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Complement Component C3 and Complement Receptor Type 3 Contribute to the Phagocytosis and Clearance of Fibrillar Aβ by Microglia

Hongjun Fu[1], **Bin Liu**[1], **Jeffrey L. Frost**[1], **Soyon Hong**[1], **Ming Jin**[1], **Beth Ostaszewski**[1], **Ganesh M. Shankar**[1], **Isabel M. Costantino**[1], **Michael C. Carroll**[2], **Tanya N. Mayadas**[3], and **Cynthia A. Lemere**[1]

[1]Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

[2]Department of Pediatrics, Harvard Medical School, Boston, MA.

[3]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

Abstract

Complement components and their receptors are found within and around Aβ cerebral plaques in Alzheimer's disease (AD). Microglia defend against pathogens through phagocytosis via complement component C3 and/or engagement of C3 cleavage product iC3b with complement receptor type 3 (CR3, Mac-1). Here we provide direct evidence that C3 and Mac-1 mediate, in part, phagocytosis and clearance of fibrillar amyloid-β (fAβ) by murine microglia *in vitro* and *in vivo*. Microglia took up not only synthetic $fA\beta_{42}$ but also amyloid cores from AD patients, transporting them to lysosomes *in vitro*. Fibrillar $A\beta_{42}$ uptake was significantly attenuated by the deficiency or knockdown of C3 or Mac-1 and scavenger receptor class A ligands. In addition, C3 or Mac-1 knockdown combined with a scavenger receptor ligand, fucoidan, further attenutated fibrillar $A\beta_{42}$ uptake by N9 microglia. Fluorescent fibrillar $A\beta_{42}$ microinjected cortically was significantly higher in C3 and Mac-1 knockout mice compared to wild-type mice 5 days after surgery, indicating reduced clearance *in vivo*. Together, these results demonstrate that C3 and Mac-1 are involved in phagocytosis and clearance of fAβ by microglia, providing support for a potential beneficial role for microglia and the complement system in AD pathogenesis.

Keywords

microglia; Aβ; complement component C3; complement receptor type 3; Mac-1; phagocytosis

INTRODUCTION

Amyloid β peptide (Aβ) is the principal component of extracellular senile plaques, one of the major pathological hallmarks of Alzheimer's disease (AD). The classical compact plaque is surrounded by dystrophic neurites, activated microglia, and reactive astrocytes (Selkoe 1991). Microglia, the principal immune effector cells in the CNS, are considered to be beneficial and/or harmful in the progression of AD, depending on the heterogeneity of microglial activation and possibly, disease stage. Although activated microglia can induce neuronal damage by releasing pro-inflammatory and toxic agents, they can also exert a

Corresponding author and contacting information: Cynthia A. Lemere, Ph.D., NRB 636F, 77 Avenue Louis Pasteur, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115; clemere@rics.bwh.harvard.edu.

protective role, possibly by phagocytosing fAβ (Chung et al. 1999; Colton 2009; Combs 2009).

Microglia express many pattern recognition receptors and dynamically survey their microenvironment by detecting exogenous pathogen-associated molecular patterns (PAMPs) or endogenous danger-associated molecular patterns (DAMPs) (Lucin and Wyss-Coray 2009) via these receptors. Among them, a number of receptors have been reported to modulate microglia-mediated phagocytosis of fAβ, such as scavenger receptor A (El Khoury et al. 1996; Paresce et al. 1996), Toll-like receptors (Liu et al. 2005; Richard et al. 2008), the receptor for advanced glycation end products (Bombois et al. 2007) (Yan et al. 1996), and the receptor complex composed of B-class scavenger receptor CD36, an integrin-associated protein (CD47) and α6β1-integrin (Koenigsknecht and Landreth 2004).

The complement system, including C1q, is also involved in the phagocytosis of fAβ by microglia (Brazil et al. 2000; Webster et al. 2000). Complement component C3 is the central component of the complement system, which provides innate immune protection against pathogens via the classical, alternative and lectin pathways. These three distinct complement activation pathways converge on the formation of C3-cleaving enzymes (i.e. C3 convertases), which cleave C3 leading to the formation of C3a, C3b and iC3b fragments. Ultimately, activation of the complement system can lead to the formation of the membrane attack complex (MAC), resulting in lysis of targeted pathogens. In addition, C3b, iC3b and C4b fragments promote phagocytosis of pathogens by binding to complement receptors such as CR1 (CD35), CR3 (Mac-1, CD11b/CD18) and CR4 (CD11c/CD18) (Bonifati and Kishore 2007; Carroll 2004; van Beek et al. 2003). The production of complement component C3 and associated complement receptors is upregulated in the brains of AD patients (Akiyama and McGeer 1990; Eikelenboom and Stam 1982; Strohmeyer et al. 2002). Our previous work (Maier et al. 2008), as well as studies from other groups (Choucair-Jaafar et al. 2011; Rogers et al. 2006; Wyss-Coray et al. 2002), have indicated that complement component C3 and complement receptor CR3/Mac-1 might be involved in microglia-mediated uptake and clearance of fAβ.

In this study, we provide direct evidence that microglia can take up and clear fAβ both *in vitro* and *in vivo*, and that complement C3 and the complement receptor CR3/Mac-1 play significant roles in this process. In addition, we report that C3 and CR3/Mac-1 may have additive or parallel effects on fAβ uptake with the class A scavenger receptor in N9 microglia. These results support the development of therapies aimed at removing Aβ by selectively activating the beneficial functions of microglia.

MATERIALS AND METHODS

Materials

The primers for PCR, cell culture related reagents, CellTracker orange, Lysotracker red, To Pro-3, Alexa Fluor-labeled second antibodies, Lipofectamine 2000 reagent, and Alexa Fluor 488 dye were purchased from Invitrogen (Carlsbad, CA). Mouse macrophage colony-stimulating factor (M-CSF) was obtained from R&D Systems (Minneapolis, MN). Synthetic human $A\beta_{1–42}$ peptide was synthesized in the UCLA Biopolymer Lab (Dr. David Teplow, Dept. of Neurology). Control siRNA and siRNA against C3 or Mac-1 were synthesized by Santa Cruz Biotechnology (Santa Cruz, CA). Fucoidan, polyinosinic acid (Poly I), low-density lipoprotein (LDL), long chain fatty acid (LCFA), and anti-β-actin antibody were purchased from Sigma (St. Louis, MO). Mouse Seroblock FcR, anti-CD68, anti-CD45 and anti-F4/80 antibodies were bought from AbD Serotec (Oxford, UK). The antibodies of PE labeled anti-LAMP-1, anti-Aβ (6E10), anti-Iba-1 and anti-LAMP-1 (1D4B) were purchased from BD Biosciences (San Jose, CA), Covance (Emeryville, CA), Wako (Richmond, VA),

and Developmental Studies Hybridoma Bank (Iowa City, IA), respectively. R1282 is a rabbit polyclonal anti-Aβ antibody that recognizes multiple forms of Aβ (gift from Dr. Dennis J. Selkoe, CND, Boston, MA). The protease inhibitor cocktail and 1x RIPA buffer were bought from Roche Applied Sciences (Indianapolis, IN) and Thermo Scientific (Rockford, IL), respectively. The mouse TH1/TH2 9-plex assay ultra-sensitive kit was purchased from Meso Scale Discovery (Gaithersburg, MD). The 5-μl Hamilton syringes and centrifugal filter devices were purchased from Hamilton Company (Reno, NV) and Millipore (Billerica, MA), respectively.

