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Ex vivo cultures of microglia from young and aged rodent brain reveal age-related changes in microglial function

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Abstract

To understand how microglial cell function may change with aging, various protocols have been developed to isolate microglia from the young and aged central nervous system (CNS). Here we report modification of an existing protocol that is marked by less debris contamination and improved yields and demonstrates that microglial functions are varied and dependent on age. Specifically, we found that microglia from aged mice constitutively secrete greater amounts of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) relative to microglia from younger mice and are less responsive to stimulation. Also, microglia from aged mice have reduced glutathione levels and internalize less amyloid beta peptide (Aβ) while microglia from mice of all ages do not retain the amyloid beta peptide for a significant length of time. These studies offer further support for the idea that microglial cell function changes with aging. They suggest that microglial Aβ phagocytosis results in Aβ redistribution rather than biophysical degradation *in vivo* and thereby provide mechanistic insight to the lack of amyloid burden elimination by parenchymal microglia in aged adults and those suffering from Alzheimer's disease.

Keywords

Microglia; Alzheimer's disease; Beta amyloid; Glutathione; Cytokine; IL-6; TNF-α; Aging

1. Introduction

A multitude of studies have implicated microglia as important players in the etiology of a number of age-related neurodegenerative diseases, including Alzheimer's disease (AD),

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Conflict of Interest
None.

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Parkinson's disease (PD) and amyotrophic lateral sclerosis (Boillee, et al., 2006,Chung, et al., 2001,El Khoury, et al., 2007,Frautschy, et al., 1998,Rogers, et al., 2002). While histological studies are essential in providing clues regarding the cells' involvement, they are limited in terms of evaluating the functions of living cells. In the past decade, protocols to isolate living microglia from postnatal animals have become available (Carson, et al., 1998,de Haas, et al., 2007,Frank, et al., 2006,Hickman, et al., 2008,Ponomarev, et al., 2005). These protocols either trap microglia using antibodies to cell-specific antigens (Hickman, et al., 2008,Tham, et al., 2003) or separate microglia using density centrifugation (de Haas, et al., 2007,Frank, et al., 2006). In both cases, the rapid isolation of microglia enables *ex vivo* experimentation of endogenous microglia in a controlled setting largely devoid of neurons, oligodendrocytes and astrocytes.

Protocols utilizing density centrifugation are advantageous to those utilizing antigen traps in terms of yield per brain (de Haas, et al., 2007). They also avoid artificial cellular reactions caused by antigen cross linking, a risk carried with the use of antibodies in trapping protocols. However, in our hands, significant amounts of non-microglial debris contaminate current density centrifugation derived cultures. In the present study, we sought to modify density centrifugation methodology to eliminate debris fields present in such cultures. With these modifications, microglial yields were preserved or slightly increased. These improvements allowed us to study microglial function and possible alterations during normal aging.

2. Material & Methods

2.1. Solutions

Dispase II (Roche, Mannheim, Germany) was reconstituted at 2U/mL in dispase buffer (0.9% HEPES, 50mM NaCl, pH 7.4) according to manufacturer's instructions. Percoll (GE Healthcare, St. Giles, UK) was diluted 1:10 with 10× phosphate-buffered saline (PBS) to create an isotonic solution. 1× PBS was added to isotonic percoll to create working solutions ranging from 75% to 25% percoll.

2.2. Animals

Debris reduction experiments were performed with non-transgenic C57BL/6 mice and mice expressing GFP under the fractalkine receptor promoter (Jung, et al., 2000). Experiments were performed using young (1–2 month old) and aged (14–16 month old) male C57BL/6 mice. The mice were housed at 22°C in a controlled 12hr light/dark cycle and provided food and water ad libitum. Animals were euthanized by exsanguination using transcardiac perfusion with PBS under deep anesthesia with sodium pentobarbital (50mg/kg body weight). This method of euthanasia is consistent with the recommendations of the Panel of Euthanasia of the American Veterinary Medical Association. After perfusion, the brain (telencephalon, cerebellum and midbrain) was rapidly removed.

