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Amyloid- β 42 protofibrils are internalized by microglia more extensively than monomers

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Abstract

One pathological hallmark of Alzheimer's disease (AD) is the accumulation of amyloid- β peptide (A β) in the affected brain. While there are numerous deleterious effects of A β accumulation, there is general agreement that a sustained inflammatory response to aggregated A β contributes to progressive neurodegeneration in AD and microglial cells play a significant role in this process. Our laboratory and others have shown that small soluble aggregates of A β activate a microglia-mediated inflammatory response. One component of the response involves internalization of extracellular A β , and this process is likely very sensitive to A β structure. In this study we analyzed the proclivity of microglia for internalization of A β 42 monomers and protofibrils using fluorescently-labeled A β . Both A β 42 species were labeled directly via amino linkage with an Alexa Fluor 488 tetrafluorophenyl ester (AF488-TFP) and then isolated individually by chromatography. A β 42 protofibrils retained their size and morphological properties after labeling but monomers had a much higher stoichiometry of labeling compared to protofibrils. Primary murine microglia internalized AF488-A β 42 protofibrils rapidly and in significant amounts compared to AF488-A β 42 monomers. Microglial internalization of protofibrils was dependent on time and concentration, and corresponded with tumor necrosis factor α secretion. In competition studies, unlabeled A β 42 protofibril internalization, detected by immunostaining, did not diminish AF488-protofibril uptake. Internalized AF488-A β 42 protofibrils were found widely dispersed in the cytosol with some lysosomal accumulation but little degradation. These studies highlight the sensitivity that microglia exhibit to A β structure in the internalization process and emphasize their affinity for soluble A β protofibrils.

Keywords

Amyloid-beta protein; aggregation; protofibrils; microglia; uptake; internalization; inflammation

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1. Introduction

Microglial cells play an important role in Alzheimer's disease-related (AD) neuroinflammation by responding to accumulations of amyloid- β peptide (A β) (Heneka, et al., 2015). Activated microglia and proinflammatory cytokines such as tumor necrosis factor α (TNF α) and interleukin 1- β (IL-1 β) can be observed clustering around extracellular A β plaques in the human AD brain and in transgenic AD mice (Apelt and Schliebs, 2001; Dickson, et al., 1993; McGeer, et al., 1987). One key problem in AD is that the phagocytic microglia are not completely efficient at removing the plaques, however they may work in some manner to restrict plaque growth (Meyer-Luehmann, et al., 2008). The form of A β present in, or surrounding, the deposits likely affects the way microglia respond to the peptide accumulation.

Soluble A β peptides, primarily 40 or 42 residues in length, are released via enzymatic cleavage of the amyloid precursor protein (APP). Both peptides are ultimately found in the plaque deposits with the dense plaque core comprised of the more aggregation-prone A β 42. The biophysical mechanisms by which plaque formation occurs in the brain are not fully understood however significant *in vitro* work has increased our understanding of the A β aggregation process. These studies have shown that unstructured A β monomer will undergo non-covalent self-assembly (Jarrett, et al., 1993) to form a polydisperse mixture of soluble oligomers (Bitan, et al., 2003; Kaye, et al., 2003; Mittag, et al., 2014) and/or protofibrils (Harper, et al., 1999; Mittag, et al., 2014; Walsh, et al., 1999; Walsh, et al., 1997) and ultimately insoluble fibrils (Harper, et al., 1997). A β assembly occurs via a nucleation-dependent polymerization process (Jarrett and Lansbury, 1993) and the rate-limiting nucleation step is characterized by a lag phase followed by rapid polymerization. More recent information on plaque composition suggests that there is greater structural diversity within the plaques than previously thought. Hyman and colleagues observed a halo of oligomeric A β surrounding A β plaques after immunostaining with the oligomer-selective NAB61 antibody (Koffie, et al., 2009). Although the plaques contain fibrillar A β at the core (Terry, et al., 1964), these data suggest that microglial cells may interact with multiple A β species within the plaque environment.

Numerous studies have demonstrated the ability of microglia (primary and cell lines) to internalize and traffic A β to the lysosome (Ard, et al., 1996; Chu, et al., 1998; Halle, et al., 2008; Li, et al., 2012; Mandrekar, et al., 2009; Paresce, et al., 1997; Sheedy, et al., 2013). Multiple receptors appear to regulate the A β internalization process including scavenger receptor (Paresce, et al., 1996), complement receptor type 3 (CR3, Mac-1), signal regulatory protein- β 1 (SIRP β 1) receptor (Gaikwad, et al., 2009), P2Y₄ receptor (Li, et al., 2013), and a receptor complex of CD36, α ₆ β ₁ integrin and CD47 (Koenigsknecht and Landreth, 2004). Many of these, and other, studies utilized either fibrillar, "soluble", or both forms of A β (Chung, et al., 1999; Fu, et al., 2012; Gaikwad, et al., 2009; Koenigsknecht and Landreth, 2004; Mandrekar, et al., 2009). The term "soluble" likely encompasses mixtures of aggregates and monomers. Cumulatively, the results indicate microglia internalization of A β is sensitive to structure or oligomerization state, and that different intracellular degradation pathways are utilized for different A β species.

A β protofibrils are well-recognized soluble intermediates in A β fibrillogenesis (Harper, et al., 1997; Harper, et al., 1999; Walsh, et al., 1999; Walsh, et al., 1997). We have previously demonstrated A β 42 protofibril formation in several physiological buffering systems including artificial cerebrospinal fluid (aCSF) (Paranjape, et al., 2012; Paranjape, et al., 2013). These structures possess a classic curvilinear morphology with lengths <100 nm, and are robust stimulators of tumor necrosis factor α (TNF α) secretion in microglia. Our earlier work utilized cytokine production, immunochemistry, and confocal microscopy imaging to show preferential activation of microglia by protofibrils (Paranjape, et al., 2012) and BV-2 microglia cell-surface binding of protofibrils (Paranjape, et al., 2013) compared to monomers and fibrils. In the current study, we expand the biophysical characterization of A β -fluorophore conjugation and demonstrate that soluble fluorescently-labeled A β 42 protofibrils are taken up rapidly by primary murine microglia, and in much greater quantity, than A β 42 monomers.

