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Microglia Demonstrate Age-Dependent Interaction with Beta-amyloid Fibrils

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Abstract

Alzheimer's disease (AD) is an age-associated disease characterized by increased accumulation of extracellular β -amyloid (A β) plaques within the brain. Histological examination has also revealed profound microglial activation in diseased brains often in association with these fibrillar peptide aggregates. The paradoxical presence of increased, reactive microglia yet accumulating extracellular debris suggests that these cells may be phagocytically compromised during disease. Prior work has demonstrated that primary microglia from adult mice are unable to phagocytose fibrillar A β 1-42 *in vitro* when compared to microglia cultured from early postnatal animals. These data suggest that microglia undergo an age-associated decrease in microglial ability to interact with A β fibrils. In order to better define a temporal profile of microglia-A β interaction, acutely isolated, rather than cultured, microglia from 2 month, 6 month, and postnatal day 0 C57BL/6 mice were compared. Postnatal day 0 microglia demonstrated a CD47 dependent ability to phagocytose A β fibrils that was lost by 6 months. This corresponded with the ability of postnatal day 0 but not adult microglia to decrease A β immunoreactive plaque load from AD sections *in vitro*. In spite of limited A β uptake ability, adult microglia had functional phagocytic uptake of bacterial bioparticles and demonstrated the ability to adhere to both A β plaques and *in vitro* fibrillized A β . These data demonstrate a temporal profile of specifically A β -microglia interaction with a critical developmental period at 6 months in which cells remain able to interact with A β fibrils but lose their ability to phagocytose it.

Keywords

inflammation; phagocytosis; receptor; cytokine; microglia; Alzheimer

Introduction

Alzheimer's disease brains compared to age matched controls demonstrate increased numbers of reactive microglia [1–4]. They are found in white and grey matter often in intimate association with A β fibril containing plaques [5–8]. Cytoplasmic projections from the microglia surround the fibrillar aggregates indicating that fibril interaction may contribute to acquisition of the reactive phenotype. [9, 10].

However, there is little histological evidence of actual microglial phagocytosis of A β fibrils [9, 10]. This lack of intracellular, lysosomal localization of phagocytosed A β is also evident in brains of AD mouse models [11, 12]. Therefore, in addition to the possibility of A β fibrils serving as activating stimuli for microglia they may also initiate an aborted phagocytic response. These observations are confounded by a very clear response of cultured microglia from rodents [13–25] and humans [26] to take up the fibrillar A β peptide. In addition, it is clear from both human [27] and rodent [28] A β vaccination studies that adult microglial uptake of A β first requires some modulation of the interaction. These data suggest that cultures of early postnatal brain-derived microglia may differ quite significantly from their adult counterparts with regard to A β interaction. Indeed, it is known that microglia from humans and rodents undergo a morphologic dystrophy with age [29, 30] and standard early postnatal brain derived microglia cultures are generated during periods of active proliferation *in vivo* and are maintained in a basally reactive phenotype [31–33].

Therefore, adult microglia rather than early postnatal microglia may offer a more relevant model for defining the precise microglia-A β interaction during disease. Several studies have documented that acutely isolated adult microglia from rodent brains retain a quiescent phenotype although prolonged culturing ultimately leads to activation [23, 34–38]. In order to better define whether an age-dependent change in microglial A β interaction exists, microglia acutely isolated from varying age C57BL/6 mouse brains were used to examine not only levels of putative A β receptors, but more importantly, changes in phagocytic and adhesion ability.

Materials and Methods

Materials

The anti- β -amyloid IgG antibody (sc-5399), Receptor for advanced glycation end products (RAGE) (sc-8230), lysosomal-associated membrane protein-1 (LAMP-1) (sc-8098), α -tubulin (sc-8035), CD36 (sc-9154), CD14 (sc-9150), formyl receptor like peptide 1 (FRL1) (sc-18191), extracellular signal regulated kinase (ERK2) (sc-154), β 1 (sc-8978) and α 6 (sc-10730) integrin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-CD11b and CD68 antibodies were purchased from Serotec (Raleigh, NC). Anti-toll-like receptor 2 (TLR2) antibody was purchased from Imgenex (San Diego, CA). The low-density lipoprotein receptor-related protein (LRP) 11H4 antibody clone was a kind gift from Isa Hussaini at the University of Virginia. Scavenger receptor A-1 (SRA-1) and CD47 antibodies were from R&D Systems (Minneapolis, MN). Lipopolysaccharide (LPS) was from Sigma (St. Louis, MO). FITC-*E. coli* bioparticles were purchased from Invitrogen (Carlsbad, CA).

