 4J). For medium plaques (>500 μm² and <1000 μm²), APPPS1;Cx3cr1^{+/-} and APPPS1;Cx3cr1^{-/-} mice demonstrated an average of ~7 and ~5 plaque associated microglia, respectively, which was significantly lower than ~9 plaque associated microglia in the medium plaques of APPPS1;Cx3cr1^{+/+} controls (Figure 4K). In addition, for medium plaques, APPPS1;Cx3cr1^{-/-} mice exhibited a significant decrease in plaque associated microglia when compared to APPPS1;Cx3cr1^{+/-} mice, indicating a gene dose-dependent effect. For small plaques (<500 μm²), differences were not statistically significant among the genotypes (Figure 4L). In summary, these results indicate that CX3CR1 deficiency reduces the number of microglia around Aβ deposits in a gene dose-dependent manner in the APPPS1 mouse model of AD.

CX3CR1 Deficiency Leads to Altered Expression of Inflammatory Cytokines/Chemokines

To gain insight into the potential mechanisms underlying the concomitant reduction in Aβ deposition and plaque-associated microglia, the expression levels of various cytokines and chemokines was examined by quantitative RT-PCR. Total brain RNA from four-month-old B6 controls, APPPS1;Cx3cr1^{+/+}, APPPS1;Cx3cr1^{+/-}, and APPPS1;Cx3cr1^{-/-} mice was reverse transcribed and assayed in triplicate for expression of the CCL2, CX3CL1, IFNγ, IL1β, IL4, IL6, IL10, and TNFα mRNAs using TaqMan probes. Expression of both TNFα and CCL2 mRNAs were increased in APPPS1;Cx3cr1^{+/+} mice at four months of age relative to B6 control mice (Figure 5, A and C, respectively). Notably, age-matched APPPS1;Cx3cr1^{+/-} and APPPS1;Cx3cr1^{-/-} mice demonstrated reduced expression levels of both TNFα (*P* < 0.001) and CCL2 mRNAs (*P* < 0.01) relative to APPPS1;Cx3cr1^{+/+} mice (Figure 5, A and C, respectively). By contrast, expression of IL1β mRNA was up-regulated in APPPS1;Cx3cr1^{+/-} and APPPS1;Cx3cr1^{-/-} animals (*P* < 0.05 and *P* < 0.01, respectively; Figure 5B) as previously reported in other neuroinflammatory models.¹³ Levels of mRNAs for CX3CL1, IFNγ, IL4, IL6, and IL10 did not

Figure 5. Altered expression of inflammatory markers in CX3CR1-deficient APPPS1 mouse model of AD. Relative mRNA expression of TNFα (A), IL1β (B), and MCP-1 (C) in APPPS1;Cx3cr1^{+/+} (*n* = 7), APPPS1;Cx3cr1^{+/-} (*n* = 8), and APPPS1;Cx3cr1^{-/-} (*n* = 9) mice at 4 months. The mRNA levels for each cytokine were normalized to the mRNA levels of GAPDH and expressed relative to that of nontransgenic C57BL/6J mice. Results from APPPS1;Cx3cr1^{+/-} and APPPS1;Cx3cr1^{-/-} animals were compared with results from age-matched APPPS1;Cx3cr1^{+/+} controls (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

Figure 6. CX3CL1-CX3CR1 signaling inhibits microglial phagocytosis. *Cx3cr1*^{+/+} (A and B) and *Cx3cr1*^{-/-} (C and D) primary microglia were incubated in the absence (A and C) or presence (B and D) of CX3CL1 (100 nmol/L) overnight, and fluorescent microspheres were added for 90 minutes. The cells were fixed and stained with antibody against Iba1 and visualized with Alexa 648-conjugated secondary antibody (pseudo-colored green; A–D). Single-plane confocal images are shown. CX3CL1-treated *Cx3cr1*^{+/+} microglia exhibited reduced numbers of phagocytosed microspheres per cell when compared with untreated *Cx3cr1*^{+/+} and CX3CL1-treated *Cx3cr1*^{-/-} microglia (E; **P* < 0.05). *n* = 3 for each group. Scale bar = 15 μ m.

exhibit significant differences among the genotypes relative to B6 control mice (data not shown). These results suggest that there are alterations in the expression of the chemokines/cytokines CCL2, IL1 β , and TNF α in the brains of the APPS1 mouse model of AD with CX3CR1 deficiency.