2. Results

Characterization of fluorophore-labeled A β

A β 42 protofibrils and monomers were labeled simultaneously using a unique adaptation of a previously-described procedure by LaDu and colleagues (Jungbauer, et al., 2009). A β 42 protofibrils were allowed to form in aCSF as outlined in the Methods. This A β 42 preparation typically produces a roughly equal distribution of protofibrils and monomers. The A β solution mixture was then incubated with amine-reactive Alexa Fluor® 488 (AF488) TFP ester dye (Figure 1A). Potential A β conjugation sites for AF488 were the primary amines at K16, K28, and the N-terminus. Since the typical protofibril preparation also contains monomers, both species can be labeled with fluorophore simultaneously. Size exclusion chromatography (SEC) separation of the A β 42 labeling solution on Superdex 75 yielded AF488-labeled A β 42 protofibrils and monomers (Figure 1B). Monitoring of the elution in-line with UV absorbance (A_{280} nm) produced three peaks: A β 42 protofibrils, A β 42 monomers, and the unconjugated AF488 dye peak. AF488 by itself has a contribution to the A_{280} nm absorbance that is equal to 11 % of the AF488 A_{494} nm absorbance. This contribution was taken into account in subsequent A β concentration determinations. Both A β 42 protofibrils and monomers were labeled with AF488 as fluorescence measurements of collected fractions showed the expected emission for the fluorophore in both peaks (Figure 1B).

In order to quantitate the amount of AF488 conjugated to each A β 42 species, absorbance scans (600–220 nm) were conducted on the same SEC fractions. A_{280} nm values obtained from these SEC fraction scans directly overlaid the SEC absorbance trace (data not shown) and were used in concert with A_{494} nm values to calculate both AF488 and A β concentrations as described in the Methods. These calculations allowed determination of labeling stoichiometry for A β 42 protofibrils and monomers. Qualitatively, it was observed that A β 42 monomers were labeled more efficiently than A β 42 protofibrils. This was not unexpected as A β monomers are unstructured leaving the amine groups more exposed for fluorophore conjugation. Quantitatively, for the representative data shown in Figure 1, AF488:A β stoichiometry was 0.10 for the peak protofibril fraction and 0.36 and 0.49 for the

two peak monomer fractions (#24 and #25 respectively). Overall, in $n=5$ separate labeling preparations the average AF488:A β labeling stoichiometry was 0.12 ± 0.04 standard deviation (SD) for A β 42 protofibrils and 0.45 ± 0.13 SD for A β 42 monomers. Thus, monomers were labeled at almost 4 times the efficiency of protofibrils and this difference in the extent of labeling between the two species had implications for data analysis in subsequent uptake experiments. Morphological analysis with transmission electron microscopy (TEM) showed that AF488-A β 42 protofibrils possessed structural characteristics similar to unlabeled A β 42 protofibrils with lengths centering around 50–100 nm (Figure 1D). Further characterization by dynamic light scattering (DLS) revealed AF488-labeled A β 42 protofibrils with hydrodynamic radii (R_H) ranging from 5–50 nm with an intensity-weighted mean R_H of 16 nm. This value was obtained from the regularized histogram shown in Figure 1E. These findings indicated that labeled and unlabeled A β 42 protofibrils were similar in their structural characteristics and the process of labeling did not dramatically alter the structure. This determination was consistent with that observed by LaDu and colleagues (Jungbauer, et al., 2009). Furthermore, the additional SEC purification step served to enrich the protofibril population and allowed careful separation of labeled monomeric and protofibrillar A β 42 pools. Of note, Jungbauer et al. found that while N-terminal-labeled A β 42 maintained similar aggregation properties to unlabeled peptide, limited solubility was observed for the AF488-N-terminal-labeled A β 42 particularly at physiological pH (Jungbauer, et al., 2009). In the paradigm described in the current studies, solubility issues were not encountered during SEC isolation of AF488-labeled A β 42 protofibrils and monomers in aCSF. Interestingly, despite potential labeling at K16 and K28, AF488-A β 42 monomers were able to aggregate over time and form fibrils (Figure 1F). The TEM image depicts fibrils collected by centrifugation after incubation of AF488-A β 42 monomers for 53 d at 4 °C. Subsequent fluorescence analysis confirmed that the fibrils were labeled with AF488 (data not shown). Conventional fluorescent techniques for monitoring A β aggregation, such as Thioflavin T and bis ANS, were unable to be used due to overlap and interference with the AF488 fluorophore emission.

Uptake of SEC-isolated AF488-A β 42 monomers and protofibrils in primary murine microglia

Numerous earlier reports have utilized the term “soluble A β ” to describe solutions prepared directly from reconstituted A β peptide. These solutions likely contain a mixture of monomeric and soluble aggregated A β species, thereby confounding the interpretation of the findings. For the current study, AF488-A β 42 monomers and AF488-A β 42 protofibrils were separated by SEC and the ability of primary murine microglia to take up the two distinct species was evaluated. AF488-A β 42 protofibrils were rapidly and extensively internalized by microglia compared to AF488-A β 42 monomers (Figure 2A). AF488-A β 42 protofibrils (10 μ M) were observed inside the microglia at the earliest time point (5 min), which represents an almost immediate entry into the cell. Much lower levels of AF488-A β 42 monomers (10 μ M) were found inside the microglia despite the fact that monomers were labeled with AF488 at nearly 4 times the stoichiometry of protofibrils. Internalized AF488-A β 42 protofibrils were dense and widespread within the cytosol of the microglia, yet not completely homogenous or diffuse. A closer inspection showed numerous globular, and some punctate, accumulations throughout the intracellular milieu. Microglia were able to

internalize AF488-A β 42 monomers but the amount relative to AF488-A β 42 protofibrils was strikingly low and demonstrated the overall proclivity of the microglia for protofibril internalization. Confocal images from multiple experiments of microglia treated with AF488-A β 42 monomers and AF488-A β 42 protofibrils were quantified using ImageJ software and demonstrated the significant difference in the speed and extent of uptake between the two A β 42 species (Figure 2B).