Animals

C57BL/6 mice and homozygous C3-deficient mice ($C3^{-/-}$; line B6.129S4-*C3*^{tm1Crr}/J) (Wessels et al. 1995) were obtained from The Jackson Laboratory. Mac-1-deficient mice (Mac-$1^{-/-}$; line B6. 129S4-*Itgam*^{tm1Myd}/J) were generated as described (Coxon et al. 1996). $C3^{-/-}$ and Mac-$1^{-/-}$ mice were backcrossed onto the C57BL/6 background for more than 10 generations. Mice were bred and maintained in a virus antibody-free animal facility at the Harvard Medical School. Mice were genotyped by PCR (Fig. S1) using the following primers: 5'-ATCTTGAGTGCACCAAGCC-3' and 5'-GGTTGCAGCAGTCTATGAAGG-3' (C3 wild-type); 5'-CTTGGGTGGAGAGGCTATTC-3' and 5'-AGGTGAGATGACAGGAGATC-3' (C3 mutant); 5'-TAGGCTATCCAGAGGTAGAC-3' and 5'-CATACCTGTGACCAGAAGAGC-3' (Mac-1 wild-type); 5'-TAGGCTATCCAGAGGTAGAC-3' and 5'-ATCGCCTTCTTGACGAGTTC-3' (Mac-1 mutant); 5'-CCTCTTTGTGACTATGTGGACTGATGTCGG-3' and 5'-GTGGATAACCCCTCCCCCAGCCTAGACC-3' (GFAP, an internal DNA control). All animal protocols were approved by the Harvard Medical Area Standing Committee on Animals and were in accordance with all state and federal regulations.

Tissue Culture

N9 microglial cells are immortalized murine cells that were generated by Dr. Paola Ricciardi-Castagnoli (Singapore) and kindly provided to us by Dr. Joseph El Khoury (Massachusetts General Hospital). The cells were cultured as described and have been shown previously to take up Aβ (Hickman et al. 2008). Briefly, N9 microglial cells were grown in RMPI 1640 medium containing 10% FBS. Primary microglia were prepared from the cortices of mouse pups at postnatal days 1–2 and cultured as described (Giulian and Baker 1986). In brief, cortices were trypsinized after removing the meninges and blood vessels. Cells were seeded in Poly-L-Ornithine-coated culture flask and cultured in DMEM containing 10% FBS. After a confluent monolayer of glial cells was obtained, microglia were shaken off, re-plated on culture dishes with or without the glass bottom, and cultured in growth medium with 10 ng/ml M-CSF, which did not affect the uptake of $FLfA\beta_{42}$ by microglia (Fig. S2). The culture medium was changed to serum-free medium the day before experimental manipulation of the cells.

Aβ Preparation

The preparation of Aβ fibrils was performed as described previously (Fu et al. 2006). Briefly, synthetic human $A\beta_{1–42}$ peptide was aged at 37° C for 1 week and labeled with Alexa Fluor 488 dye at room temperature for 1 h. The labeled mixture was passed through 3 k centrifugal filters twice and 100 k centrifugal filter once at 3200 g for 30 min. The final fluorescently labeled $A\beta_{1–42}$ fibrils are referred to as "$FLfA\beta_{42}$". Amyloid cores extracted from the brains of human AD patients were prepared as described previously (Shankar et al. 2008). We collected brain specimens from deceased human subjects at autopsy after obtaining informed consent from the next of kin under protocols approved by the Partners Human Research Committee at Brigham and Women's Hospital.

Congo Red Staining

The preparation of FLfAβ_{42} and the amyloid cores extracted from the brains of human AD patients were incubated with 0.2% Congo red for 20 min at room temperature and observed under an Olympus BX50 microscope using a polarizing filter.

Live Cell Imaging

N9 microglial cells or primary microglia plated on the glass-bottom of 35-mm culture dish were changed to serum-free medium the day before experiments and incubated with 0.5 μM FLfAβ_{42} in the presence of 2 μM CellTracker orange or 100 nM Lysotracker red. Following incubation, the cells were washed three times with cold 1x PBS which was then replaced with fresh serum-free medium, and observed under a Zeiss LSM 510 confocal microscope.

Flow Cytometry

Microglial cell conditioned medium was replaced with serum-free medium the day before the experiment and incubated with 0.5 μM FLfAβ_{42} for 1 h in the presence or absence of different pretreatments. Cells were washed three times with cold 1x PBS, trypsinized, and subjected to a FACS Calibur flow cytometer (BD Biosciences, CA) as described (Webster et al. 2001).

Western Blot Assay

Western blot was performed as reported previously (Maier et al. 2008). Briefly, media and cell lysates from N9 microglial cells exposed to FLfAβ_{42} or human amyloid cores were centrifuged at 15,000 g for 5 min. The pellet containing fAβ species was resuspended in 1x loading buffer and separated on 12% Bis-Tris gels. Proteins on the gels were transferred to membranes and detected with anti-Aβ (6E10, 1:1000) monoclonal antibody. β-actin was used as a loading control. As a control to exclude the possibility of fAβ sticking to the plate, FLfAβ_{42} was added to blank culture media in the absence of N9 micoglial cells for 4 h. The culture media were collected and the bottom of the culture dishes was lysed with 1x RIPA buffer. The collected blank media and lysates were subjected to the aforementioned procedures. Image J software was used to obtain the integrated intensity of each lane above 188 kDa on the blot. The selection area covered the signal and the same size was maintained for each band. The ratio of 6E10/actin in the control sample was set as 100% and the ratio of 6E10/actin integrated intensity in other groups was calculated as the percentage of that of control.

Transient Transfection

Primary microglia or N9 microglial cells were transfected with control siRNA or siRNA against C3 or Mac-1 using the Lipofectamine 2000 reagent. Cultured media were replaced with fresh medium 4 h post-transfection and cells were cultured for 24 h before experiments. Protein levels of C3 and Mac-1 were significantly reduced 24 h after transfection of C3 or Mac-1 siRNA, respectively (Fig. S3).

Immunocytochemistry

Microglial culture medium was replaced with serum-free medium. Cells were incubated with 0.5 μM FLfAβ_{42} for 1 h or 20 ng of the amyloid cores extracted from the brains of human AD patients for 4 h. Cells were washed three times with cold 1x PBS and fixed in cold methanol for 10 min. After being blocked with 3% BSA in TBS-containing 10 μg/ml Fc block, the cells were incubated with PE labeled primary anti-LAMP-1 (1:150), anti-LAMP-1 (1D4B, 1:100) or anti-Aβ (R1282, 1:1000) antibodies at room temperature for 1 h or overnight at 4° C. Next, the cells were incubated with the appropriate Alexa Fluor

secondary antibodies (1:250) and nuclei were visualized by TO Pro-3 staining. Images of the stained cells were captured using a Zeiss LSM 510 confocal microscope.

Stereotaxic Brain Microinjection

Two microliters FLfAβ_{42} at 0.3 mg/ml (0.6 μg) and 2 μl of the vehicle control were microinjected into the frontal cortex on opposites sides of the brain of 8–9-month-old adult female C57BL/6, C3$^{-/-}$ or Mac-1$^{-/-}$ mice in a stereotaxic apparatus (Stoelting, IL) via a 5-μl Hamilton syringe. The injection coordinates, with respect to Bregma, were +2.8 mm anterior, 1.2 mm lateral, and −1.3 mm ventral to the skull. The rate of injection was 0.5 μl/min, after which the cannula was left in place for an additional 5 min to allow for diffusion. Animals were kept on a warming pad until they had fully recovered from anesthesia and were housed individually to prevent damage to the scalp sutures until they were sacrificed for tissue processing. All appropriate measures were taken to minimize pain and discomfort in experimental animals according to the animal surgery procedures approved by the Harvard Medical Area Standing Committee on Animals. Two hours or five days after surgery, the mice were cardio-perfused with PBS and the brains were removed and fixed in 4% PFA for 24 h. Brains were sectioned coronally at 20 μm on a Leica CM1850 cryostat.

Immunohistochemistry

Frozen mouse sections were incubated with anti-Iba-1 (1:200), anti-CD68 (1:200), anti-CD45, anti-F4/80, and anti-LAMP-1 (1D4B, 1:100) antibodies overnight at 4° C. After washing with PBS, sections were incubated with appropriate Alexa Fluor second antibodies and nuclei were visualized by TO Pro-3 staining. The injected FLfAβ was detected with the excitation wavelength at 488 nm. Images were obtained as a z-series stack using a Zeiss LSM 510 confocal microscope.