CX3CR1 Signaling Inhibits Microglial Phagocytosis

Our observations that reduced amyloid deposition in CX3CR1-deficient mice, along with less microglia per plaque, were somewhat counterintuitive. One potential explanation is that CX3CL1-CX3CR1 signaling could alter the phagocytic capacity of microglia. To examine this possibility, primary microglia from *Cx3cr1*^{+/+} (Figure 6, A and B) and *Cx3cr1*^{-/-} animals (Figure 6, C and D) were assessed for their ability to phagocytose fluorescent microspheres. On average, *Cx3cr1*^{+/+} microglia phagocytosed ~4 microspheres per cell (Figure 6, A and E)

Figure 7. Enhanced A β phagocytosis in CX3CR1-deficient mice. Five- to 7-month-old *Cx3cr1*^{+/+} (*n* = 4; A) and *Cx3cr1*^{-/-} (*n* = 4; B) mice were injected with fibrillar A β ₁₋₄₂ (red; HiLyte 555-conjugated) or with control A β ₄₂₋₁ peptide (*n* = 6; C). Sections (30 μ m) containing the needle track were immunostained with an antibody against Iba1 and visualized with Alexa 648-conjugated secondary antibody (pseudo-colored green; A–C). From confocal Z-stacks spanning 20–30 μ m in depth, we obtained maximum projections (A–C) and slices in the x- and y-plane showing A β internalization (A and B). The number of microglia (E) and percent phagocytic microglia (D) within 50 μ m of the needle track were quantified. *Cx3cr1*^{-/-} microglia exhibited enhanced A β phagocytosis when compared with control *Cx3cr1*^{+/+} microglia (**P* < 0.01; D), whereas the number of microglia around the injection site did not significantly differ between genotypes (E). Furthermore, as expected, control A β ₄₂₋₁ peptide injection triggered significantly reduced microglial reaction compared to fibrillar A β ₁₋₄₂ injection (C and E; **P* < 0.05).

whereas CX3CL1-treated *Cx3cr1*^{+/+} microglia phagocytosed only ~2 microspheres per cell (Figure 6, B and E; *P* < 0.05). *Cx3cr1*^{-/-} microglia demonstrated equivalent phagocytic responses either with (Figure 6, C and E) or without fractalkine treatment (Figure 6, D and E), indicating that CX3CL1-mediated inhibition of microglial phagocytic capacity required CX3CR1 signaling. These results suggest that alterations in CX3CL1-CX3CR1 signaling can lead to altered phagocytic capabilities of microglia and could account for the apparent discrepancy between the reduced numbers of microglia per plaque and reduced A β deposition observed in CX3CR1-deficient animals.

CX3CR1 Deficiency Enhances A β Phagocytosis

Our *in vitro* data suggested that CX3CL1-CX3CR1 signaling inhibits microglial phagocytic capacity. To examine whether loss of CX3CL1-CX3CR1 signaling could lead to enhanced uptake of A β *in vivo* and thus account for findings in the CX3CR1-deficient AD mouse models, fibrillar A β ₁₋₄₂ was stereotactically injected into the cortices of *Cx3cr1*^{+/+} (Figure 7A) and *Cx3cr1*^{-/-} animals (Figure 7B). On average, 46% of *Cx3cr1*^{+/+} microglia within 50 μ m of the needle track contained fibrillar A β , whereas 66% of *Cx3cr1*^{-/-} microglia contained A β (Figure 7D; *P* < 0.01). Notably, however, both *Cx3cr1*^{+/+} and *Cx3cr1*^{-/-} microglia accumulated in similar numbers near the fibrillar A β ₁₋₄₂ injection sites (Figure 7E; *P* < 0.05), suggesting that there was no significant alteration in the ability of microglia to migrate to the A β injection site. Stereotaxic injections with a control A β ₄₂₋₁ peptide