The time-dependent microglial uptake of AF488-A β 42 protofibrils correlated with production of TNF α by the microglia (Figure 2C). The time course for TNF α production was similar to what we have observed previously for unlabeled A β 42 protofibrils and monomers (Paranjape, et al., 2012; Paranjape, et al., 2013; Terrill-Usery, et al., 2014) wherein protofibrils, but not monomers, are robust stimulators of microglial TNF α secretion. Although uptake of AF488-A β 42 protofibrils occurs rapidly, there appears to be sufficient cell-surface interaction to trigger signaling pathways for TNF α synthesis and secretion.

Comparisons between AF488-labeled and unlabeled A β

Amino-labeling of A β 42 may interfere with some of the cellular processes that recognize and traffic A β 42 species, particularly in light of the higher AF488 labeling stoichiometry for A β 42 monomers. However, unlabeled A β 42 monomers displayed the same characteristics as AF488-labeled A β 42 monomers and were not taken up readily by primary murine microglia (Figure 3A–B). Internalization of unlabeled A β 42 protofibrils by primary microglia was rapid and extensive similar to AF488-labeled protofibrils (Figure 3C–D) confirming that the presence of the AF488 label did not hinder or modulate microglial A β internalization. We have previously shown avid binding of A β 42 protofibrils, but not monomers, to BV-2 microglia (Paranjape, et al., 2013). In order to confirm that the AF488 label did not block protofibril interactions with the microglial cell surface, binding studies were conducted using both immunostaining and direct fluorescence confocal microscopy. Both unlabeled and AF488-labeled A β 42 protofibrils accumulated around the BV-2 cell surface (Figure 3E–F). The cumulative findings indicated that labeling of either A β 42 monomers or A β 42 protofibrils did not alter cellular interactions with the peptides.

Alternative approaches to confocal microscopy were employed to further ascertain and confirm differences in microglia internalization between AF488-labeled A β 42 protofibrils and A β 42 monomers. Immuno-dot blot analyses were conducted on lysates prepared after exposure of the primary microglia cells to A β for increasing times. Microglial cells were thoroughly washed before cell lysis including an acid wash to remove cell surface-bound A β . The findings were similar to those obtained from confocal microscopy and showed the extensive uptake of protofibrils compared to monomeric A β (Figure 4A). Due to the low levels of internalized monomers, cell lysates were also probed with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody as an immunoblot lysate control (Figure 4A). Densitometry quantitation of cumulative dot blot data from three separate experiments demonstrated the time-dependent uptake of A β 42 protofibrils compared to A β 42 monomers (Figure 4B). The dot blot analysis also provided information on the stability of internalized A β 42 protofibrils and susceptibility to cellular degradation. No loss of internalized A β 42

protofibril material was noted during the longer 6 and 24 h exposure times (Figure 4A–B). This indicated that although A β 42 protofibrils were taken up rapidly by microglia, they were quite resistant to proteolysis.

The AF488 labeling of A β 42 protofibrils and A β 42 monomers also permitted measurement of primary microglia uptake by fluorescence plate reader. After exposure of adherent primary microglia to AF488-A β 42 protofibrils and monomers for increasing times, PBS and acid-washed microglia were assessed for intracellular AF488-A β 42 accumulation. Percent fluorescence uptake was determined by the 535 nm emission intensity of internalized AF488-A β divided by the 535 nm emission intensity of the AF488-A β remaining in the medium after treatment of microglia. This was done to normalize the fluorescence between the higher stoichiometry-labeled monomers and lower stoichiometry-labeled protofibrils. The fluorescence plate reader findings supported those from confocal microscopy and immuno-dot blot showing the rapid and extensive uptake of protofibrillar A β 42 (Figure 4C).

Concentration-dependence of AF488-A β 42 monomers and protofibrils for microglial uptake

Exposure of the primary murine microglia for 10 min to a range of AF488-A β 42 monomers and protofibril concentrations again showed the predilection of the microglia for A β 42 protofibril uptake compared to A β 42 monomers (Figure 5A). Even at the lowest concentration tested (500 nM), observable levels of AF488-A β 42 protofibrils were visible within the microglia. Microglial uptake of protofibrils was concentration-dependent with the highest levels found at 5–10 μ M. It was apparent from our studies that microglia respond rapidly, and internalize, soluble A β 42 protofibrils, but not A β 42 monomers. Fluorescence intensities were quantified from images obtained from multiple concentration-dependent primary microglia uptake experiments and the results indicated that internalization of A β 42 protofibrils did not saturate within the concentration range and cell density used for these experiments (Figure 5B).