2.3. Reduction of debris produced by brain homogenization

Each brain was washed in cold 1× PBS, then minced using small scissors. Brain tissue was gently dissociated by immersion into 10mLs (per brain) of dispase II solution (2U/mL),

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trypsin solution (0.05%) or by grinding within a tissue homogenizer (glass Potter, Braun, Melsungen, Germany). Dissociated brain tissue was placed within a 50mL conical tube and laid horizontally in an orbital shaker set to shake for 1hr, 37°C at 150rpm. Remnant tissue chunks were further homogenized by rapidly triturating with a 10mL pipette (BD Biosciences, Boston, MA) with a wide bore to prevent cell shearing. This was carried out with a fully charged pipette aid (Drummond). Enzyme activity was halted by diluting the resultant homogenate 1:1 with cold 10% fetal bovine serum (FBS) in 1× PBS. Meninges and clumped cells were removed with 70μm filtration (BD Biosciences, Boston, MA) to obtain a suspension of single cells.

2.4. Preparation of discontinuous percoll gradients

The homogenate was spun 1000 × g for 10min at 4°C. The supernatant was discarded and the pellet of an individual brain was resuspended in 6mLs of 75% isotonic percoll (high percoll) (GE Healthcare, Buckinghamshire, U.K). Three mLs of this mixture was then aliquoted into a 15mL polystyrene tube. Five mLs of 35% isotonic percoll (low percoll) was layered atop the high concentration percoll at a rate of 150μl/sec to create a distinct interface between the percoll layers. To replicate gradients described in the literature, 25% percoll was utilized for low percoll. 1× PBS was layered atop the low concentration percoll. The resultant discontinuous gradient was then allowed to settle on ice for 15 minutes allowing most of the homogenate to naturally rise towards its isopycnic position. The gradient was then centrifuged at 800 × g for 45min in a HS-4 swinging bucket rotor (Thermo Fisher Scientific, Waltham, MA) set to 4°C. We did not notice changes in microglial yields with high acceleration or the application of the brake. However, yields were significantly diminished if the gradients are not processed immediately following centrifugation. To process the gradients, the volume of the PBS layer and the low concentration percoll layer were rapidly aspirated. A band of microglia (usually 0.5–1.5mL), captured between the low concentration and high concentration percoll layers was then collected and diluted in 50mL of 1× PBS. This was centrifuged at 1000 × g for 10min at full acceleration and brake. The supernatant was quickly decanted and the cell pellet resuspended in DMEM culture media containing 10% FBS. We also added 0.15μg/mL granulocyte monocyte colony stimulating factor (GM-CSF, R & D Systems, Minneapolis, MN), although this is not required for culturing microglia.

2.5. Immunochemistry

Isolated cells were grown in culture media overnight. Cells were then washed, fixed in 4% paraformaldehyde and processed for immunofluorescence of microglial antigen Iba1 (1:500, Wako, Richmond, VA), microglial antigen Cd11b (1:1000, Serotec, Raleigh, NC), astrocyte antigen GFAP (1:1000, Dako Corporation, Carpinteria, CA), and neuronal antigen NeuN (1:500, Millipore, Bellirica, MA). Cells were rinsed and incubated with goat anti-rabbit Alexa 488 (Invitrogen, Carlsbard, CA) and goat anti-mouse Alexa 568 (Invitrogen, Carlsbard, CA). For dendritic cell control experiments, cells were fixed in acetone and blocked for endogenous avidin/biotin (Vector: Burlingame, CA) and stained with dendritic cell antigen Cd11c (Pharmingen, San Diego, CA) and biotin mouse anti-hamster (Pharmingen, San Diego, CA). Normal mouse spleen sections were utilized as a positive

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control. Cells were photographed with an Olympus DP71 camera mounted on an Olympus BX60 microscope.

2.6. Cell viability

Microglial mitochondrial respiratory activity, a measure of cell viability, was determined using a colorimetric MTT (methylthiazolyldiphenyl-tetrazolium bromide) assay (Bioassay Systems, Hayward, CA). This was compared to a reference value of HEK-293 cells, a highly viable immortal cell line, and dying cultures treated with 1% Triton X-100, a toxic reagent.