triggered significantly reduced microglial reaction in both the *Cx3cr1*^{+/+} and *Cx3cr1*^{-/-} brains (Figure 7, C and E; $P < 0.05$), indicating that the microglial responses were specific to fibrillar A β ₁₋₄₂. In addition, the *Cx3cr1*^{-/-} microglia in the A β ₁₋₄₂ injected animals exhibited a different morphology, with a more rounded appearance, than either *Cx3cr1*^{+/+} A β ₁₋₄₂ injected animals or A β ₄₂₋₁ injected controls (compare Figure 7B to 7A and 7C), which is consistent with an altered phagocytic capacity of these cells. These data demonstrate that blocking CX3CL1-CX3CR1 signaling leads to enhanced uptake of fibrillar A β within microglia and could thus explain the reductions in A β deposition observed in CX3CR1-deficient APPPS1 and R1.40 animals.

Discussion

The findings presented here offer insights into the role of microglial CX3CR1 signaling in the development of amyloid pathology in the APPPS1 and R1.40 mouse models. Previous studies showed that in the CNS, CX3CL1 is expressed by neurons and signals to CX3CR1 on microglia.^{13,14} Furthermore, recent reports implicated impaired CX3CL1-CX3CR1 signaling in neurodegeneration. First, in various models of neurodegeneration, including the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease; a transgenic mouse model of amyotrophic lateral sclerosis; and induction of neuroinflammation via systemic lipopolysaccharide administration, CX3CR1 deficiency resulted in increased neuronal cell death and worsening of phenotypes via mechanisms cell-autonomous to microglia.¹³ Second, *Cx3cr1* knockout mice exhibited age-dependent subretinal microglial accumulation and retinal degeneration.^{37,38} Third, the V249I and T280M polymorphisms in *CX3CR1* are associated with altered risk for age-related macular degeneration in humans.^{17,18} Taken together these results support a role of CX3CL1-CX3CR1 signaling to microglia in modulating neurodegenerative disease phenotypes.

The present studies tested the hypothesis that CX3CR1 deficiency would alter AD phenotypes in transgenic mouse models of the disease. Unexpectedly, CX3CR1 deficiency in two different mouse models of AD leads to an amelioration of the amyloid pathology in both the rapid onset APPPS1 (Figure 1) as well as the gradual onset R1.40 (see Supplemental Figure S2 at <http://ajp.amjpathol.org>) mouse models of AD. To examine the effects of CX3CR1 deficiency on neurons, TUNEL and cleaved caspase 3 staining of brain sections from CX3CR1-deficient APPPS1 and R1.40 animals revealed no evidence for any gross neurodegeneration (data not shown) while staining with antibodies for dystrophic neurites (CT15 and AT8) revealed a reduction in staining in both the APPPS1;*Cx3cr1*^{-/-} (see Supplemental Figure S3 at <http://ajp.amjpathol.org>) and R1.40;*Cx3cr1*^{-/-} animals (data not shown) that appears to reflect the reduction in A β deposition observed in these mice.

Significantly, we also observed a gene dose-dependent effect of CX3CR1 deficiency, with heterozygous *Cx3cr1* mice exhibiting an intermediate phenotype. This

suggests that alterations in CX3CR1 signaling could influence human AD pathogenesis, a hypothesis that could be tested via genetic association studies with *CX3CR1* variants implicated in age-related macular degeneration or via therapeutic strategies aimed at reducing CX3CR1 signaling. The finding of a heterozygous phenotype also has implications for previous studies in the field, as heterozygous *Cx3cr1*^{GFP/+} mice have been used to study microglial dynamics in AD mouse models.^{3,5}

Multiple mechanisms could account for the reduction in A β deposition observed in the CX3CR1-deficient mouse models of AD. To determine whether CX3CR1 deficiency altered APP production or processing, we showed that the levels of steady-state APP and APP CTFs remained unchanged in CX3CR1 deficient APPPS1 and R1.40 mice (Figure 2 and see Supplemental Figure S4 at <http://ajp.amjpathol.org>). Given that CX3CR1 is exclusively expressed by microglia within the brain and the effects of CX3CR1 deficiency are autonomous to microglia,¹³ we postulated that the effect of CX3CR1 deficiency on A β deposition was mediated via alterations within microglia.