Cytokine Multiplex ELISA

Five days after the microinjection of 0.6 μg FLfAβ_{42} into the frontal cortex of mouse brains, the mice were cardio-perfused with PBS and the brains were removed and homogenized in 1x RIPA buffer containing a protease inhibitor cocktail. The cytokine profile in the brain homogenates was measured using the MSD mouse TH1/TH2 9-plex assay ultra-sensitive kit according to the manufacturer's instruction. IFN-γ, IL-1β, IL-2, KC/GRO, IL-12p70 and TNF-α are M1 microglial activation markers; and IL-4, IL-5 and IL-10 are M2 microglial activation markers.

Statistical Analysis

Data were expressed as mean ± SEM. Significance was assessed with Student's *t*-test or one-way ANOVA followed by Dunnett post-hoc test using Prism 4.0 GraphPad Software (San Diego, CA). A value of $p < 0.05$ was considered significant.

RESULTS

Microglia Take up fAβ and Transport It to Lysosomes Both *In Vitro* and *In Vivo*

Fluorescently labeled fibrillar Aβ_{42} (FLfAβ_{42}) was verified by Congo red staining, which revealed classic yellow-green birefringence when observed under polarized light (Fig. S4). N9 immortalized murine microglial cells or primary microglia from C57BL/6 mouse pups were incubated with FLfAβ_{42} for different periods of time. By confocal microscopy, most of the FLfAβ_{42} colocalized with the cytosolic indicator (CellTracker Orange) (Fig. 1A), suggesting that both N9 microligal cells and primary microglia took up synthetic fAβ. Furthermore, flow cytometry demonstrated that microglial cells took up FLfAβ_{42} in a concentration- (Fig. 1B, C) and time-dependent manner (Fig. 1D, E, F). Primary microglia

from wild-type mice took up more FLfAβ_{42} than N9 microglial cells (Fig. 1F), possible due to differences in sensitivity and/or expression of cell surface receptors for FLfAβ_{42}. Notably, there was no significant difference in FLfAβ_{42} uptake before and after quenching of cell surface binding with 0.25% trypsin-EDTA or 1 mg/ml trypan blue (data not shown), indicating that most of the detected FLfA$\beta_{42}$was intracellular and not cell-surface associated. In addition, 88.8% of the FLfAβ_{42}added to the N9 microglial cells was detected in the cell lysate by Western blot (Fig. 2A). We only calculated fAβ above 188 kDa to reflect high MW, fibrillar Aβ. The uptake of fAβ was confirmed by a control study in which very little FLfAβ_{42} was found to stick to the culture dish after 4 h incubation with blank culture medium in the absence of N9 microglial cells. Most of the FLfAβ_{42} was still present in the blank culture medium (Fig. S5), confirming that the fAβ_{42}did not stick to the plate at the concentration used in our study.

N9 microglial cells were also treated with amyloid cores extracted from human AD brain, which contains abundant pathological fAβ (Fig. S4). Twenty-four hours after treatment, a high molecular weight band, corresponding to 90.3% of the original amount of fAβ in the cores added to the culture dish, was detected in the cell lysate (Fig. 2C), confirming that microglia take up pathological amyloid cores *in vitro*.

Next, we asked whether microglia take up fAβ *in vivo*. Five days after surgery, we found abundant Iba-1 (a resident microglia marker) and CD68 (an activated microglia/macrophage marker) positive staining within and/or closely surrounding the microinjected FLfAβ_{42}. Most of the Iba-1- and CD68-positive cells, in the injection site contained FLfAβ (Fig. 3A, B), suggesting that these microglia were activated and induced to take up fAβ *in vivo* within 5 d after surgery. Notably, there were far fewer Iba-1- and CD68-positive microglial cells in the vehicle-injected contralateral hemisphere (Fig. S6), indicating that the microinjected FLfAβ_{42}, instead of the injection itself, induced the microglial activation. There was no positive staining for CD45 or F4/80 5 d after surgery although the positive controls worked for both of CD45 and F4/80 (data not shown), suggesting that only those microglia of a specific activation state took up fAβ.

Using live cell imaging and immunocytochemistry, we found that most of the FLfAβ_{42} colocalized with the staining of lysosomal markers in live (Fig. S7A, B) and fixed microglia (Fig. S7C, D), respectively, *in vitro*. Amyloid cores taken up by N9 microglia colocalized with LAMP-1 (Fig. S8A, B). Furthermore, the microinjected FLfAβ_{42} partially colocalized with LAMP-1 staining *in vivo* (Fig. 3C). Thus, both *in vitro* and *in vivo* data indicate that microglia take up fAβ and transport it to lysosomes, possibly for degradation. The degradation of FLfAβ_{42} or amyloid cores was directly assessed by Western blot. Six hours after washout, the amount of FLfAβ_{42} or amyloid cores in the cell lysate was dramatically reduced by 53.48% and 33.36% respectively ($p < 0.01$), and neither was detected in the washout media (Fig. 2A–D), suggesting that microglia can degrade the internalized fAβ, without re-secreting it into the culture media.

Phagocytosis of fAβ by Primary Microglia Is Attenuated by the Deficiency or Knockdown of C3 or Mac-1

To investigate the mechanism of fAβ uptake by microglia, we focused on complement component C3 and the complement receptor type 3, Mac-1. Primary microglia from wildtype C57BL/6, C3−/− and Mac-1−/− mice were exposed to FLfAβ_{42} for 1 h. The mean fluorescent intensity of the FLfAβ_{42} signal (Fig. 4A, B) and the percentage of microglia taking up FLfAβ_{42} (Fig. 4C) by $C3^{-/-}$ or Mac-$1^{-/-}$ microglia were significantly reduced by 38.6% and 27.9% ($p < 0.01$), respectively, compared to wild-type microglia. Next, we transfected primary microglia with siRNA against C3 or Mac-1, which significantly reduced FLfAβ_{42} uptake by 71.0% and 48.7% ($p < 0.01$), respectively, compared to fAβ uptake by

microglia transfected with control siRNA (Fig. 4D). Thus, both the deficiency and the knockdown of C3 or Mac-1 attenuated microglia-mediated phagocytosis of fAβ. The addition of recombinant complement C3a dramatically increased the uptake of FLfAβ_{42} by $C3^{-/-}$ microglia (Fig. S9), further confirming the role of C3 in the uptake of fAβ by microglia.

C3 and Mac-1 May Act in Parallel with the Class A Scavenger Receptor in Modulating Phagocytosis of fAβ by N9 Microglia

Scavenger receptors have been reported previously to mediate phagocytosis of fAβ by microglia (El Khoury et al. 1996; Paresce et al. 1996). We, too, found that a 1 h pretreatment with fucoidan, a ligand of class A and B scavenger receptors, significantly inhibited phagocytosis of FLfAβ_{42} by N9 microglial cells. Using specific ligands for different classes of scavenger receptors, we found that FLfAβ_{42} uptake was reduced by 93.1% ($p < 0.01$) in N9 microglial cells following pretreatment with polyinosinic acid (a specific ligand for the class A scavenger receptor), but not by pretreatment with low-density lipoprotein (LDL, a specific ligand for the class B-I scavenger receptor) or long-chain fatty acid (LCFA, a specific ligand for the class B-II scavenger receptor) (Fig. 5A). Thus, in our study, microglia-mediated phagocytosis of fAβ was only affected by inhibiting class A but, not class B scavenger receptors.

Interestingly, combining C3 or Mac-1 siRNA transfection of N9 cells with fucoidan pretreatment significantly reduced the uptake of FLfAβ_{42} by 47.1% and 73.9% ($p < 0.01$), respectively, when compared to N9 microglia transfected with control siRNA and pretreated with fucoidan (Fig. 5B). Unlike transfected primary microglia, N9 cells transfected with siRNA against C3 or Mac-1 had less than a 10% reduction in the uptake of FLfAβ_{42} compared to N9 cells transfected with control siRNA in the absence of fucoidan ($p > 0.05$, Fig. 5B). These results suggest that complement C3 and/or the receptor Mac-1 may act in parallel with the class A scavenger receptor in N9 microglia-mediated phagocytosis of fAβ.