Uptake of AF488-A β 42 protofibrils is not diminished by competition with unlabeled A β 42 protofibrils

Although microglia internalization of A β 42 protofibrils did not appear to saturate, it was possible that the internalization process was receptor-mediated or driven by transport machinery with specific binding sites that may limit the number of protofibrils that can be internalized. It was reasoned that if the number of internalization or transport sites were saturated, competition experiments between AF488-labeled, and unlabeled, A β 42 protofibrils would reveal this. However, images obtained after exposure of the microglia to solution mixtures containing 10 μ M AF488-A β 42 protofibrils and 0, 5, or 10 μ M unlabeled A β 42 protofibrils were not significantly different (Figure 6A). Additional, and multiple, experiments at 5 μ M AF488-A β 42 protofibrils also did not show competitive inhibition by unlabeled A β 42 protofibrils (Figure 6B). Specifically the presence of unlabeled A β 42 protofibrils did not diminish the uptake of AF488-A β 42 protofibrils indicating that the process was either not competitive or non-saturable at the concentrations used in these experiments.

Cellular localization of AF488-A β 42 protofibrils following microglial internalization

Numerous studies have demonstrated partial localization of A β within lysosomes (Halle, et al., 2008; Li, et al., 2012; Liu, et al., 2010; Mandrekar, et al., 2009). Our initial studies using Lyso-Tracker Red to image lysosomes showed significant internalization of AF488-A β 42 protofibrils but no lysosome-A β 42 protofibril colocalization (data not shown). Due to weak Lyso-Tracker confocal fluorescence signals, another lysosomal marker, Lamp1, was targeted by immunostaining. The greater sensitivity of the Lamp1 assay revealed some colocalization of AF488-A β 42 protofibrils with lysosomal compartments. However, significant amounts of AF488-A β 42 protofibrils were not in the lysosomes and many lysosomes were devoid of protofibrils (Figure 7).

3. Discussion

The question of how the aggregation of A β impacts its biological activity has been front and center for almost three decades. Answering these questions has been hindered in many circumstances by the use of unseparated solution mixtures that may contain fibrillar A β material as well as a variety of soluble A β species (both monomeric and aggregated). In this report, we fluorescently labeled an A β preparation containing both A β 42 protofibrils and monomers and then chromatographically separated the two A β species. This allowed direct comparison of microglial internalization propensity and kinetics between A β 42 protofibrils and A β 42 monomers. Primary microglia significantly favored internalization of A β 42 protofibrils as compared to A β 42 monomers both in total amount and speed, demonstrating that an aggregated form of A β is readily and preferentially taken up by microglia within the milieu of soluble A β . Furthermore, new insights were gained during the amine-dependent AF488 labeling of A β . Protofibrils were labeled less effectively than monomers, likely due to the restricted accessibility of lysine residues 16 and 28 in the β -sheet conformation.

We have previously investigated the microglia-A β 42 interaction in BV-2 murine microglial cells. That particular study indicated a preferential binding of A β 42 protofibrils to the cell surface compared to A β 42 monomers and A β 42 fibrils (Paranjape, et al., 2013). An important observation from that earlier study, relative to the current investigation, was that primary microglia were far more active at internalizing A β than BV-2 cells. However, the propensity to interact preferentially with protofibrillar A β 42 was consistent in both studies. There is not a consensus in the field on the relationship between A β structure and microglial internalization. Several studies have reported internalization of fibrillar A β (Chung, et al., 1999; Fu, et al., 2012; Gaikwad, et al., 2009; Koenigsknecht and Landreth, 2004; Mandrekar, et al., 2009), while others have demonstrated internalization of soluble or oligomeric A β (Chung, et al., 1999; Mandrekar, et al., 2009). The findings are not always consistent as Pan et al. showed that oligomeric A β is not internalized by microglia, yet interferes with fibrillar A β phagocytosis (Pan, et al., 2011). Less is known about protofibrillar A β , but one study found that protofibrils, but not fibrils, were internalized by microglia in the AD CRND8 mouse model (Liu, et al., 2010).

Mammalian cells ingest material by either phagocytosis (larger particles such as bacteria, yeast, and cellular debris) or fluid-phase pinocytosis (smaller particles) (Conner and Schmid, 2003). Pinocytosis can occur through one of five routes: macropinocytosis, clathrin- or

caveolin-mediated endocytosis, and clathrin- or caveolin-independent endocytosis. Macropinocytosis is associated with particles that are more than 1 μm in size, while the other four mechanisms are associated with smaller particles ranging in size from 60–120 nm (Conner and Schmid, 2003). Clathrin and caveolin are proteins that promote the formation of vesicles at the cell surface. These vesicles are then pinched off from the membrane as they enter the cell. During fluid-phase pinocytosis, membrane ruffles form on the membrane surface of microglial cells and when the ruffles close some of the extracellular medium is taken into the cell (Swanson and Watts, 1995). The resulting vesicle is fully inside the cell in less than 15 minutes (Swanson and Watts, 1995). During our microglia internalization studies, we have observed A β 42 protofibrils appear to accumulate around the microglia membrane ruffles. This is evidenced by hollow circular A β 42 structures localized at the cell surface in some of the images. Furthermore, the rapidity of A β 42 protofibril internalization by microglia is consistent with fluid-phase pinocytosis. Landreth and coworkers have demonstrated distinct mechanisms of uptake for fibrillar A β by receptor-mediated nonclassical phagocytosis (Koenigsknecht and Landreth, 2004) and soluble A β (primarily monomer and small oligomers) by macropinocytosis (Mandrekar, et al., 2009).