Analysis of microglial markers demonstrated a gene dose-dependent reduction in both the numbers of Iba1-positive microglia surrounding individual A β deposits (Figure 4) as well as staining for the microglial activation marker CD68 (Figure 3) in CX3CR1-deficient APPPS1 mice. Thus, our results showed a reduction in the amount of A β deposition and alterations in the activation of microglia surrounding the A β deposits. These observations were unexpected, as it seemed more likely that reduced amyloid burden might be mediated by augmented microglial activation.

In addition to alterations in microglial activation, we also observed a substantial reduction in astrogliosis in CX3CR1-deficient APPPS1 animals (see Supplemental Figure S5 at <http://ajp.amjpathol.org>), suggesting that there is an altered neuroinflammatory milieu within these animals. However, this was not unexpected given that there is reduced A β deposition in these animals. In addition, because CX3CR1 is exclusively expressed by microglia in the murine CNS,^{13,15} the reduced astrogliosis is by definition a secondary response to primary alterations in microglia. Thus, while we cannot formally exclude the role of astrocytes in the reduced A β deposition observed in CX3CR1-deficient animals, the current studies point to enhanced A β phagocytosis by *Cx3cr1*^{-/-} microglia as the more proximal and significant contributor.

Interestingly, we also found selective alterations in the levels of brain mRNAs for specific cytokines/chemokines depending on the genotype of the animal (Figure 5). Similar to previous findings in AD brains and mouse models,³⁹⁻⁴² the levels of CCL2 and TNF α mRNAs were elevated in the APPPS1;*Cx3cr1*^{+/+} mouse brain. Notably, CX3CR1 deficiency reduced the levels of CCL2 and TNF α mRNA to that observed in the non-transgenic controls, while brain levels of IL1 β mRNA were increased.

In addition, *in vitro* phagocytosis assay revealed that CX3CL1-CX3CR1 signaling regulates the phagocytic capacity of microglia (Figure 6). CX3CL1 treatment of primary microglia led to a 50% reduction in the number of

phagocytosed microspheres in wild-type *Cx3cr1*^{+/+} primary microglia. Importantly, the phagocytic capacity of *Cx3cr1*^{-/-} primary microglia did not change with CX3CL1 treatment, indicating that the reduced microglial phagocytosis observed is mediated by CX3CR1 signaling. We did not observe an enhancement of phagocytosis in *Cx3cr1*^{-/-} compared to untreated *Cx3cr1*^{+/+} microglia.

Finally, we confirmed that microglial A β phagocytosis was enhanced in CX3CR1-deficient APPPS1 animals (Figure 7). Sixty-six percent of *Cx3cr1*^{-/-} microglia around fibrillar A β ₁₋₄₂ injection sites were phagocytic, whereas only 46% of *Cx3cr1*^{+/+} microglia were phagocytic. However, *Cx3cr1*^{+/+} and *Cx3cr1*^{-/-} microglia accumulated in similar numbers around the injection sites, suggesting that microglial migration was not impaired by CX3CR1 deficiency.

In the AD brain, microglia accumulate at the site of newly formed A β deposits via migration or local proliferation and may help restrict plaque growth.^{4,5} We hypothesize that the drastic reduction in A β deposition in CX3CR1 deficient R1.40 and APPPS1 animals is due to alterations in the ability of CX3CR1 deficient microglia to i) remove A β , and/or ii) accumulate around A β deposits via proliferation and/or migration. The postulated sequence of events is that A β accumulation is associated with neuronal stress and CX3CL1 proteolytic release,⁴³ leading to reduced phagocytosis by *Cx3cr1*^{+/+} but not *Cx3cr1*^{-/-} microglia.