Tissue Culture

Acute microglia were derived from postnatal day 0, 2–4 month, 6–8 month or 12–17 month C57BL/6 mice as previously described [23]. Briefly, postnatal day 0 mice were killed via decapitation and adult mice were killed via CO₂ asphyxiation and exsanguination and perfused with PBS with Ca²⁺. Cortices were isolated, finely minced and filtered through 140 and 70 μ m filters. Filtered tissue was digested with DNase I and collagenase (Worthington Biochemical, Lakewood, NJ) before being separated on a percoll gradient (GE Healthcare, Piscataway, NJ). The microglia layer was collected and the cells were then counted and either lysed for protein analysis or used immediately for experiments. The protocol provides cultures that are >97% pure based upon CD68 immunoreactivity [23]. Since approximately 100,000–200,000 microglia are isolated per brain a single experimental condition was typically a pool of 10 brains to allow for sufficient cellular signal or protein amounts to be

quantified and analyzed. Cultured microglia were derived from postnatal day 0–2 (P0) pups and isolated from mixed cultures at 14 days *in vitro* as previously described [39].

Western Blotting

Isolated cells were collected and lysed using ice cold RIPA buffer (20mM Tris, pH 7.4, 150mM NaCl, 1mM Na₃VO₄, 10mM NaF, 1mM EDTA, 1mM EGTA, 0.2mM phenylmethylsulfonyl fluoride, 1% Triton, 0.1% SDS, and 0.5% deoxycholate) with protease inhibitors (AEBSF 104mM, Aprotinin 0.08mM, Leupeptin 2.1mM, Bestatin 3.6mM, Pepstatin A 1.5mM, E-64mM). To remove insoluble material cell lysates were sonicated and centrifuged (14,000 rpm, 4°C, 10 min) and the method of Bradford was used to quantify protein concentrations [40]. Proteins were resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes for Western blotting, and antibody binding was detected via enhanced chemiluminescence (GE Healthcare, Piscataway, NJ). For a single condition of a Western blot, the cells from approximately 10 brains are pooled to provide enough protein to be detectable. Therefore, the graph values are average data (+/–SD) from 30–50 animals analyzed from 3–5 unique Western blots. Western blots were quantified using Adobe Photoshop software. Optical density of bands were normalized against their respective loading controls and averaged (+/–SD).

Phagocytosis assay

Peptide phagocytosis was quantitated by measuring the uptake of FITC-conjugated A β 1–42 (rPeptide, Athens, GA). The peptide was fibrillized according to manufacturer protocol and as previously described [23]. Briefly, the peptide was dissolved in 2mM NaOH at 1mg/mL. The peptide was sonicated for 30 seconds and diluted with 10 $\times$ PBS and water to a final concentration of 50 μ M A β . The peptide was allowed to fibrillize 24hr at 37 degree C before use. Acutely isolated microglia or macrophage were incubated with A β 1–42 fibril aggregates or FITC-*E. coli* bioparticles (positive control, 0.125mg/ml) in 96-well plates for 6 hours. To quench the FITC signal from extracellular peptide or bioparticles, medium was removed and the cells were rinsed with 0.25mg/ml trypan blue in PBS. Application of trypan serves to quench any remaining FITC signal on the plate as well as any bound to the external leaflet of the plasmalemma. Intracellular fluorescence was read (480 nm excitation and 520 nm emission) via fluorescent plate reader (Bio-Tek, Winooski, VT).

Phagocytosis inhibition

Acutely isolated microglia were preincubated with varying concentrations of inhibitors: Fucoidan, a general scavenger-type receptor inhibitor [41] (Sigma), A β 1-11 an RHD-containing peptide and β 1 integrin receptor inhibitor [42](Bachem), or 4N1K, a CD47 inhibitor [43] (Bachem) for 30 minutes and then incubated with 1 μ M FITC-A β 1–42 fibrils for 2 hours.

Cell viability assay

To determine cell viability after 2 hour stimulations, cellular release of lactate dehydrogenase (LDH) into the media and total cellular LDH were measured using a commercial nonradioactive assay (Promega, Madison, WI). Absorbance measurements were taken at 490 nm. Optical densities corresponding to LDH activity from treated conditions were normalized to no treatment controls to generate % control LDH release values. To compare acutely isolated microglia viability after 7 DIV cells were cultured in DMEM/F12 with 10% heat-inactivated FBS, 5% horse serum and antibiotics (1.5 μ g/mL penicillin/streptomycin/neomycin) for 7 days. Cells were fixed in 4% paraformaldehyde and immunostained using an anti-CD68 antibody. Antibody binding was visualized using Vector

VIP as the chromagen and CD68 immunoreactive cells were counted and averaged $\pm$ SD from 3 wells per condition counting 4 random fields per well.

