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# Mechanism mediating oligomeric Aβ clearance by naïve primary microglia

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The accumulation of soluble oligomeric amyloid-β peptide (oAβ) proceeds the formation of senile plaques and contributes to synaptic and memory deficits in Alzheimer's disease (AD). The mechanism of mediating microglial oAβ clearance remains unclear and thought to occur via scavenger receptors (SRs) in microglia. SRs respond to their ligands in a subtype-specific manner. Therefore, we sought to identify the specific subtypes of SRs that mediate oAβ internalization and proteases that degrade oAβ species in naïve primary microglia. The component of oAβ species were characterized by western blot analysis, analytical ultracentrifugation analysis, and atomic force microscopy. The oAβ species remained soluble in the medium and microglial lysates during incubation at 37 °C. SR-A, but not CD36, mediated oAβ internalization in microglia as suggested by the use of subtype-specific neutralizing antibodies and small interfering RNAs (siRNAs). Immunoprecipitation analysis showed that oAβ interacted with SR-A on the plasma membrane. After internalization, over 40% of oAβ vesicles were trafficked toward lysosomes and degraded by cysteine proteases, including cathepsin B. The inhibitors of proteasome, neprilysin, matrix metalloproteinases, and insulin degrading enzyme failed to protect internalized oAβ from degradation. Our study suggests that SR-A and lysosomal cathepsin B are critical in microglial oAβ clearance, providing insight into how microglia are involved in the clearance of oAβ and their roles in the early stages of AD.


## Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the elderly (Blennow et al., 2006). Amyloid-β peptide (Aβ) monomers, derived from the proteolysis of amyloid precursor protein (APP), assemble into different conformations including oligomeric, fibrillar and aggregated forms (Haass and Selkoe, 2007; Roychaudhuri et al., 2009). Cerebral levels of Aβ do not correlate with the cognitive deficits in AD patients; however, the accumulation of soluble oAβ contributes to synaptic and memory deficits prior to the formation of senile plaques in the early stages of AD (Haass and Selkoe, 2007). The neutralization of oAβ by systemic active immunization rescues oAβ-attenuated long-term potentiation (LTP) (Klyubin et al., 2005; Walsh and Selkoe, 2007). Multiple species of oAβ are known to contribute to synaptic damage and memory decline (Walsh and Selkoe, 2007). Aβ dimers and trimers reduce the density of dendritic spines and attenuate LTP (Shankar et al., 2008; Townsend et al., 2006). Microinjection of Aβ dodecamers leads to behavioral deficits in water maze tests (Lesne et al., 2006). The amyloid cascade hypothesized that insufficient clearance and/or overproduction of Aβ could contribute to AD pathogenesis (Selkoe, 2001; Wang et al., 2006). Therefore, the insufficient clearance of oAβ in the early stages of AD is more relevant to the synaptic damage and cognitive deficits than the accumulation of fibrillarAβ (fAβ) years later.

Microglia, the resident brain macrophage, monitor the microenvironment of the CNS and carry out innate immune responses by pattern recognition receptors, including scavenger receptors (SRs) (Block et al., 2007; Husemann et al., 2002). Different subtypes of SRs differentially activate macrophage and microglia upon binding with toxic protein and pathogens (Block et al., 2007; Moore and Freeman, 2006). Scavenger receptor type-A (SR-A), scavenger receptor type-B1 (SR-B1) and CD36 have been identified as receptors of insoluble fAβ/aggregated Aβ in microglia (Coraci et al., 2002; El Khoury et al., 1996; Husemann et al., 2002; Paresce et al., 1996). Therefore, it is well established that SRs participate in the uptake of insoluble fAβ/aggregated Aβ.

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Although microglia have the highest capability for soluble oAβ uptake compared with neurons and astrocytes (Mandrekar et al., 2009), oAβ receptors in microglia has not been identified. It has been shown that fucoidan, a general inhibitor of SRs, reduces oAβ uptake (Shimizu et al., 2008; Tahara et al., 2006). The binding of oAβ to specific subtypes of SRs may induce different microglial responses as suggested by the distinct activation profiles of microglia induced by neurotoxic stimuli through individual pattern recognition receptors (Block et al., 2007; Moore and Freeman, 2006). Therefore, this study aimed to identify the specific subtypes of SRs that mediates microglial oAβ uptake.

Furthermore, the cellular compartment of internalized oAβ and its degradation kinetics remains unclear. Neprilysin (NEP) and matrix metalloproteinases (MMPs) degrade Aβ monomers in primary microglia (Jiang et al., 2008). Inhibitors of NEP and insulin degradation enzymes (IDE) added to the medium attenuate the degradation of $I^{125}$-labeled oAβ by measuring free $I^{125}$ in the medium (Shimizu et al., 2008). However, it is unclear whether extracellular or intracellular proteases degrade oAβ and whether free $I^{125}$ is released from Aβ monomers (Shimizu et al., 2008). The levels of trimers and dodecamers of oAβ were not altered in NEP and AD double transgenic mice, suggesting that NEP is not the major oAβ degradation protease (Meilandt et al., 2009).

Cathepsin B (CatB), a lysosomal cysteine protease, generates C-terminal truncations of Aβ (Mueller-Steiner et al., 2006). Genetic deletion of CatB in AD transgenic mice enhances the plaque load, Aβ1–42 levels, and neuronal deficits (Mueller-Steiner et al., 2006). The cystatin C knockout, an endogenous inhibitor of cysteine proteases, such as CatB, reduces the levels of soluble Aβ as well as minimize cognitive deficits (Sun et al., 2008). Furthermore, the beneficial effects of cystatin C knockout were abolished on CatB-null mice. This *in vivo* evidence clearly suggests that CatB is important for degrading soluble Aβ. However, the role of microglia in the degradation of soluble Aβ has not been elucidated.