Microglial Removal of A β

Recent *in vivo* imaging studies using mouse models of AD have shown that microglia may help restrict the growth of senile plaques.^{4,5} However, considerable evidence suggests that *in vivo* A β removal is critically dependent on microglial activation status.^{33,44-47} Microglia can exist in multiple activation states that are determined by the local stimuli for activation and reflected in production of specific cytokines and chemokines.⁴⁸ Within the AD brain, there is a chronic activation of microglia associated with inflammatory cytokines including TNF α that can substantially block the ability of the microglia to remove^{47,49,50} or degrade A β .^{51,52} Recent studies demonstrated that overexpression of IL1 β , an inflammatory cytokine leads to reduced A β pathology in mouse models of AD.^{9,53}

One partial explanation for our results is that CX3CR1 deficiency alters the microglial activation status and enhances A β clearance. In support of this possibility, TNF α mRNA levels were selectively down-regulated in CX3CR1 deficient APPPS1 animals, and TNF α has been shown to directly reduce microglial expression of A β -degrading enzymes such as insulin-degrading enzyme and neprilysin.^{51,52} Furthermore, IL1 β mRNA levels were significantly increased in a gene dose-dependent manner in the CX3CR1-deficient APPPS1 animals, and previous studies demonstrated that IL1 β overexpression enhances A β clearance.^{9,53} Finally, mRNA levels of MCP-1, which promotes microglia-mediated A β oligomerization,⁵⁴ was reduced in CX3CR1-deficient APPPS1 ani-

mals and thus could lead to reduced A β oligomerization/deposition. These observations suggest that CX3CR1 deficiency leads to a cytokine environment that harnesses the beneficial effects of microglial activation in response to A β . Notably, increased IL1 β levels were also observed in *Cx3cr1* knockout mice in response to lipopolysaccharide injections, where it led to enhanced neurotoxicity.¹³ Indeed, the effect of CX3CR1 deficiency appears to depend on the nature of the CNS insult as recent studies demonstrated that *Cx3cr1* knockout mice exhibit smaller infarcts after transient middle cerebral artery occlusion.^{19,20}

Another relevant observation is that CX3CR1 signaling directly blunts microglial phagocytosis and might prevent effective A β clearance. In support of this hypothesis, our *in vitro* and *in vivo* data demonstrate that CX3CL1-CX3CR1 signaling inhibits microglial phagocytosis, including fibrillar A β . Notably, CX3CL1 is found in high concentrations in both normal and diseased CNS,⁵⁵ and our results suggest that CX3CL1-CX3CR1 signaling establishes a permissive environment for A β deposition in the AD brains and transgenic mouse models by down-regulating the microglial phagocytic response toward A β . Enhanced phagocytosis in the absence of CX3CR1 signaling could also help to explain the heterogeneity of phenotypes in CX3CR1-deficient CNS disease models. Depending on the nature of the CNS insult, increased microglial phagocytosis may ameliorate⁴⁶ or worsen pathology.⁵⁶

Finally, a recent study provided evidence that gancyclovir-mediated depletion of microglia expressing thymidine kinase had no demonstrable effect on A β pathology in two mouse models of AD.⁵⁷ While these studies suggest that relatively short term depletion of microglia had only negligible effect on A β deposition, numerous other studies have suggested that altering the proliferation,⁵⁸ signaling,^{8,9,11} and migration^{7,59} of microglia directly impact development of A β pathology. Taken together, these data suggest multiple redundant pathways that regulate A β clearance and deposition in different disease states and experimental conditions.