C3- or Mac-1-Deficiency Reduces the Clearance of Microinjected fAβ *In Vivo*

To verify the roles of C3 or Mac-1 in the clearance of fAβ *in vivo*, we compared the amount of cortically microinjected FLfAβ_{42} remaining in the brains of C57BL/6, $C3^{-/-}$ or Mac-$1^{-/-}$ mice 5 d after surgery. We found that the fAβ fluorescent signal remaining in the brains of $C3^{-/-}$ or Mac-$1^{-/-}$ mice was significantly higher by 76.5% ($p < 0.05$) and 113.3% ($p < 0.01$), respectively, compared to wild-type mice (Fig. 6A, B), demonstrating that C3- or Mac-1-deficiency significantly reduced the clearance of microinjected fAβ *in vivo*.

C3- or Mac-1-Deficiency Modestly Alters the Cytokine Profile *In Vivo*

To explore whether C3- or Mac-1-deficiency affects the microglial activation state induced by fAβ *in vivo*, we measured both M1 and M2 microglial activation markers in brain homogenates 5 d after microinjecting FLfAβ_{42} into the frontal cortex of C57BL/6, $C3^{-/-}$ or Mac-$1^{-/-}$ mice. While most of the M1 (IFN-γ, IL-1β, IL-2, KC/GRO, IL-12p70 and TNF-α) and M2 (IL-4, IL-5 and IL-10) microglial activation markers were mildy elevated in both $C3^{-/-}$ and Mac-$1^{-/-}$ mice compared to wild-type C57BL/6 mice (Supplemental Table 1), only the increase in IL-10 in $C3^{-/-}$ mice compared to wildtype mice reached significance ($p = 0.0164$).

DISCUSSION

Microglia are competent phagocytes and are thought to be capable of binding and phagocytosing fAβ *in vitro* (Ard et al. 1996; Bard et al. 2000; Paresce et al. 1996). However, *in vivo* data on microglia-mediated phagocytosis of fAβ remain controversial (Bolmont et al.

2008; Grathwohl et al. 2009; Stalder et al. 2001), probably due to the use of different methods to detect fAβ uptake. In this study, we used both fluorescently labeled synthetic fAβ and isolated amyloid cores from human AD patients to investigate microglial uptake of fAβ. As shown by confocal microscopy and Western blot, microglia took up both synthetic fAβ (*in vitro* and *in vivo*) and human AD amyloid cores (*in vitro*). In addition, internalized fAβ colocalized with microglial markers (Iba-1 and CD68) and lysosomal markers (lysotracker red and LAMP-1) *in vivo*, suggesting that microglia may take up fAβ and transport it to lysosomes, possibly for degradation. Indeed, the degradation of fAβ by microglia was confirmed by Western blot analysis. These results are consistent with previous reports that late endosomes/lysosomes may play a role in the degradation of intracellular fAβ (Chung et al. 1999; Majumdar et al. 2007).

Investigating how fAβ gets into microglial cells may help identify putative targets that could potentially be modulated to promote phagocytosis and clearance of fAβ by microglia. Our previous study (Maier et al. 2008) and studies from other groups (Choucair-Jaafar et al. 2011; Rogers et al. 2006; Webster et al. 2000; Wyss-Coray et al. 2002) suggest that complement component C1q, C3 and Mac-1 may be involved in the clearance of fAβ via phagocytosis. Here, we further examined the roles of complement C3 and complement receptor Mac-1 in modulating phagocytosis of fAβ. We found that both the intracellular fluorescent fAβ signal and the percentage of microglia taking up fAβ were significantly reduced when C3 or Mac-1 were absent or knocked down. Further, the addition of the recombinant human complement component C3a significantly and concentration-dependently enhanced the uptake of fAβ by $C3^{-/-}$ microglia (Fig. S9). These results provide direct evidence that C3 and Mac-1 are involved in the phagocytosis of fAβ by microglia. The siRNA-mediated knockdown of C3 or Mac-1 in primary microglia resulted in a much larger reduction of fAβ uptake than complete knockout of either protein. This may due to the compensatory increases in other complement components or fAβ-binding receptors in those complete knockout animals. Alternatively, it may also due to the inter-assay variation. Targets coated with complement C3 activation product iC3b may be specifically recognized and phagocytosed by Mac-1 on macrophage or microglia (van Beek et al. 2003). *In vivo*, C3 deficiency likely leads to an absence of complement opsonization of fAβ and thus a reduction in fAβ uptake by Mac-1-bearing microglia. Since it is unlikely that microglia obtained from $C3^{-/-}$ mice can synthesize C3, the underlying mechanisms by which C3 and Mac-1 promote fAβ uptake *in vitro* in the absence of serum are less clear. We speculate that C3 expressed by microglia might be activated by fAβ directly or indirectly to generate C3b or iC3b, and thus facilitate fAβ binding to, and internalization by Mac-1. Alternatively, Mac-1 may recognize fAβ directly and promote the phagocytosis of fAβ. Mac-1 was found to colocalize with Aβ plaques in the brain, suggesting that Aβ may activate microglia partly through binding to Mac-1 itself (Strohmeyer et al. 2002). Furthermore, preliminary data from binding and immunoprecipitation assays both favor the hypothesis that Aβ binds to Mac-1 receptors (Zhang et al. 2011).

A number of other receptors, as described in the Introduction, have been reported to modulate microglia-mediated phagocytosis of fAβ. We confirmed that ligands for the class A scavenger receptor, such as fucoidan and poly I, significantly attenuated fAβ uptake, however, ligands for class B-I (LDL) or B-II (LCFA) did not have this inhibitory effect. Our data indicate that the class A but not the class B-I or B-II scavenger receptor on the surface of microglia is responsible for the binding and uptake of fAβ. Although the class B-II scavenger receptor CD36 was shown previously to be a candidate receptor for fAβ (Bamberger et al. 2003; Coraci et al. 2002), we were unable to confirm this result in our cell culture system. Differences in experimental conditions used by us and other groups, including detection methods, inhibiting ligands or antibodies, etc. may explain this inconsistency. Because complement C3, Mac-1 and scavenger receptor A may all be

involved in phagocytosis of fAβ, we asked whether they act in parallel to facilitate microglial uptake of fAβ. We found that knocking down C3 or Mac-1 via transfection of siRNA against C3 or Mac-1 significantly enhanced the ability of fucoidan to inhibit phagocytosis of fAβ by N9 microglia, suggesting that different Aβ binding receptors may work in parallel to induce microglia-mediated phagocytosis of fAβ.

Are C3 and/or Mac-1 involved in the clearance of fAβ by microglia *in vivo*? To answer this question, we microinjected fluorescently labeled fAβ into the frontal cortex of 8–9 month-old C57BL/6, $C3^{-/-}$ and Mac-$1^{-/-}$ mouse brains and compared the amount of FLfAβ remaining 5 d after the surgery. The fluorescent intensities of FLfAβ were significantly higher in $C3^{-/-}$ and Mac-$1^{-/-}$ mouse brain sections than wild-type mouse brain sections, demonstrating that C3- or Mac-1-deficiency reduced the clearance of fAβ *in vivo*. In contrast to our results, there are reports that microglia are inefficient or unable to clear fAβ deposits *in vivo* (Grathwohl et al. 2009; Stalder et al. 2001). Using serial electron microscopy and 3-D analyses, Stalder and colleagues found that fAβ did not get into the cytoplasm of microglia in 15–20 mo-old aged APP transgenic (APP23) mice. Grathwohl et al. crossed APP/PS-1 or APP23 mice with CD11b-HSVTK mice, in which nearly complete ablation of microglia was achieved for up to 4 weeks after ganciclovir application in 3-mo old APP/PS-1 and TK mice or in 17-mo old APP23 and TK mice. There was no significant effect of the absence of microglia on formation and maintenance of amyloid plaques. It should be noted that APP/PS-1 and APP23 transgenic mice develop robust amyloid pathology and abundant microglial activation as early as 2 mo and 6–8 mo of age, respectively. We speculate that at this later time point, it might be too late to observe the phagocytosis of fAβ by microglia possibly because the over-activation of microglia could have changed their anti-inflammatory and phagocytic functions to pro-inflammatory state with disease progression. The expression of Aβ-binding receptors and Aβ-degrading enzymes are significantly decreased in aging APP transgenic mice, whereas those of pro-inflammatory cytokines are increased (Hickman et al. 2008). Unlike the aforementioned previous studies in APP transgenic mice, our current study used 8–9 month-old wild-type C57BL/6 and complement-deficient mice that do not over-express human APP nor generate fAβ and sAβ. The injection of fAβ into murine cortex can only determine the response of naïve microglia to an acute stimulus and might not completely model the chronic nature of AD. The lack of steady accumulation of fAβ with aging may account, in part, for the differences in our results. Also, it is possible that aging may play a role on the uptake and degradation of fAβ. Further studies are underway.