In the course of the *in situ* fluorescence plate reader experiments we found that a significant percentage of the A β 42 protofibrils were not taken up by the microglia under those concentration and time conditions. Some of this may be explained by A β adsorption to plate surfaces and A β association with the cell surface, which was removed by the acid wash (~8% for protofibrils at 24 h, very little for monomers, data not shown). Nevertheless this suggests that the rate of A β 42 protofibril uptake by microglia may be limited in some manner. The fluorescence plate reader findings indicated that the amount of internalized A β protofibrils continued to increase over time as measured by fluorescence plate reader. Although the immuno-dot blot data (Figure 4B) appears to reach a steady level, this is likely due to the smaller range of the dot blot assay with respect to densitometry quantitation. The concentration-dependent experiments in Figure 5 indicated that further increases in [A β 42 protofibril] increased the amount of internalized A β . Figure 4C suggests there is plenty of available A β 42 protofibrils (>95%) at an initial concentration of 2 μM . However, an increase in [A β 42 protofibrils] in the medium surrounding the microglia to 5 μM and 10 μM (Figure 5) favored increased uptake based on membrane gradient dynamics. The competition experiments in Figure 6 confirmed that inclusion of unlabeled A β 42 protofibrils with AF488-A β 42 protofibrils prior to microglia treatment did not diminish the amount of internalized AF488-A β 42 protofibrils and saturation was not achieved. Further investigation into the mechanism of uptake microglia internalization of A β protofibrils will be needed to fully understand the transport or diffusion process.

Internalization by microglia and trafficking to intracellular lysosomal compartments has been shown to be a key pathway for aggregated A β (Chu, et al., 1998; Fu, et al., 2012; Halle, et al., 2008; Li, et al., 2012; Liu, et al., 2010; Mandrekar, et al., 2009; Paresce, et al., 1997). This process may or may not be degradative (Chu, et al., 1998; Paresce, et al., 1997). Our studies indicated that some, but only a small percentage, of A β 42 protofibrils were trafficked to the lysosome. This finding, and the observation that A β 42 protofibrils are quite stable (Coalier, et al., 2013), may help to explain our observation that A β 42 protofibrils were not degraded and cleared after internalization by primary microglial cells.

Our investigations over a number of years have pointed to soluble A β 42 protofibrils as robust stimulators of microglia compared to fibrils and monomers. We have conducted numerous studies of protofibril properties and the ensuing cellular response. While there are more studies of “oligomeric” A β , there are fewer on microglial-protofibril interactions. Due to the substantial interest in soluble A β aggregates such as protofibrils and oligomers, it is important to discern what is being studied when a solution of A β is described as “soluble” since these solutions likely contain multiple A β species. Few investigations rigorously separate A β mixtures into soluble aggregates and monomers, thus confusing the interpretation of the results. For our studies, the critical factor in ascertaining the difference between the two A β species was the isolation of each population by SEC. Our studies demonstrated rapid and widespread internalization of A β 42 protofibrils by microglia and the difference in microglia uptake was striking between A β 42 protofibrils and monomers. The internalization of A β by microglia is an important part of AD and these *in vitro* findings shed light on the sensitivity of microglia to distinct A β species.

4. Experimental procedures

4.1. A β peptide preparation

A β 1–42 (A β 42) was obtained from ERI Amyloid Laboratory, LLC (Oxford, CT) (formerly W. M. Keck Biotechnology Resource Laboratory) in lyophilized form and stored at -20°C . A β 42 peptide was dissolved in 100% hexafluoroisopropanol (HFIP) (Sigma-Aldrich, St. Louis) at 1 mM, separated into aliquots in sterile microcentrifuge tubes, and evaporated uncovered at room temperature overnight in a fume hood. The following day the aliquots were vacuum-centrifuged to remove any residual HFIP and stored in desiccant at -20°C .

4.2. A β labeling

A β 42 protofibrils were prepared as previously described (Paranjape, et al., 2012). Briefly, lyophilized A β 42 (1 mg) was dissolved in 50 mM NaOH to yield a 2.5 mM A β 42 solution followed by dilution to 250 μM A β 42 in prefiltered artificial cerebrospinal fluid (aCSF, 15 mM NaHCO₃, 1 mM Na₂HPO₄, 130 mM NaCl, 3 mM KCl, pH 7.8). The A β 42 solution was incubated at room temperature for 30 min–4 h to allow the formation of protofibrils. For unlabeled A β 42 preparations, the solution was centrifuged at 18,000g for 10 min and the supernatant was chromatographically separated. For fluorophore labeling of A β 42, 500 μL of the A β protofibril preparation was added to a vial containing 100 μg of Alexa Fluor® 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester (AF488-TFP) dry powder (Life Technologies), mixed gently by pipet, and transferred to a 1.8 mL glass vial containing a small magnetic stir bar. A second 500 μL aliquot of A β 42 solution was added to the dye solution to bring the final volume to 1 mL. The solution was gently stirred for 1 h at 25°C , transferred to a siliconized Eppendorf tube, and centrifuged and separated as described above.

4.3. Size exclusion chromatography

Both unlabeled and fluorophore-labeled A β centrifugation supernatants were fractionated on a Tricorn Superdex 75 10/300 GL column (GE Healthcare) using an AKTA FPLC system (GE Healthcare). Prior to injection of A β , the Superdex 75 column was coated with sterile

bovine serum albumin (Sigma) to prevent any non-specific binding of A β to the column matrix. Following a 1 mL loading of the sample, A β was eluted at 0.5 mL min⁻¹ in aCSF and 0.5 mL fractions were collected and immediately placed on ice. Elution was monitored in-line by UV absorbance (A₂₈₀). A β concentrations were determined as described below.

4.4. Absorbance measurements and concentration calculations

Chromatography samples were scanned from 600 to 220 nm on a Cary 50 (Varian) UV/Visible spectrophotometer and absorbance values at 280 nm (A_{280, tot}) and 494 nm (A_{494, AF488}) in particular were obtained. Since A β and the AF488 label (A_{280, AF488} = A_{494, AF488} × 0.11) both contribute to A_{280, tot}, A β concentrations were obtained after subtraction of the A_{280, AF488} contribution. This was done using A_{494, AF488} and A_{280, tot} from the absorbance scans and the following calculation derived from Beer's Law ($A = \epsilon \cdot c \cdot l$).

$$C_{A\beta} = [A_{280, tot} - (A_{494, AF488} \times 0.11)] \div [\epsilon_{280, A\beta} \times l] \quad (\text{Equation 1})$$

where l is the path length (1 cm) and $\epsilon_{280, A\beta}$ is the extinction coefficient at 280 nm for A β (1450 M⁻¹ cm⁻¹). AF488 concentrations were determined using an extinction coefficient at 494 nm of 71,000 M⁻¹ cm⁻¹. The AF488:A β 42 stoichiometry was also determined from the absorbance-determined concentrations calculations.