Tissue adhesion assay

Human tissue was obtained from the University of Washington Brain Bank. Flash frozen AD or age-matched control brain temporal cortex was sectioned (10 μ m) onto 0.05mg/mL poly-L-lysine coated glass coverslips. The tissue was air dried and sterilized via 30 min. UV irradiation at room temperature then rinsed with DMEM/F12 media prior to the addition of microglia. Microglia isolated from postnatal day 0 or 6–8 month microglia (as described above) were prelabeled with a fluorescent dye according to the manufacturer's instructions (Cyquant Proliferation Assay, Invitrogen) or 3 μ M DAPI (10 min) prior to adhesion. Pre-labeled microglia were seeded at a density of 70,000 cells/well onto 24 well plates containing the glass coverslips with AD or control brain sections in serum free DMEM/F12. The microglia were allowed to adhere to the AD or control brain tissue with gentle rocking (20rpm) for 1 hour (25 degree C). The cells and tissue were rinsed 3 times with PBS (25 degree C) then fixed with 4% paraformaldehyde and immunostained using an anti-A β antibody (sc-5399) to visualize plaques. Antibody binding was visualized using texas red-conjugated secondary antibody. The number of adherent microglia on A β immunoreactive plaques from 2 sections per each AD or control brain, per treatment condition, counting 4 random fields per section were averaged $\pm$ SD. Plaque adherent microglia were counted only if a portion of the DAPI or Cyquant signal from microglia visually overlapped the A β immunoreactivity. Experiments were repeated 4 independent times using freshly isolated microglia pooled from approximately 10 brains per age group for each experiments using three different AD or control brain cases.

A β fibril adhesion assay

A β 1–42 peptide (Bachem, Torrance, CA) was fibrillized as previously described [23]. Briefly, peptide was dissolved in dH₂O to a final concentration of 500 μ M and incubated at 37 degree C for 7 days. Fibrillized peptide (0.125nmole/mm²) was plated into black walled-clear bottom 96 well plates (BD Falcon) and allowed to dry. Microglia were collected from approximately 10 animals of postnatal day 0 and 6 months of age and pooled per age and labeled with a fluorescent dye according to the manufacturer's instructions (Cyquant Proliferation Assay, Invitrogen). Pre-labeled microglia were seeded at a density of 70,000 cells/wells onto A β or tissue culture plastic only (control) wells with replicates of 8 per age group. Cells were allowed to adhere for 1 hour (25 degree C) with gentle rocking (20rpm) then each well was rinsed 3 times with PBS (25 degree C) and the bound/adherent microglia were quantified via fluorescent plate reader (346/460). Experiments were repeated 3 independent times pooling microglia from 10 animals per age group for each experiment.

Microglia phagocytosis of human tissue assay

Flash-frozen temporal cortex tissue from age-matched control and AD brains (n=3 per group) tissue was sectioned (10 μ m) onto 0.05/mg/mL poly-L-lysine coated glass coverslips. The tissue was air dried and sterilized via 30 min. UV irradiation at room temperature then rinsed prior to the addition of 35,000 microglia/coverslip in DMEM/F12 with 10% heat-inactivated FBS, 5% horse serum and antibiotics (1.5 μ g/mL penicillin/streptomycin/neomycin). Fresh media was replenished 1 day after plating and every third day until tissue and microglia were fixed in 4% paraformaldehyde at either day 3 or 7 *in vitro*. The cultures were fixed at each time point and immunostained (sc-5399) to visualize A β immunoreactive plaques in 3 sections per brain, per condition, from 4 random 20 $\times$ fields and averaged $\pm$ SD. Cultures were stained with anti-mouse specific CD68 (MCA1957XZ, Serotec) to visualize the added exogenous microglia.

Statistical Analysis

Average values $\pm$ SD were calculated and statistically significant differences were determined using one-way ANOVA with Tukey post hoc comparison or upaired t-test.