It should be noted that in our previous study, we found no significant changes in plaque deposition or gliosis in APP/$C3^{-/-}$ mice until 17 months of age (Maier et al. 2008), which seems to be inconsistent with our current finding that $C3^{-/-}$ microglia cannot respond to an acute fAβ stimulus *in vivo*. Our hypothesis is that there may be some compensatory mechanisms existing in the APP/C3-deficient mice, including compensatory up-regulation of other complement component (e.g. C1q) and/or complement receptor (e.g. CR3) which have been reported be involved in the phagocytosis of fAβ (Brazil et al. 2000; Webster et al. 2000; Zhang et al. 2011). Also, the slow, gradual accumulation of cerebral Aβ in APP/$C3^{-/-}$ mice may reach a critical threshold due to aging and/or total Aβ deposition after which the microglia are no longer able to phagocytose Aβ. Lastly, the acute microinjection studies in the current study may be influenced to some degree by the response to the injection itself, even though only low-level glial activation was observed in the vehicle-injected contralateral cortex.

Due to the heterogeneity of microglial activation and differential effects of different cytokines on microglial uptake of fAβ (Colton 2009; Koenigsknecht-Talboo and Landreth 2005; Morgan et al. 2005), we hypothesized that the clearance of amyloid plaques might

also be dependent on the activation state of microglia. There are at least two main types of microglial activation states (i.e. M1 and M2). The M1 state is associated with a proinflammatory state involving release of Th1 cytokine profile such as IFN-γ, IL-1, IL-2, IL-6, IL-12, and TNF-α; while the M2 state is associated with an anti-inflammatory and more phagocytic state with a Th2 cytokine profile such as IL-4, IL-5, IL-10, and TGF-β1 (Morgan et al. 2005). In this study, we found that most of the M1 and M2 microglial activation markers were modestly elevated in the $C3^{-/-}$ and Mac-$1^{-/-}$ mice injected with fAβ, although only the increase in IL-10 in the $C3^{-/-}$ mice was significant compared to wildtype mice (Supplemental Table 1). The ratio of M1/M2 was not significantly different in the complement-deficient mice compared to wildtype mice. This insignificance might be due to the small numbers of animals and high variability in our study. Whereas an acute stimulus (fAβ) was provided to adult mice in this study, future studies using chronic stimuli in aged mice may better define how C3- or Mac-1-deficiency affects the microglial activation state induced by fAβ *in vivo*, which could result in the alteration of fAβ clearance by microglia.

In summary, we demonstrate that complement component C3 and the complement receptor type 3, Mac-1, are involved in the phagocytosis and clearance of fAβ by microglia *in vitro* and *in vivo*. They may act in parallel with the class A scavenger receptor in microglial uptake of fAβ. Our study provides insights into the molecular mechanisms involved in fAβ phagocytosis by microglia that may be exploited to develop new AD therapeutics aimed at increasing Aβ clearance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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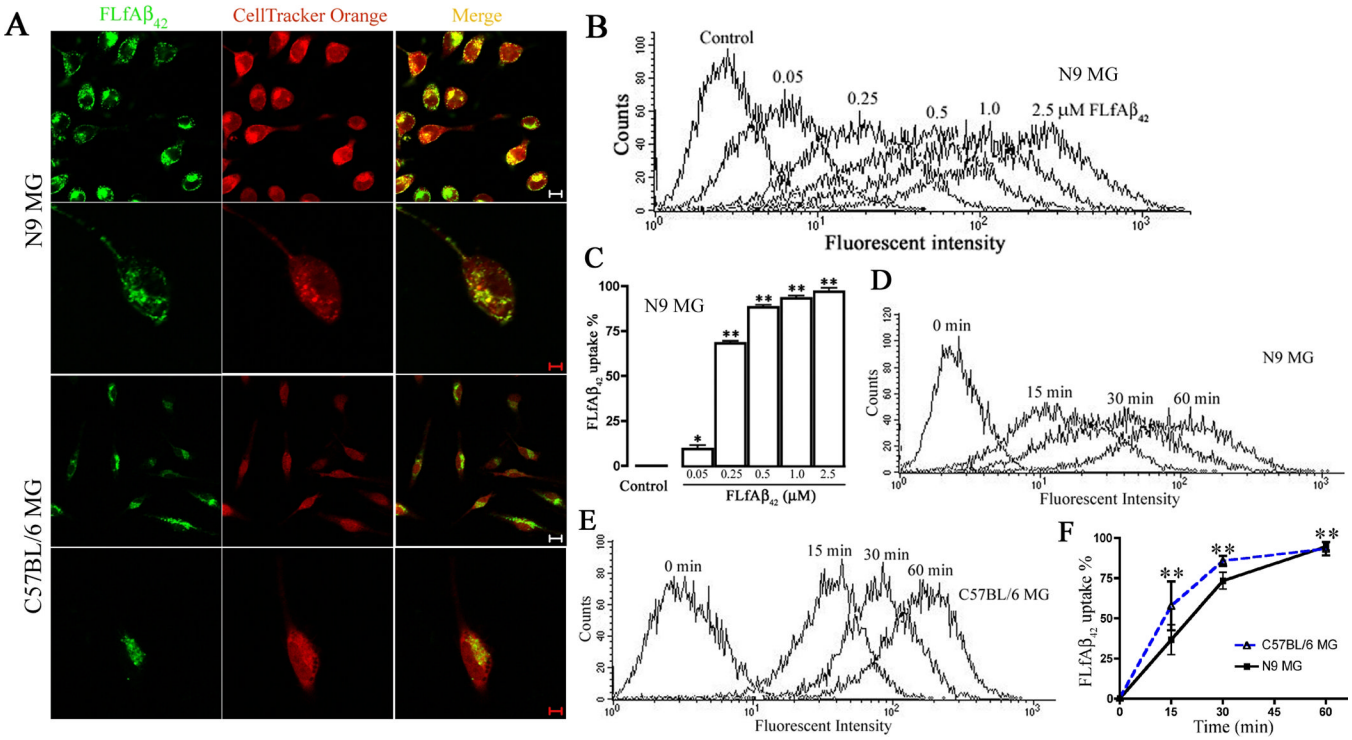

Fig. 1.

Microglia take up FLfAβ$_{42}$ in a concentration- and time-dependent manner. **A**: N9 microglial cells (N9 MG) and primary microglia (C57BL/6 MG) from C57BL/6 mouse pups were incubated with 0.5 μM FLfAβ$_{42}$ and 2 μM CellTracker Orange for 1 h and observed using a confocal microscope. **B, C**: N9 MG were incubated with different concentrations of FLfAβ$_{42}$ for 1 h and the percentage of cells taking up FLfAβ$_{42}$ was measured using a FACS Calibur flow cytometer. **D–F**: N9 MG and C57BL/6 MG were incubated with 0.5 μM FLfAβ$_{42}$ for 0–60 min and the percentage of cells taking up FLfAβ$_{42}$ was measured using the flow cytometer. The data (C, F), expressed as a percentage of the microglia taking up FLfAβ$_{42}$ in total measured microglia, represent the means ± SEM of three separate experiments from each (B, D, E); * $p < 0.05$, ** $p < 0.01$ *versus* control (one-way ANOVA followed by Dunnett post-hoc test). White scale bar = 10 μm; red scale bar = 5 μm.