4.5. Fluorescence measurements

AF488-labeled A β 42 fluorescence was determined in chromatography fractions using a Cary Eclipse fluorescence spectrophotometer. Emission scans (500–600 nm) were obtained with an excitation wavelength of 495 nm. The resulting curves were integrated from 505–550 nm to produce relative fluorescence units (RFU). *In situ* cellular fluorescence plate reader measurements were carried out using a Perkin Elmer Victor 3 multi-mode instrument. Excitation and emission filters were 485 nm and 535 nm, respectively, with a 0.1 second signal averaging time.

4.6. Dynamic light scattering

Hydrodynamic radius (R_H) measurements were taken at room temperature with a DynaPro Titan instrument (Wyatt Technology, Santa Barbara, CA). Samples (30 μ l) were placed into a quartz cuvette and light scattering intensity was collected at a 90° angle using a 5-second acquisition time. Particle diffusion coefficients were calculated from auto-correlated light intensity data and converted to R_H with the Stokes-Einstein equation using Dynamics software (version 6.12.0.3). Histograms of percent intensity vs. R_H were generated by data regularization and intensity-weighted mean R_H values (nm) were derived from the regularized histograms.

4.7. Primary microglia isolation

Primary murine microglia were obtained from wild-type (WT) C57BL/6 (Harlan Laboratories). Microglia were isolated as previously described (Esen and Kielian, 2007; Paranjape, et al., 2012) from 3–4 day old mouse pups. Briefly, brains were isolated under

sterile conditions, minced, and trypsinized. The brain tissue was then resuspended in complete DMEM growth medium containing 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 µg/mL amphotericin-B, OPI medium supplement (oxalacetate, pyruvate, insulin) (Sigma-Aldrich), and 0.5 ng/ml recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) (Life Technologies). The cell suspension was filtered, centrifuged, resuspended in complete medium and seeded into 150 cm² flasks. Cells were cultured at 37 °C in 5% CO₂ until confluent (1–2 weeks) and microglia were selectively harvested from the adherent astrocyte layer by shaking of the flask for 6 h or overnight at 37 °C in 5% CO₂ and collection of the medium.

4.8. Microglial uptake

For cell uptake studies, WT primary murine microglia, obtained as described above, were seeded in sterile glass-bottom Mat-Tek P35GC-1.5–14-C culture dishes (MatTek Corp., Ashland, MA) or 96-well cell culture plates overnight at a density of 5×10^5 cells/ml in complete growth medium. Prior to cell treatment, medium was replaced with growth medium lacking FBS and GM-CSF (assay medium). Cells were then incubated at 37 °C in 5% CO₂ for the indicated times with AF488-labeled, unlabeled Aβ₄₂, aCSF control, or AF488 dye fraction. Following incubation, the conditioned medium was collected and stored at –20 °C for subsequent analysis by enzyme-linked immunosorbent assay (ELISA). Adherent microglial cells were rinsed twice with assay medium and then fixed with 3.7 % formaldehyde for confocal microscopy analysis. For combination fluorescence plate and dot blot analysis, microglia were plated in Nunc F96 Nunclon sterile 96-well black plates. A more thorough wash protocol was utilized for these studies. This protocol involved removal of medium from treated cells followed by rinsing each well 3X with 100 µL sterile PBS. An acid wash (100 µL) was performed using a solution of 0.5 M NaCl and 0.2 M acetic acid for 15 minutes at 4 °C, then the cells were rinsed 2X with PBS. All PBS and acid washes were moved to another plate for both fluorescence and dot blot analysis. For intracellular cell extract preparation, microglia were lysed for 15 min at 25 °C with 35–50 µL radio immunoprecipitation assay (RIPA) buffer containing 150 mM NaCl, 20 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), and protease inhibitor cocktail (Sigma-Aldrich) at a 10x dilution. In some cases, multiple microglia wells were combined to yield higher concentration lysates. Microglial lysates were stored at –20 °C for further analysis.

4.9. BV-2 microglia cell culture and cell binding assay

BV-2 murine microglial cells were cultured as previously described (Paranjape, et al., 2012) in Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L glucose) (Hyclone) containing 50 U/ml penicillin, 50 µg/ml streptomycin, 50 µM β-mercaptoethanol, and 5 % fetal bovine serum (FBS, Hyclone). The Aβ-BV-2 microglial binding assay was conducted as previously described (Paranjape, et al., 2013). BV-2 murine microglia cells (0.2 ml, 5×10^5 cells/mL) were plated in individual MatTek dishes overnight. Cells were treated with either AF488-Aβ or unlabeled Aβ (5 µM) or aCSF buffer control and incubated for 30 min at 25 °C. Cells were then washed 3x with PBS containing 0.05 % Tween 20 (PBST) before and after fixing with 3.7 % formaldehyde, and after each step in the immunoassay for unlabeled Aβ

detection. All incubations were conducted with gentle shaking at 25 °C. Samples treated with unlabeled A β were incubated for 1 h each with blocking buffer (10 % w/v dried milk in PBST), anti-A β antibody Ab9 (1:5000 dilution in PBST with 5 % w/v dried milk), and donkey anti-mouse IgG antibody conjugated to Northern Lights (NL) 493 (R&D Systems). After a final wash step, 1 mL PBS was applied to the wells to avoid cellular dehydration.