Results

Microglia demonstrated an age-dependent decrease in ability to phagocytose A β fibrils *in vitro*

To avoid the confound of prolonged culturing conditions and determine whether a temporal decrease in ability of microglia to phagocytose A β fibrils existed, microglia from varying age mice were acutely isolated and A β uptake quantified. Microglia isolated from postnatal day 0 and 2 month old mouse brains significantly phagocytosed A β fibrils although this ability was lost by 6 months of age (Fig. 1). The fact that 6 month old microglia were still competent to take up bacteria, however, demonstrated that a critical period existed between 2 and 6 months of age regarding changes in A β -specific microglial interaction in these cells. To determine whether this temporal phagocytic ability could be generalized to other phagocytic cells, resting peritoneal macrophage were isolated from 6 month old mice and assayed for their ability to take up A β fibrils. In contrast to their 6 month old microglia counterparts, macrophage were competent at phagocytosing A β thus demonstrating that brain resident microglia are unique from peripheral macrophage with respect to their A β interaction phenotype (Fig. 1).

Microglia exhibited age-associated alteration in expression levels of CD36, an A β interacting protein

One possibility explaining the decreased ability for A β uptake by adult microglia could be the result of altered expression of A β interacting proteins. To examine this, microglia were acutely isolated from postnatal day 0, 2–4 month, 6–8 month, and 12–17 month old animals for comparison to 6 month resting peritoneal macrophage and cultured microglia. Although only a subset of putative A β interacting proteins were analyzed due to limitations in sample amount, quantification of Western blot analysis demonstrated that only CD36 out of a number of putative A β interacting proteins demonstrated an age dependent decrease in protein levels with significantly less protein at 12–17 months compared to all other ages as well as to cultured microglia [16, 17, 20, 44–60] (Fig. 2). This change in at least one potential A β receptor protein level indicated that microglial phenotype changes with age. However, these data also suggested that a change in expression levels of a reported A β receptor was likely not sufficient to explain the loss of A β uptake by 6 months of age. In addition, these findings helped to support the idea that cultured microglia or postnatal day 0 microglia can not substitute for aged microglia 12–17 months of age when attempting to define the specific nature of A β interactions.

Postnatal day 0 microglia utilized CD47 for A β uptake

However, given the fact that at least one A β receptor protein level difference was noted between cultured microglia and postnatal day 0 microglia versus 12–17 month microglia (Fig. 2) it was worthwhile to determine what receptors the acutely isolated microglia used to take up A β fibrils since the majority of A β fibril phagocytosis studies have been reported from cultured cells. For example, cultured microglia have three A β interacting proteins that have been identified as particularly important in A β interactions, CD36, CD47, and α 6 β 1 integrin [17]. To determine whether a similar multi-receptor biology was necessary in the acutely isolated cells, postnatal day 0 microglia were incubated with and without receptor competing agents for scavenger-type receptors including CD36, CD47, and α 6 β 1 integrin; fucoidan, 4N1K, and (A β 1–11) RHD-containing peptide, respectively. Postnatal day 0

microglia took up A β peptide into LAMP-1 positive vesicles validating phagocytic uptake (Fig. 4). This uptake was attenuated by fucoidan and 4N1K treatment but not the RHD-containing peptide, suggesting a role for scavenger receptors and CD47 but not β 1 integrins in A β uptake into acutely isolated postnatal day 0 cells (Fig. 3). However, since the concentration of fucoidan used was somewhat toxic to microglia during treatment, varying concentrations of fucoidan and 4N1K were used to determine the dose-dependency of either agent to attenuate A β phagocytosis (Fig. 4). Unlike 4N1K, fucoidan only altered A β uptake at concentrations that were toxic to microglia indicating that its inhibitory actions might be partially due to decreased viability (Fig. 4). It is important to point out that although a significant 4N1K-dependent inhibition of A β uptake at 100 μ g/mL was observed, these findings should be interpreted with caution with the consideration that cells even at this concentration might be undergoing some sort of initial cellular compromise related to death which was observed at the 5 fold higher 500 μ g/mL concentration. However, with regard to fucoidan use, no non-toxic concentration had any effect on the ability of the microglia to take up A β allowing us to hypothesize that fucoidan effects on diminishing phagocytic ability were unrelated to specific effects on CD36 but more likely related to toxicity in our hands.

Postnatal day 0 microglia demonstrated increased ability to clear A β peptide plaques from AD brains compared to adult microglia although both age groups adhered to A β