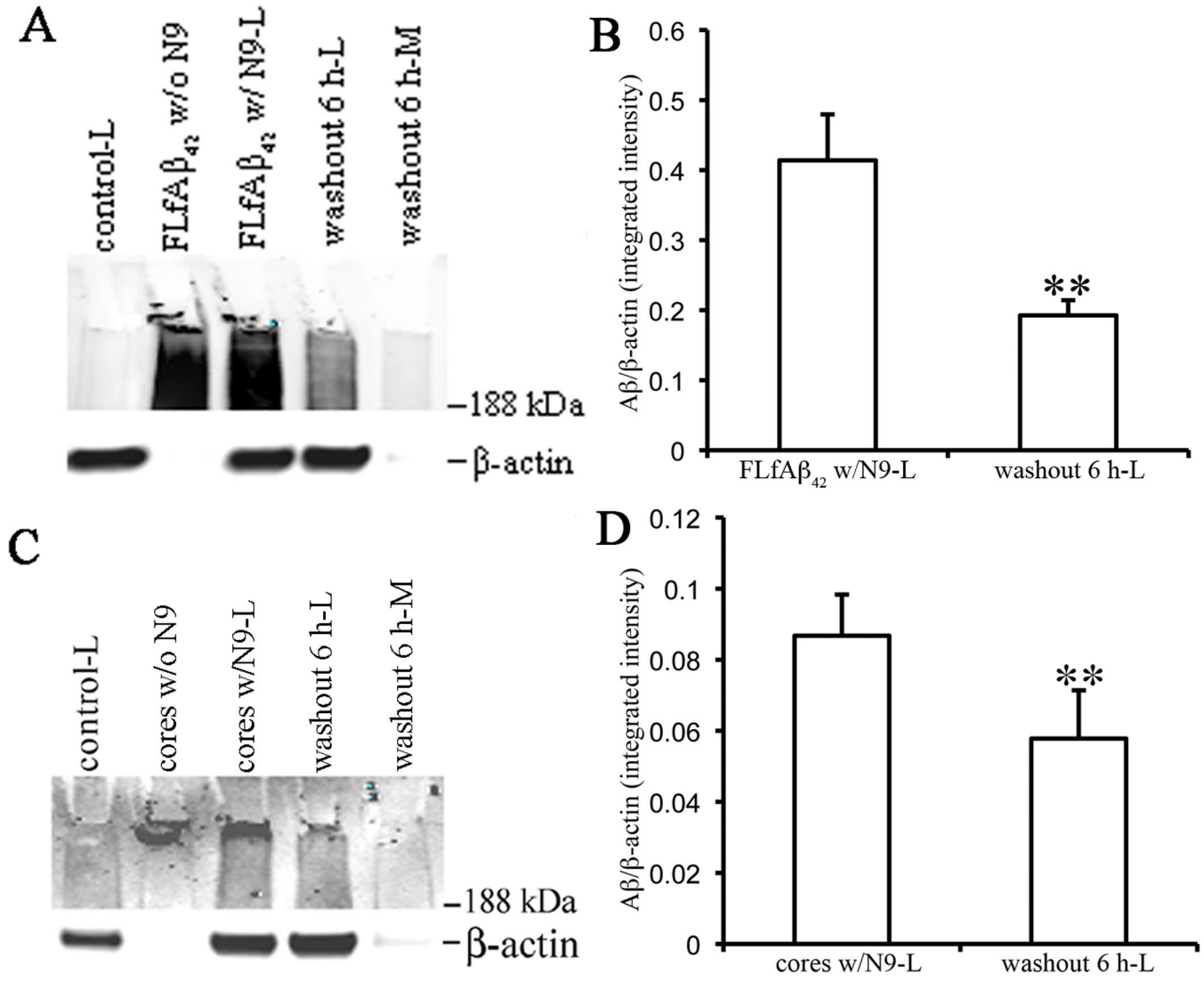

Fig. 2.

Microglia take up and degrade FLfAβ_{42} and human amyloid cores. **A, B**: N9 microglial cells (N9) were incubated with vehicle (control) or 0.5 μM FLfAβ_{42} for 4 h. **C, D**: N9 microglial cells were incubated with vehicle (control) or 20 ng human amyloid cores for 24 h. The cells were lysed immediately or washed with cold PBS and lysed 6 h later. The media were collected before being lysed. The FLfAβ_{42} and human amyloid cores in the cell lysates (-L) and washout 6 h media (-M) were detected by Western blot using anti-Aβ antibody, 6E10. "FLfAβ_{42} w/o N9" and "cores w/o N9" mean that FLfAβ_{42} or human amyloid cores were added to a culture plate containing blank culture medium without N9 microglial cells, indicating the starting amount of FLfAβ_{42} or human amyloid cores. Aβ in the blank culture medium and at the bottom of culture plates was also collected after 4 h for detection by Western blot. The data, expressed as the ratio of Aβ/β-actin in terms of integrated intensity, represent the means ± SEM of three separate experiments; ** $p < 0.01$ *versus* FLfAβ_{42} w/ N9-L in (B) or cores w/N9-L in (D) (Student's *t*-test).

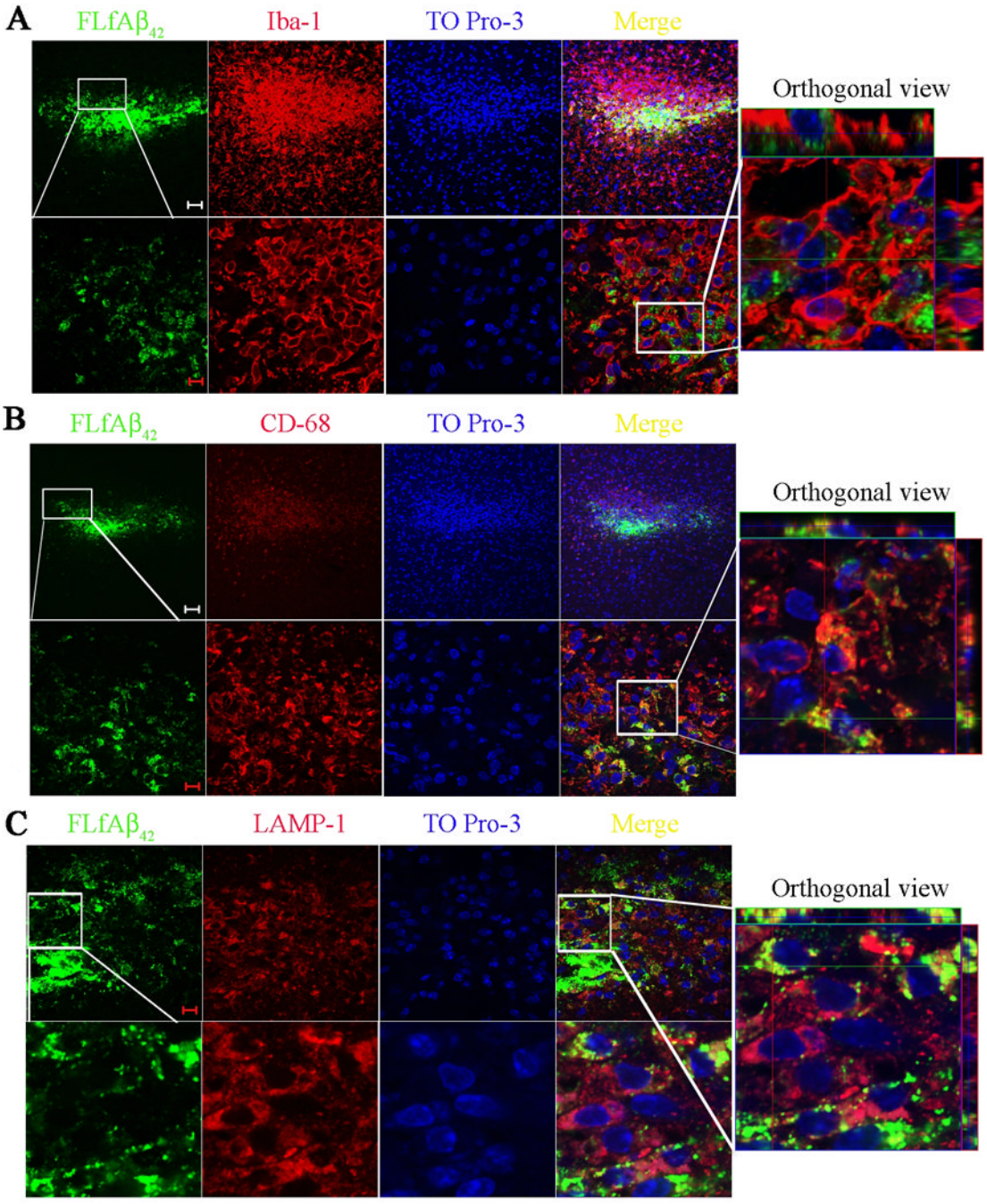

Fig. 3.