4.10. Confocal microscopy

Cells were treated in Mat-Tek dishes and fixed as described above, followed by three washes with PBST. Cell nuclei were visualized by incubation for 5 min with 0.2 mL of 0.3 μ M 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). After a final wash step, 1.0 mL PBS was applied to the wells to avoid cellular dehydration. LysoTracker studies were done prior to cell treatment by adding 0.100 mL of assay medium containing 0.100 μ M LysoTracker and incubating 30 min at 37 °C in 5% CO₂. Microglia treated with AF488-labeled A β were imaged directly without further manipulation. For microglia treated with unlabeled A β , fixed cells were immunostained as described above in Section 4.8. Lysosomal Lamp1 detection was done by immunostaining as described for unlabeled A β but with a 1:500 dilution of anti-Lamp1 primary antibody (Novus Biologicals) into PBS containing 1% BSA and a 1:100 dilution of anti-mouse IgG-NL637 secondary antibody (R & D Systems). Excitation wavelengths used were 405 nm (DAPI), 488 nm (AF488-labeled A β 42 and anti-mouse IgG-AF488 secondary antibody), 561 nm (LysoTracker Red, Invitrogen), and 639 nm for anti-mouse IgG-NL637. Both two-dimensional and Z-stack images were obtained with a Zeiss LSM 700 confocal microscope at 40X magnification using ZEN 2009 imaging and analysis software. For comparison experiments, all optical and electrical settings were fixed at identical levels for each image to ensure that fluorescence intensity measurements were not influenced by different settings. Confocal fluorescence images were quantitated with ImageJ software using a procedure detailed by (Ansari, et al., 2013). Briefly, a region of interest was drawn around each cell using a polygon drawing tool. Area and integrated density were measured for each cell. Background fluorescence was measured by averaging 5 random areas between cells in the field. The corrected total cell fluorescence (CTCF) was calculated as follows: [integrated density of total cell area – (total cell area x mean background fluorescence)].

4.11. Dot blot

All steps in the dot blot were done at 25 °C on a platform rotary shaker set at 70 rpm. A nitrocellulose membrane was soaked in water and allowed to dry completely. Microglial cell lysates (1.7 μ L) were spotted onto the membrane, allowed to dry for 20 min, and blocked with phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 5% milk with gentle shaking for 1 hr. The membrane was washed 3x for 5 min with PBS containing 0.2% Tween at pH 7.4 (PBST). The membrane was treated with mouse anti-A β primary antibody (Ab9, 1:5000 dilution) followed by anti-mouse IgG-HRP secondary antibody (1:1000) with a multi-wash step in between. Both antibodies were diluted in PBS containing 1% milk and 0.1% Tween prior to membrane application. After washing, the membrane was treated with ECL western blotting substrate solution (Pierce), and washed for 1 min with accelerated shaking at 120 rpm. The membrane was subsequently dried and exposed to film for 60 seconds in an autoradiography cassette. The film was developed, fixed, washed in water, and

dried for scan analysis. Duplicate dot blot membranes were probed for GAPDH as a control intracellular protein. The procedure only differed in the primary mouse anti-GAPDH antibody (1:1000 dilution). The primary and secondary antibody diluents in the GAPDH procedure were PBS containing 1% milk and 0.1% Tween. Densitometry was performed on dot blot film images using ImageJ software.

4.12. ELISA

Levels of murine TNF α were determined by ELISA as previously detailed (Udan, et al., 2008). Briefly, 96-well plates were coated overnight with monoclonal anti-mouse TNF α capture antibody (R&D Systems), washed with PBS containing 0.05% Tween-20 and blocked with PBS containing 1% bovine serum albumin (BSA), 5% sucrose and 0.05% NaN₃ following by a wash step. Successive treatments with washing in between were done with samples or standards, biotinylated polyclonal anti-mouse TNF α detection antibody (R&D Systems) in 20 mM Tris with 150 mM NaCl and 0.1% BSA, streptavidin-horseradish peroxidase (HRP) conjugate, and equal volumes of HRP substrates 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide. The reaction was stopped by the addition of 1% H₂SO₄ solution. The optical density of each sample was analyzed at 450 nm with a reference reading at 630 nm using a SpectraMax 340 absorbance plate reader (Molecular Devices, Union City, CA). The concentration of TNF α in the experimental samples was calculated from a mouse TNF α standard curve of 15–2000 pg/ml. When necessary, samples were diluted to fall within the standard curve.

4.13. Electron microscopy

SEC-purified AF488-A β 42 protofibrils or fibrils (10 μ L) were applied to a 200-mesh formvar-coated copper grid (Ted Pella, Inc.). Samples were allowed to adsorb for 10 min at 25 °C, followed by removal of excess sample solution. Grids were washed three times by placing the sample-containing side down on a droplet of water. Heavy metal staining was done by incubation of the grid on a droplet of 2 % uranyl acetate (Electron Microscopy Sciences, Hatfield, PA) for 5 min, removal of excess solution, and air drying. Affixed samples were visualized with a JEOL JEM-2000 FX transmission electron microscope operated at 200 keV.

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Abbreviations used

AD	Alzheimer's disease
Aβ	amyloid- β protein
aCSF	artificial cerebrospinal fluid
HFIP	hexafluoroisopropanol
PBS	phosphate-buffered saline

SEC	size exclusion chromatography
TEM	transmission electron microscopy
TNFα	tumor necrosis factor α

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Highlights

- A β 42 protofibrils and monomers were simultaneously labeled, then separated.
- A β 42 monomers were labeled at higher stoichiometry than protofibrils.
- Microglia internalized A β 42 protofibrils very rapidly and extensively.
- Purified A β 42 monomers were not readily taken up by microglia.
- Significant colocalization of A β 42 protofibrils with lysosomes was not observed.