In this study, neutralizing antibodies and sequence-specific small interfering RNAs (siRNAs) targeting SR-A, SR-B1, and CD36 were used to identify the specific subtypes of SRs that mediate oAβ internalization. Inhibitors of lysosomal proteases and proteasome were used to study the intracellular oAβ degradation machinery. Furthermore, the involvements of NEP, MMPs, and IDE in oAβ degradation were examined. The cellular compartments of the internalized oAβ as well as its degradation kinetics were monitored by confocal microscopy and real-time recording. The mechanism of oAβ clearance in naïve primary microglia revealed by this study may clarify how microglia clear oAβ and shed light in the microglial dysfunctions contributing to the insufficient clearance and accumulation of oAβ in the early stages of AD.

## Materials and methods

### *Reagents*

Synthetic Aβ1–42 and fluorescein amidite (FAM)-labeled Aβ1–42 were purchased from American Peptide (Sunnyvale, CA) and Biopeptide (San Diego, CA), respectively. Antibodies against SR-A and CD36 were purchased from AbD Serotec (Oxford, United Kingdom). Anti-SR-B1 antibody was purchased from Novus Biologicals (Littleton, CO). Anti-early endosome antigen 1 (EEA1) was purchased from Abcam (Cambridge, MA). Anti-Rab11 was purchased from Zymed Laboratories (Burlingame, CA). DQ-BSA and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). Anti-Aβ antibody 6E10 and 4G8 were purchased from Signet (Dedham, MA). Chloroquine, bafilomycin A1 (Baf A1), leupeptin, aprotinin, pepstatin, E64d, Ca-074 Me, MG132, epoxomicin, phosphoramidon, thiorphan, and insulin were purchased from Sigma-Aldrich (St. Louis, MO).

### *Preparation and biochemical characterization of oAβ*

oAβ was prepared as described and then centrifuged at 14,000*g* at 4 °C for 10 min to remove fibrillar and aggregated Aβ (Chromy et al., 2003; Dahlgren et al., 2002). A diluted solution of oAβ was spotted onto a mica slide and scanned using an Agilent® 5400 atomic force microscope (Molecular Imaging Corporation, Tempe, AZ) as described previously (Huang et al., 2009). Analytical ultracentrifugation (AUC) analysis was performed at 60,000 rpm using a Beckman An-50 Ti rotor (Chou et al., 2005). Freshly dissolved Aβ and oAβ at 100 μM were loaded into the centerpiece, individually. An absorbance wavelength of 280 nm was chosen for peptide detection, which was monitored in continuous mode. Multiple scans at different time intervals were fitted to a continuous size distribution model using the SEDFIT program (Brown and Schuck, 2006; Schuck, 2000).

### *Primary mouse microglia cell culture*

Primary mouse microglia were prepared as described (Shie et al., 2005). Briefly, mixed glial cultures were derived from the cortices of neonates on postnatal day 1. Cortices were dissociated by papain and endonuclease followed by trituration. Dissociated cells were grown in DMEM with 10% low-endotoxin fetal bovine serum (FBS) at 37 °C in a 5% $CO_2$ humidified atmosphere. After a 14-day incubation, microglia were separated from astrocytes by gentle agitation. The purity of primary microglia was $93.3 \pm 2.4\%$.

### *siRNA transfection*

ON-TARGET plus SMART pools of duplex siRNAs, containing a mixture of four sequence-targeted siRNAs, against SR-A (cat no. L-042239), SR-B1 (cat no. L-045065), CD36 (cat no. L-062017), and siGLO Red RNA duplex (cat no. D-001630) were purchased from Dharmacon (Lafayette, CO). The negative control siRNA (NC siRNA, cat no. 4390843) was purchased from Ambion (Austin, TX). Primary microglia ($2 \times 10^5$) were transfected with 150 nM SR-A siRNA, 150 nM SR-B1siRNA, 200 nM CD36 siRNA, 200 nM NC siRNA , and 50 nM siGLO Red RNA duplex using Lipofectamine 2000, individually. The knockdown effect of siRNA was examined 48 h after transfection. More than three independent experiments were performed.

### *Immunocytochemistry*

After the indicated treatment, fixed microglia were incubated in primary anti-Aβ antibody 6E10 (1:500), 4G8 (1:500), anti-SR-A antibody (1:500), anti-CD36 antibody (1:300), anti-EEA1 antibody (1:100), and anti-Rab11 antibody (1:100) overnight at 4 °C, individually. Microglia were incubated with secondary antibodies conjugated to either AlexaFluor 488 or 594 (1:500). The coverslips were mounted in Vitashield (Vector Laboratories, Burlingame, CA) and observed using a Zeiss fluorescent microscope (Axioplan II) or a Leica confocal microscope (TCS SP2). Relative fluorescence intensity was quantified using MetaMorph software 7.1. The individual fluorescence intensities in more than 100 cells were analyzed in each experiment. The experiments were repeated at least three times.

### *Western blot analysis*

Cells were lysed in lysis buffer containing 20 mM Tris–HCl (pH 7.4), 137 mM NaCl, 1% Triton X-100, 1 mM sodium deoxycholate, 1 mM EDTA , 1 mM EGTA, 1 mM $NA_3VO_4$, 50 mM NaF, and protease inhibitor cocktail. After electrophoresis, proteins were transferred onto PVDF membranes (NEN Life Science Products, Boston, MA). After blocking, the membranes were incubated overnight at 4 °C with primary antibodies for SR-A at a 1:1,000 dilution and β-actin at a 1:5,000 dilution. After incubation with secondary antibody (chicken