Primary wildtype microglia take up microinjected FLfAβ_{42} and transport it to lysosomes *in vivo*. **A–C**: Five days after the stereotaxic injection of 0.6 μg FLfAβ_{42} into the frontal cortex, wildtype C57BL/6 mouse brains were sectioned and incubated with anti-Iba-1 (A), anti-CD68 (B) or anti-LAMP-1 (C) antibodies. After washing with PBS, sections were incubated with appropriate Alexa Fluor second antibodies and nuclei were visualized by TO Pro-3 staining. Images were obtained as a z-series stack using a Zeiss LSM 510 confocal microscope. White scale bar = 50 μm; red scale bar = 10 μm.

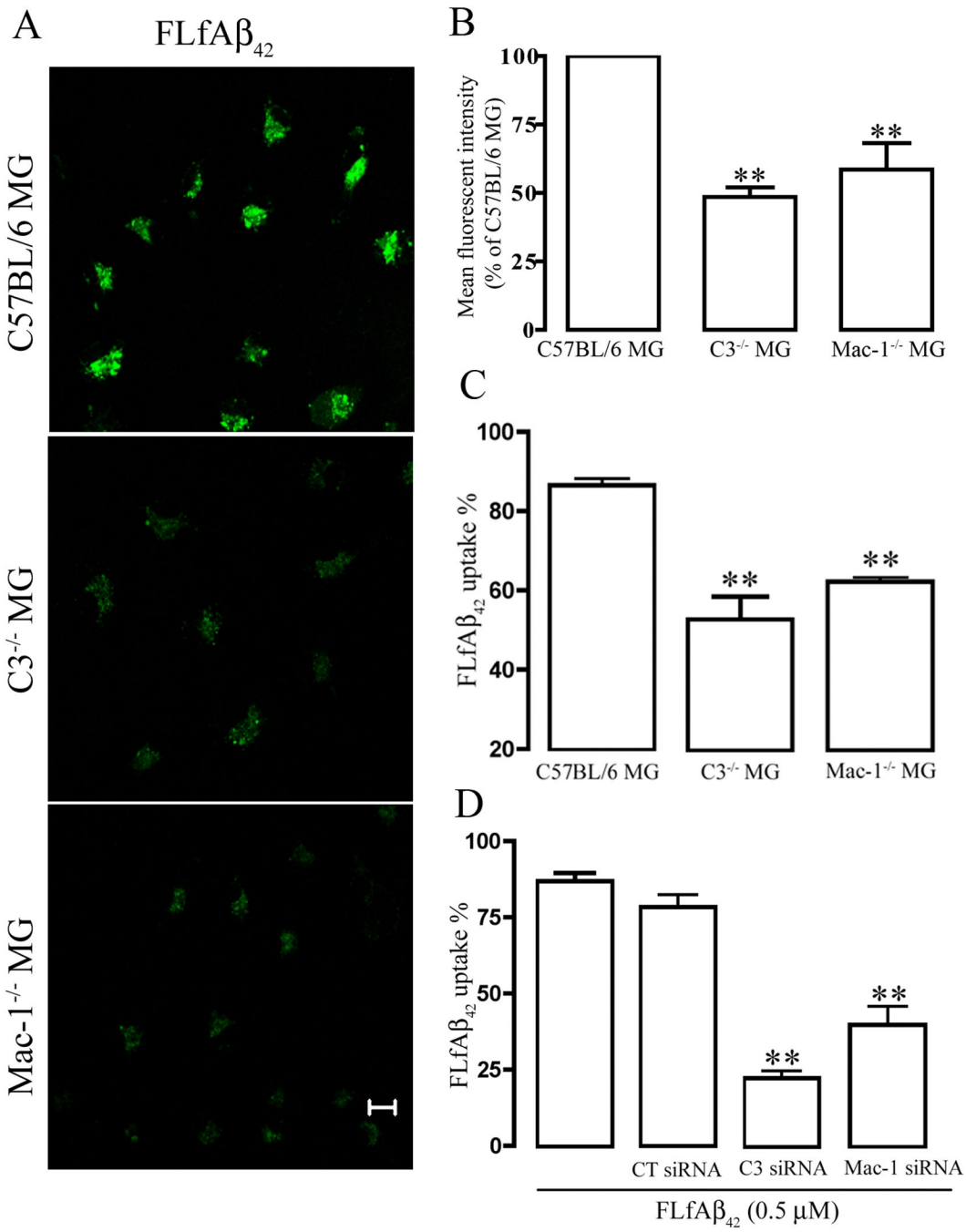

Fig. 4.

The deficiency or knockdown of C3 or Mac-1 attenuates the uptake of FLfAβ_{42} by primary microglia. **A–C**: Primary microglia from C57BL/6, C3$^{-/-}$ and Mac-1$^{-/-}$ mouse pups were incubated with 0.5 μM FLfAβ_{42} for 1 h and observed using a confocal microscope (A). The mean fluorescent intensity of the ingesting microglia (B) was compared. The percentage of cells taking up FLfAβ_{42} (C) was measured using a flow cytometer. **D**: Primary microglia from C57BL/6 mouse pups were transfected with C3, Mac-1, or control (CT) siRNA. Cells were treated 24 h later with 0.5 mM FLfAβ_{42} for 1 h and the percentage of cells taking up FLfAβ_{42} was measured using flow cytometry. The data, expressed as a percentage of microglia taking up FLfAβ_{42} relative to total microglia, represent the means ± SEM of three

separate experiments; ** $p < 0.01$ *versus* C57BL/6 MG in (B, C) or *versus* the group treated with CT siRNA in (D) (one-way ANOVA followed by Dunnett post-hoc test). Scale bar = 10 μm.