Figure 1.

Preparation and characterization of AF488-Aβ42. Panel A. Structure of the AF488 TFP compound structure (Life Technologies). Panel B. AF488-labeled protofibrils, monomers and unincorporated dye were separated on Superdex 75 and eluted in aCSF. The elution trace (blue line) was acquired from in-line $Abs_{280\text{ nm}}$ readings, and peak fraction fluorescence (red circles) was determined by integrating the AF488 emission from 505–550 nm. Panel C. SEC elution trace (blue line) overlaid with peak fraction $Abs_{494\text{ nm}}$ measurements (red circles) subsequently used in stoichiometry calculations. Panel D. Protofibrils (10 μL, 38 μM) applied to a copper formvar grid and imaged by TEM at a magnification of 59,000, scale bar = 50 nm. Panel E. AF488-Aβ42 protofibrils were analyzed by DLS directly after SEC isolation and a representative regularized histogram of percent intensity versus R_H is presented. Panel F. TEM of AF488-Aβ42 fibrils (10 μL, 37 μM) that formed during incubation of AF488-Aβ42 monomer fractions for 53 days at 4 °C. Magnification = 18,000, scale bar = 50 nm.

Figure 2.

AF488-Aβ42 protofibrils are internalized by primary microglia in greater quantities than AF488-Aβ42 monomers. Primary microglia were plated in Mat-Tek cell culture dishes and treated with 10 μM AF488-Aβ42 monomers and protofibrils (green) at 37 °C for the times indicated. Cells were washed and fixed with 3.7 % formaldehyde as described in the Methods, and then stained with DAPI (blue). Panel A. Representative confocal images of time-dependent microglial exposure to AF488-Aβ42 (Panel A) were acquired at 40X magnification using identical power and pinhole settings for each fluorophore with a Zeiss LSM 700 laser scanning confocal microscope. Scale bar = 10 μm. Panel B. Quantitation of the AF488 fluorescence intensity (fluor int) in the confocal images with ImageJ as described in the Methods was carried out on data from multiple fields of two separate experiments utilizing identical conditions. Panel C. Tumor necrosis factor α (pg/ml) secretion measured by ELISA from samples in Panel A.

Figure 3.

AF488-labeling of Aβ42 does not interfere with microglia uptake or surface interaction. Primary microglia (A–D) or BV-2 microglia (E, F) were treated with 5 μM unlabeled Aβ42 (A, C, E) or AF488-Aβ42 (B, D, F) monomers or protofibrils, washed, fixed and stained with DAPI (blue). AF488-Aβ42 samples were imaged directly (green), while unlabeled samples were immunostained as described in Methods using an AF488-conjugated secondary antibody (green). Scale bar = 10 μm.

Figure 4.

Aβ42 protofibrils are internalized by microglia in a time-dependent manner and to a greater extent than Aβ42 monomers. Primary microglia were incubated in a Nunc black 96-well cell culture plate and treated with 2 μM AF488-Aβ42 for the times indicated. Microglia cells were washed extensively, including an acid wash to remove external cell-surface-associated Aβ42, prior to fluorescence plate reading and lysate preparation. Panel A. Representative immuno-dot blot analysis of microglia lysates using anti-Aβ (top) or anti-GAPDH (bottom). Panel B. Densitometry quantitation (in pixel intensity) using ImageJ of dot blots from 3 separate time-dependent uptake experiments. Panel C. Following extensive washing and prior to microglial lysate preparation, internalized AF488-Aβ42 protofibrils or monomers were measured by fluorescence plate reader. The fluorescence data for the internalized AF488-Aβ42 represents the averages and standard error from 3 separate experiments and is shown as a percentage of the total AF488-Aβ42 fluorescence present in the microglia assay medium for each condition.

Figure 5.

Internalization of AF488-Aβ42 by primary microglia is concentration-dependent. Panel A. Primary microglia were treated as described in Figure 2 legend with AF488-Aβ42 monomers or protofibrils (green fluorescence) for 10 min at the concentrations indicated. Cells were counterstained with DAPI (blue) and confocal fluorescence images (40X) were captured as described. Panel B. Quantitation of the fluorescence intensity in the A488 channel for the images in panel A and 2 additional concentration-dependent experiments. For each field, the integrated density of fluorescence measured with ImageJ was divided by the number of cell nuclei.

Figure 6.

Competition with unlabeled Aβ42 protofibrils does not significantly reduce internalization of AF488-Aβ42 protofibrils. Panel A. Primary microglia were treated with solution mixtures of AF488-labeled (L) and unlabeled (U) Aβ42 protofibrils at the concentrations depicted for 15 min at 37 °C. Cells were fixed, stained with DAPI (blue), and imaged (40X magnification) as described earlier in Fig 1. Panel B. In a separate set of experiments (n=2), primary microglia were treated with lower concentrations of AF488-labeled Aβ42 protofibrils (5 μM) for 10 min at 37 °C. Ratios depicted in panel B are in μM. Confocal images were obtained and uptake (fluorescence intensity) was quantified using ImageJ as previously described.

Figure 7.

Partial colocalization of AF488-A β 42 protofibrils with lysosomes. Primary microglia were cultured as described in Methods and treated with 5 μ M A488-A β for 30 min at 37 $^{\circ}$ C, washed, fixed, and permeabilized with 0.5 % Triton X-100. Cells were immunostained with 6E2 anti-Lamp1 primary and anti-mouse IgG-NL637 secondary antibody. Three different fields are shown and white arrows indicate lysosomes devoid of A488-A β 42

protofibrils. Yellow arrows indicate areas of colocalization for A488-A β 42 protofibrils and lysosomes. Green: A488-A β ; Red: Lamp1; Blue: DAPI. Scale bar = 10 μ m.

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