In order to verify that these differences in age-associated A β uptake extended beyond the use of *in vitro* fibrillized A β , microglia acutely isolated from postnatal day 0 and 6–8 month animals were allowed to adhere to AD and control brain temporal cortex section for 3 and 7 days to quantify any changes in A β immunoreactive plaque load. Only the postnatal day 0 microglia decreased plaque number significantly at 7 days *in vitro* (Fig. 5). This was entirely consistent with their ability to take up the synthetic peptide *in vitro* (Fig. 1). However, the number of CD68 immunoreactive, surviving microglia at 7 days *in vitro* was significantly less from cells derived from 6–8 month old versus postnatal day 0 mice suggesting some of the decreased clearance of A β plaque load from tissue sections may be due to decreased microglia numbers. One possibility for attenuated plaque clearance by the adult microglia could be a decreased ability to interact with the plaque peptide. As a means of assessing whether microglia lose their ability to recognize the A β fibril containing plaques with age, microglia acutely isolated from postnatal day 0 and 6–8 month animals were allowed to adhere to A β plaques in sections of AD or control brains and quantified. In spite of decreased A β fibril phagocytic ability (Fig. 1, 5), 6–8 month old microglia demonstrated a significantly greater ability to adhere to A β peptide positive plaques in AD brains when compared to postnatal day 0 microglia (Fig. 6B). Interestingly, there was no difference in the ability of postnatal day 0 and 6–8 month old microglia to adhere to A β immunoreactive plaques from control brains (Fig. 6B) or *in vitro* fibrillized A β (Fig. 6C). Moreover, significantly more microglia from 6–8 month old microglia adhered to A β immunoreactive plaques from AD brains versus control brains. This suggests that there may be differences in the composition of the A β immunoreactive plaques from control versus AD brains such that a component of the AD plaques promotes greater adhesion compared to non-disease plaques and *in vitro* A β . Collectively, these data suggest that adult microglia are not compromised in their ability to recognize A β fibrils but are instead likely deficient in the subsequent steps of phagocytic uptake required for this peptide conformation.

Discussion

These results indicate that mouse microglia have a developmentally critical period between the ages of 2 and 6 months in which the ability to phagocytose specifically A β fibrils is lost. This appears to be unique to microglia-A β interaction since adult microglia retained the

ability to phagocytose bacterial components and adult peripheral macrophage retained their ability to phagocytose A β . Due to a limitation in animal numbers we necessarily pooled a span of ages from 2–4 months, 6–8 months and 12–17 months but still found that a simple change in expression levels of several reported A β receptor proteins was not sufficient to explain the loss in phagocytic ability observed by 6 months of age. Indeed, adult microglia were still able to adhere to A β fibril-containing plaques and *in vitro* fibrillized A β verifying that the loss of ability may not be due to altered expression of the appropriate interacting proteins. However, microglia did demonstrate decreased expression of at least one putative A β -interacting protein, CD36. This is similar to work from another group which utilized mRNA analysis from isolated microglia. Particularly, the prior study demonstrated CD36 mRNA levels decreased in 8 and 14 month microglia compared to 1.5 and 3 month microglia isolated from wild type C57BL6/J mice [61]. These prior findings fit well with our current protein analysis demonstrating that 12–17 month microglia have significantly less CD36 protein levels than postnatal day 0 microglia. Because each experimental condition required approximately 10 animals for each age, at least 30–50 animals had to be collected for each age/condition in order to quantify protein changes. Although a larger list of microglial ages and inclusion of macrophage as well as stimulated and unstimulated conditions would offer a more comprehensive list of A β receptor changes the study was not principally aimed at emphasizing a specific change in a putative receptor level as the critical point responsible for the deficiency of microglial A β uptake with age and was focused on microglia rather than peripheral macrophage. Future efforts would, however, benefit from additional comparisons of peripherally infiltrated macrophage compared to resident microglia at each age group with and without various stimulatory conditions. Nevertheless, the current data again raises the question of which cell type is the most relevant choice for *in vitro* study of A β interaction since acutely isolated microglia at any age differed considerably from typical cultured microglia and macrophage with regard to expression of numerous putative A β interacting proteins.

It is possible that there are yet unidentified proteins that are required for A β -microglia interaction that were not assessed and the reason for limited uptake in adult microglia is a consequence of age-dependent changes in receptor expression. Indeed, an age and disease dependent change in microglial phenotype including expression of A β interacting proteins has been reported from microglia isolated from an APP/PS1 mouse model of disease [61]. Moreover, a requirement for a pre-conditioning stimulus has also been reported by others as a necessity for facilitating monocyte/microglia A β interaction supporting the idea of a phenotype change [62]. This suggests that further identification of the specific temporal profiles of age-dependent changes in microglial phenotype is warranted. Moreover, defining the mechanism(s) involved in regulating this change in microglial phenotype may be exploitable to manipulate or retain A β phagocytic ability in older age cells. However, as already suggested, since 6 month old microglia were able to interact/adhere to A β fibrils it appears that the cells recognize A β to some degree but are not able to complete the phagocytic process. Although there appeared to be a slight attenuation of viability of 6 month microglial viability by 7 days *in vitro* compared to postnatal day 0 microglia the apparent decrease in 6 month old microglia plaque clearance at 7 days corresponded well to their inability to phagocytose A β fibrils when acutely isolated. Therefore, a fundamental decrease in particularly A β phagocytosis ability is representative of this age. Perhaps their phagocytic problem was not due to changes in receptor levels but instead some compromise in their phagocytic machinery mechanism. It is important to point out that the plaque counts performed to generate the data for Figures 5 were based upon A β immunoreactivity alone. We did not distinguish diffuse versus compact or cored depositions and therefore we can not determine whether increased plaque clearance by postnatal day 0 microglia was preferential for any particular form of plaque. However, since the postnatal day 0 but not 6 month old microglia were capable of phagocytosing *in vitro* fibrillized A β we hypothesize that some of