Microglial Proliferation/Migration

In mouse models of AD, the numbers of plaque-associated microglia increase over time and restrict the growth in plaque volume.^{4,5} However, in other situations, excess microglial accumulation appears to exacerbate A β pathology.⁷ In CX3CR1-deficient animals, there was a gene dose-dependent reduction in number of microglia associated with A β deposition. We postulate that our observations could be explained by the following: i) enhanced microglial clearance of A β , so that the retention of cells per plaque was reduced; ii) alterations in microglial proliferation/cell death; and/or iii) alterations in migration of microglia/macrophages to A β deposits.

Microglia surrounding A β deposits undergo extensive turnover in a mouse model of AD, exhibiting both apoptosis and proliferation.⁶⁰ Interestingly, CX3CR1 signaling inhibits microglial apoptosis via Fas-mediated mech-

anisms *in vitro*.³⁴ Future studies will be needed to determine whether the reduction in microglial number associated with A β deposition in CX3CR1-deficient animals is due to alterations in microglial cell death or proliferation. However, our preliminary analysis suggests that microglial proliferation and cell death were unaltered in APPPS1 mice deficient for CX3CR1 as assessed via bromodeoxyuridine incorporation and cleaved caspase 3 labeling, respectively (data not shown).

Another possibility is altered migration of either microglia or peripheral monocytes/macrophages to A β deposits in the CX3CR1-deficient animals. Various genetically modified mouse models have convincingly demonstrated the role for other chemokines/cytokine pathways in altering the accumulation of mononuclear phagocytes (monocytes, macrophages, and microglia) and A β pathology, including CCR2⁵⁹ and IL1 β .⁹ However, in the present studies, despite the reduction in the number of microglia around the A β deposits in CX3CR1-deficient APPPS1 animals, there was a significant reduction in A β deposition, consistent with an enhanced capacity of microglia to remove A β . Accordingly, the simplest interpretation of our data are that fewer *Cx3cr1*^{-/-} microglia were associated with each plaque due to their enhanced phagocytic capacity, leading to the associated findings of reduced A β deposition and fewer microglia per plaque.

A recent study (published while the current study was under review) suggested that CX3CR1 deficiency can prevent neuronal loss in the 3xTg mouse model of AD.⁶¹ A 1.8% loss of neurons per month observed in the 3xTg mouse model by two-photon microscopy was prevented in the CX3CR1 deficient 3xTg animals. However, this study examined 3xTg animals at 4–6 months, an age at which there is no extracellular deposition of A β but instead abundant intracellular A β immunoreactivity. At the single age examined, the levels of A β remain relatively unchanged in these animals, although their assessment of insoluble A β appears to show a non-significant reduction in the knockout mice.

Fuhrmann et al argue that these studies show that the lack of CX3CR1 does not “interfere with the A β -phagocytosing activity of microglia.” However, our studies demonstrate that CX3CR1 deficiency reduces *extracellular* A β deposition in two different mouse models of AD via a mechanism that likely involves enhanced phagocytic clearance of extracellular A β fibrils. Thus, the apparent discrepancy in the results of the two studies may be reflective of the cellular location of the A β aggregates and is supported by our findings that the steady-state levels of A β in predepositing CX3CR1-deficient APPPS1 and R1.40 animals remained unchanged (data not shown).

Furthermore, we did not observe any obvious alterations in neurodegeneration in the APPPS1;*Cx3cr1*^{-/-} and R1.40;*Cx3cr1*^{-/-} animals, consistent with previous studies demonstrating no overt neurodegeneration in either the APPPS1 or R1.40 animals.^{21,36} Notably, similar types of studies also did not detect robust neuronal loss in aged 3xTg animals,⁶² and it remains to be determined how the neuronal loss observed by Fuhrmann et al via two photon microscopy relates to the amyloid toxicity

observed in the 3xTg, APPPS1 and R1.40 mouse models. Notably, the reduction in A β deposition in the CX3CR1-deficient APPPS1 and R1.40 mice also led to a reduction in the formation of dystrophic neurites, which perhaps could, in turn, impact local neurodegeneration. Clearly, additional studies are warranted to further define the role of CX3CL1/CX3CR1 signaling in AD pathogenesis

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