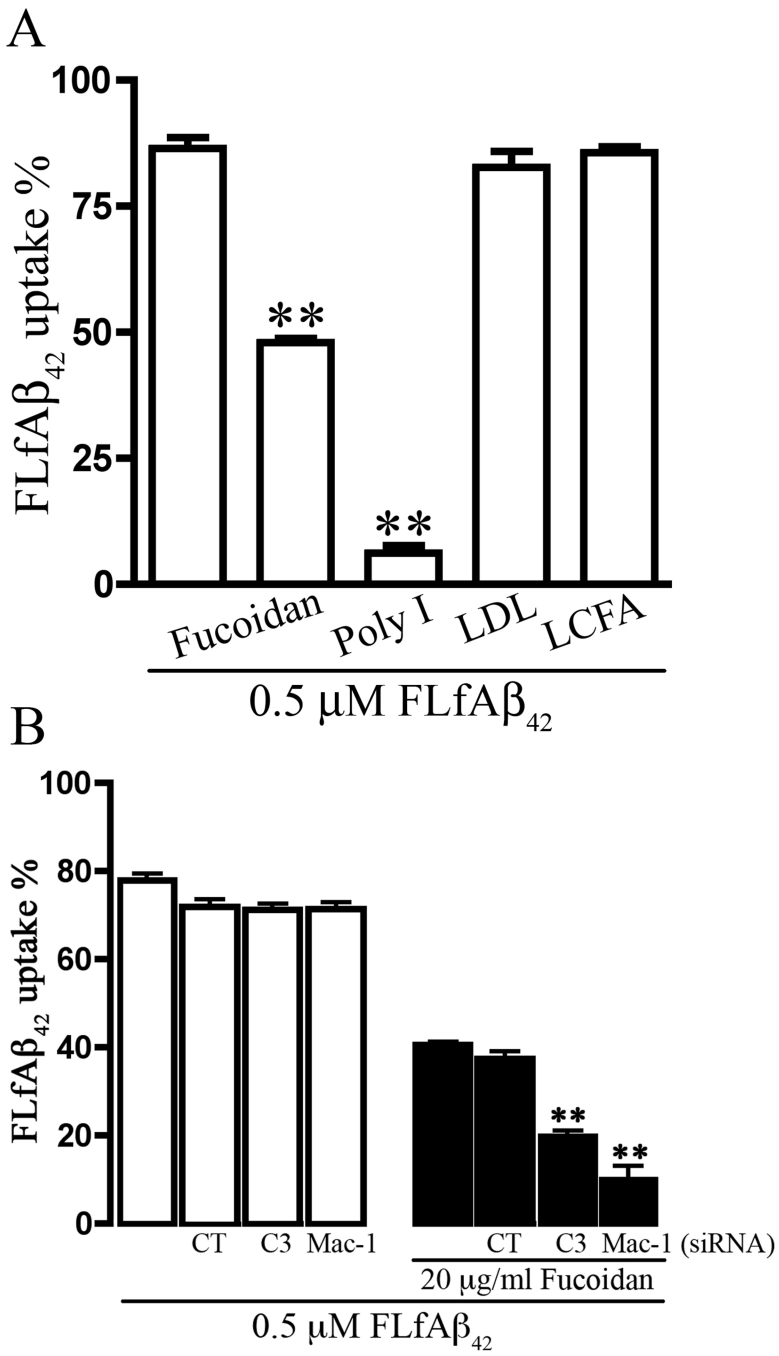

Fig. 5.

Class A scavenger receptor ligands attenuate the uptake of $FLfA\beta_{42}$ by N9 microglia, which is enhanced by the knockdown of C3 or Mac-1. **A**: N9 MG were incubated with 0.5 μM $FLfA\beta_{42}$ in the presence or absence of a 1 h pretreatment with 20 μg/ml Fucoidan, 200 μg/ml polyinosinic acid (Poly I), 100 μg/ml low density lipoprotein (LDL), or 200 μg/ml long chain fatty acid (LCFA). **B**: N9 MG were transfected with control (CT) siRNA, C3 or Mac-1 siRNA. Twenty-four h after transfection, cells were pretreated with or without 20 μg/ml Fucoidan for 1 h and then incubated with 0.5 μM $FLfA\beta_{42}$ for 1 h. The percentage of cells taking up $FLfA\beta_{42}$ was measured using by flow cytometry. The data, expressed as a percentage of microglia taking up $FLfA\beta_{42}$ relative to total microglia, represent the means ±

SEM of three separate experiments; * $p < 0.05$, ** $p < 0.01$ *versus* the group treated with FLfAβ_{42} alone in (A) or *versus* the group treated with CT siRNA, fucoidan and FLfAβ_{42} in (B) (one-way ANOVA followed by Dunnett post-hoc test).

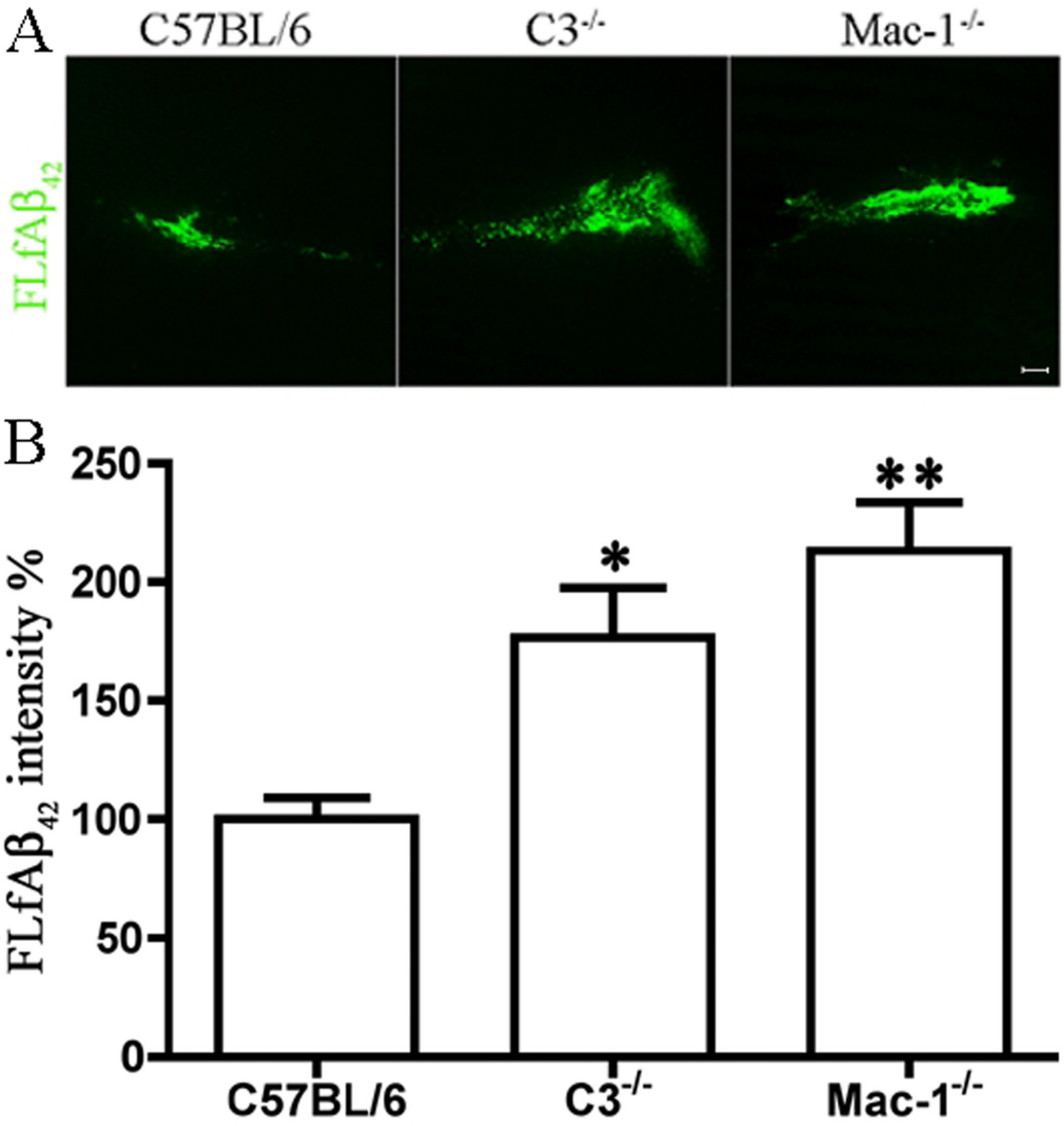

Fig. 6.

The deficiency of $C3^{-/-}$ or $Mac^{-/-}$ attenuates the clearance of microinjected FLfAβ_{42} in frontal cortex 5 days after the surgery. **A, B**: Brains were sectioned coronally at 20 microns on a cryostat. The mean fluorescent intensities of microinjected FLfAβ_{42} 5 days after the surgery were calculated (n = 5 each group) as shown in (B). The data, expressed as a percentage of the control (i.e. C57BL/6 mice), represent the means ± SEM of 5 microinjected mice; * $p < 0.05$, ** $p < 0.01$ *versus* control (Student's *t*-test). Scale bar = 100 μm.