the enhanced clearance of plaques includes fibrillar A β . An intriguing possibility is that intracellular events leading to formation or shuttling of A β fibril stimulated endosomal or lysosomal compartments are compromised.

We hypothesize that this loss of phagocytic ability may directly contribute to reduced clearance of A β from the brain with age providing a mechanism for allowing fibrillar plaques to begin accumulating. It is reported that mouse models of AD have significant increases in plaque deposition at approximately 6 months of age that may be directly related to a change in microglial phenotype [63]. In agreement with this notion, a prior study has demonstrated that wild type microglia undergo an increase in mRNA levels of YM-1, an alternative activation marker, from 6 to 18 months of age while a transgenic AD line expressing human mutant APP and PS1 demonstrated significantly higher levels of YM-1 mRNA compared to wild type microglia already at 6 months [64]. Moreover, the transgenic but not wild type microglia increased mRNA levels of classic activation markers such as TNF α at 18 months compared to 6 months particularly in non-plaque associated microglia which correlated with increased levels of oligomeric A β in the brain. This correlates well with our prior work demonstrating that acutely isolated murine microglia demonstrate an age-dependent decrease in A β fibril but not oligomer stimulated TNF α secretion [23]. Jimenez et al. also demonstrated that a decrease in A β phagocytic ability occurred from 6 months to 18 months in the transgenic brains based upon confocal microscopy immunolabeling [64]. Our current findings of an age-dependent change in A β phagocytic ability in acutely isolated microglia (Figure 1) corresponds well with the histologic changes observed by Jimenez et al. [64]. Although we did not examine changes in expression of alternative activation markers such as YM-1, Jimenez et al. found that microglial YM-1 mRNA levels appeared to be accelerated in the transgenic mouse microglia at 6 months compared to wild type animals. It is possible that interaction with A β peptide or other inflammatory stimuli in the disease model brain drives the cells into an early alternative activation state unique to the transgenic model that is superimposed upon any age associated changes in ability to phagocytose or be stimulated by A β fibrils.

It is also of interest that 6 month old microglia demonstrated greater ability to adhere to A β immunoreactive plaques compared to postnatal day 0 microglia but only from AD brains and not control brain plaques or *in vitro* A β . One intriguing possibility for this is that the plaque composition varies between AD and control brains and some component besides fibrillar A β of the AD plaques, in particular, provides a better substrate for 6 month versus postnatal day 0 microglia. These findings are similar to those reported prior in which rat microglia demonstrated increased binding to senile plaques rather than non-senile plaques on AD brain sections indicating again that the particular composition of plaques may influence the degree of microglia interaction [65]. Another possibility for differences in the abilities of the postnatal day 0 and 6 month old microglial to adhere to the plaques from AD and control brains may be due to the fact that plaque detection was based upon A β immunoreactivity alone. Therefore, it is possible, for instance, that increased adhesion of 6 month old microglia to particularly AD brain plaques is based upon a particular A β conformation or composition. Future efforts to dissect out these subtleties will resolve this age-dependent phenotype. Histologic examination of endogenous microglia in AD brains also supports the idea that plaque components besides A β influence microglial association. Veerhuis et al. reported that microglia in AD brains were clustered specifically around those plaques that contained fixed complement factor C1q and serum amyloid P [66]. Perhaps one reason that 6 month old rather than postnatal day 0 microglia demonstrated greater adherence to A β immunoreactive plaques in AD versus control brains and no difference when adhered to A β fibrils *in vitro* is that the older microglia interacted with a particular component of AD plaques in addition to A β .

Although these results indicate that microglia isolated from 6 month mouse brains have deficient ability to clear both *in vitro* generated A β fibrils and A β fibril containing plaques, other groups have reported that adult microglia have the ability to phagocytose A β fibrils once isolated from the brain [36]. It is possible that even our cells would develop A β phagocytic ability with the appropriate growth conditions *in vitro* but the effort of this work was to minimize the length and conditions of *in vitro* growth to model as closely as possible, the conditions *in vivo*. Of course, it is not possible even with this study to state that the cells are accurately modeling their precise *in vivo* phenotype. However, the lack of phagocytic potential upon acute isolation certainly models histological observations from diseased brains [9, 10] much closer than the typical prolonged early postnatal culture models which present a clear paradox of aggressive phagocytic potential versus the *in vivo* presentation.

It is important to keep in mind that the results are focused on understanding age-dependent changes specific for microglia interactions with A β . Numerous studies have now demonstrated that peripheral blood-derived macrophage appear to be critical for efficient removal of A β fibrils *in vivo* using rodent transgenic models of disease [67–74]. Indeed, these results confirm that adult macrophage but not microglia are competent at A β fibril phagocytosis. Although macrophage infiltration into the brain may be an important component of the immune changes that occur during Alzheimer's disease the focus in this study was on age dependent changes in microglial-A β interaction rather than to characterize the changes that also occurred in peripheral immune cell interaction with A β . Indeed, we appreciate that the resting peritoneal macrophage used in this study will not have the same phenotype as blood-derived macrophage that have recently entered the brain. Therefore, our intent was simply to show that at 6 months of age when microglia lose their phagocytic ability it is not simply an overall loss of immune cell function but something unique to microglia-A β interaction that is age-dependent. However, future efforts to understand microglial behavior during age and disease must also include a component of adult macrophage either alone or in combination with adult microglia to more accurately model the complex multi-phagocyte environment surrounding plaques.

Finally, the data suggests one possible explanation for the problem of phagocytic uptake in adult microglia could simply be dysfunction of CD47 since postnatal day 0 microglia required CD47 function for uptake. Therefore some deficiency or alteration in the CD47 stimulated signaling response may occur between 2 and 6 months of age that mediates the change. Admittedly, CD47-A β interaction is only one of nearly a dozen reported receptors that are used for mediating adhesion and uptake in early age microglia [16, 17, 20, 44–60]. However, the fact that CD47 rather than scavenger-type receptors and β 1 integrins was required for A β uptake in these cells suggests that this particular *in vitro* model of acute microglia isolation rather than using prolonged serum-cultured cells offers unique A β fibril interaction mechanisms that may be more reflective of *in vivo* conditions. This system offers the opportunity to compare several different receptor-dependent interactions as well as their associated signaling pathways to systematically elucidate the changes that occurs with age to explain decreased ability to phagocytose A β fibrils.

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Figure 1.

Microglia demonstrated decreased ability to phagocytose Aβ fibrils with age. Acutely isolated microglia from (A) postnatal day 0, (B) 2 month, (C) 6 month, or (D) peritoneal macrophage from 6 month mice were incubated with 500nM FITC-Aβ or 0.125mg/mL FITC-bioparticles (positive control) for 6 hours. Intracellular relative fluorescence units (RFU) were quantitated and averaged via plate reader (480nm excitation and 520nm emission) and averaged (+/-SD). Graphs are representative of 4 independent experiments analyzed by t-test or one-way ANOVA (*p<0.001 from control). (E) Postnatal day 0 microglia were incubated with 500nM FITC-Aβ for 6 hours then fixed and immunostained

using anti-CD11b antibody and texas red-conjugated secondary antibody with DAPI counterstain for nuclei. Intracellular A β (arrows) were visualized via FITC conjugation.

Figure 2.

Selected Aβ receptor protein levels were compared between cultured and acutely isolated mouse microglia with age. Microglia were isolated from mixed cultures at 14 days *in vitro* (cultured) and stimulated 24hr with or without 25ng/mL LPS (cultured+LPS) or acutely from postnatal day 0 (P0) 2–4 month, 6–8 month, or 12–17 month old mice and Western blotted for quantitation of optical densities (O.D.) of selected proteins (α6 integrin, β1 integrin, TLR2, CD36, CD47, SRA-1, FPRL1, LRP, and RAGE) normalized against their respective loading controls (ERK2). Microglia were isolated from 10 animals per age group and pooled for each experiment. Experiments were repeated 3–5 times (totaling 30–50 animals per condition) and averaged $\pm$ SD. Statistical significance was determined via one-way ANOVA (*p<0.05 from P0).

Figure 3.

Acutely isolated microglia from postnatal day 0 mice take up Aβ fibrils in a CD47 but not a scavenger receptor or β1 integrin-dependent manner. Postnatal day 0 (P0) acutely isolated microglia were incubated with specific antagonists for scavenger-type receptors (300μg/mL Fuco), CD47 (100μg/mL 4N1K), or β1 integrin (100μg/mL RHD-containing peptide). Cells were incubated with and without antagonists for 30 minutes, 37°C, then incubated with 1μM FITC-Aβ 1–42 for 2 hours. (A) After incubation, relative intracellular fluorescence units (RFU) were read (480nm excitation and 520nm emission) via plate reader. (B) To determine cell viability after the 2 hour stimulation, cellular release of lactate dehydrogenase (LDH) was measured from culture media. Graphs are average values (±SD) and

representative of 5 independent experiments analyzed via one-way ANOVA (* $p < 0.001$ from A β control).

Figure 4.

A CD47 receptor antagonist dose-dependently attenuated the ability of acutely isolated postnatal day 0 microglia to take up A β fibrils. Microglia from P0 C57BL/6 pups were acutely isolated from brains and cultured for 6 hours with 1 μ M FITC-A β . (A) Cells were fixed and immunostained using an anti-LAMP-1 antibody. Antibody binding was visualized using a texas red conjugated secondary antibody. (B,C) Alternatively, microglia were pretreated with 10, 100, or 500 μ g/mL Fucoidan or 4N1K for 30 min. prior to the 6 hour 1 μ M A β -FITC stimulation. (B) After the 6 hour stimulation, relative fluorescence intensity units (RFU) from phagocytosed peptide in each condition were quantitated via plate reader (480 nm excitation and 520nm emission) and averaged ($\pm$ SD). (C) Cell viability after the 6

hour stimulation was determined by quantitating LDH released into the media normalized to cellular LDH levels and graphed as percent control release. Graphs are representative of three independent experiments analyzed via one-way ANOVA. (% $p<0.01$; * $p<0.05$; ** $p<0.001$ from control; \$ $p<0.01$; & $p<0.05$; # $p<0.001$ from A β).

Figure 5.

Postnatal day 0 but not 6 month old microglia decreased numbers of Aβ plaques when co-cultured with AD brain tissue. (A) Human temporal cortex from age-matched control or AD tissue (n=3) was cultured with or without acutely isolated microglia (35,000 cells/well) from P0 or 6 month C57BL/6 mice for 3 or 7 days *in vitro* (DIV). The cultures were fixed at each time point and immunostained with anti-mouse CD68 antibody to visualize exogenous murine microglia as well as with anti-Aβ antibody to detect Aβ containing plaques. Plaque numbers were counted, averaged $\pm$ SD, analyzed via one-way ANOVA, and graphed. (*p<0.001 from 7 day no microglia AD tissue only). (B) To compare microglia viability after 7 DIV, microglia from acutely isolated postnatal day 0 and 6 month old mice were

cultured without tissue for 7 days. Cells were fixed, immunostained using an anti-CD68 antibody, and counted and averaged $\pm$ SD (* p <0.005 from P0). Graphs are representative of 3 independent experiments analyzed via t-test. (C) Representative images are shown of postnatal day 0 microglia cultured on an AD brain section for 7 days *in vitro* double-stained with anti-mouse CD68 and texas red-conjugated secondary antibody along with anti-A β antibody and FITC-conjugated secondary antibody to visualize A β uptake into the cells.

Figure 6.

Microglia isolated from 6 month and postnatal day 0 microglia were adherent to A β plaques and A β fibrils *in vitro*. Microglia from P0 or 6–8 month old C57BL/6 mice were acutely isolated from brains, labeled with 3 μ M DAPI (blue nuclei), and seeded onto unfixed AD or age-matched control cryosections (10 μ m) for 1hr. The tissue was rinsed to remove unbound cells and the remaining cells and tissue were fixed and plaques immunostained using an anti-A β antibody. (A) A representative image of AD plaque-adherent P0 and 6 month microglia is shown (arrows). Scale bar, 100 μ m. (B) The number of microglia adherent to A β immunoreactive plaques were averaged $\pm$ SD, analyzed via one-way ANOVA and graphed. Data is representative of 4 independent experiments. (C) Alternatively, 0.125nM/mm²

fibrillized A β was coated onto a 96 well plate and dried. Microglia from postnatal day 0 or 6 month animals were isolated, prelabeled with 3 μ m DAPI, and allowed to adhere to tissue culture plastic only (Control) or A β coated wells for 1 hour. The wells were rinsed to clear any unbound microglia and the fluorescence intensity was quantitated via plate reader (346 nm excitation and 460 nm emission), averaged $\pm$ SD and analyzed by one-way ANOVA. The data shown is representative of 3 independent experiments.