2.7. Microglial stimulation

In order to compare the inflammatory reaction of microglia in young and aged brains, cells were isolated from 2 and 14 months old mice, as described above, and seeded in 96-well tissue culture plates (Corning Incorporated, Corning, NY) at a density of 3×10^5 cells/well. The cultures were incubated overnight at 37°C with 5% CO_2 and saturated humidity. The next day, cells were stimulated by replacing the original culture media with media containing 2% FBS and immunostimulatory agents in different concentrations. Two highly potent immune stimuli were selected, i.e. lipopolysaccharide (LPS, (*Escherichia coli* 055:B5) (Sigma, St. Louis, MO), a toll-like receptor 4 (TLR4) agonist and PamCSK3 (Invitrogen, Carlsbard, CA), a TLR2 agonist. LPS and PamCSK3 were added at a concentration of 10–100ng/mL (LPS) or 0.1– 1μg/mL (PamCSK3). Control conditions were included, containing no stimuli. After 24hrs of incubation, the media of stimulated microglia were collected and centrifuged for 10min at 20°C and 1200rpm. The supernatants were used for IL-6 and TNF-α enzyme-linked immunosorbent assays (ELISA). For every condition, cytokine levels were calculated in three different wells, while each experiment was performed in quadruplicate.

2.8. IL-6 ELISA

Mouse IL-6 secreted protein levels were determined with a general sandwich ELISA protocol. Briefly, an enhanced protein binding ELISA plate (Nunc, Rochester, NY) was incubated overnight at 4°C with the capture antibody, rat anti-mouse IL-6 (BD Bioscience Erembodegem, Belgium). After blocking the non-specific binding for 2hrs, standards (BD Bioscience Erembodegem, Belgium) and samples were added for 2hrs at room temperature. Subsequently, biotinylated rat anti-mouse IL-6 (BD Bioscience Erembodegem, Belgium) was used as a detection antibody. Following incubation with a Streptavidin-Horseradish Peroxidase conjugate (Dako Cytomation, Heverlee, Belgium), a TMB substrate (BD Bioscience Erembodegem, Belgium) was applied to the plate. Finally, optical densities (OD) were read between 450–570nm, using a spectrophotometer (Powerwave X Select) and concentrations were calculated. The detection limit of the assay was 10pg/mL.

2.9. TNF-α ELISA

Mouse TNF-α secreted protein levels were measured using a commercially available ELISA kit (eBioscience, San Diego, CA), according to the manufacturer's instructions. Concentrations were determined according to the OD values, measured using a

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spectrophotometer (Powerwave X Select) at a wavelength between 450–570nm. The detection limit of the assay was 8pg/mL.

2.10. Glutathione measurements

Total glutathione (reduced and oxidized) was measured in microglia using a glutathione reductase enzymatic recycling assay (Cayman Chemical, Ann Arbor, MI) that is based on the colorimetric conversion of nitrobenzoic acid to 5-thio-2-nitrobenzoic acid (Tietze, 1969). Briefly, microglia from the brains of young or aged mice were lysed immediately following isolation and prepared for glutathione measurements according to manufacturer's instructions. Glutathione levels within the range of standards were attained by combining microglia from four brains. Therefore to attain three repetitions, 12 mice per age group were assayed. All samples were normalized to total protein using bicinchonic acid (BCA) colorimetric assay (Pierce, Rockford, IL).

2.11. Aβ42 fate analysis

Aβ42 lyophilized protein with and without a FITC conjugate (rPeptide, Bogard, GA) was resuspended to 1mg/mL in 1% NH_4OH and stored at −20°C according to manufacturer's directions. To visualize internalized amyloid beta in living cells, Aβ42-FITC was diluted to 4μg/mL in DMEM and added to microglia for 3hrs. The cells were then stained with DAPI nuclear counterstain (1:1000) for 5min. and imaged. For enhanced subcellular resolution of internalized amyloid beta, cells (exposed to non-conjugated Aβ42) were fixed as described above for immunocytochemistry using antibodies against amyloid beta (6E10, 1:2000, Signet Laboratories, Dedham, MA) and the lysosome associated protein, Lamp 1 (1:2, gift from Dr. Notterpek, University of Florida). To determine the aggregation state of Aβ42 in stock solutions used in internalization and fate experiments, samples were diluted in Laemmli sample buffer containing 2% sodium dodecyl sulfate (SDS) and loaded in 4–20% TG-SDS gels (Invitrogen, Carlsbad, CA) for standard SDS-PAGE. Immunoblots were probed with 6E10 at a dilution of 1:5000. 6E10 has affinity to individual Aβ42 peptides and therefore monomers, oligomers and higher order multimers of Aβ42 are distinguishable by size difference. Gel blots were photographed using a Fugi imaging system (Fugifilm Life Science, Stamford, CT).

RS chambers (Nunc, Roskilde, Denmark) that contain a hybrid of glass and polystyrene surfaces have reduced non-specific interaction with Aβ and were therefore chosen for fate analysis experimentation. To further reduce non-specific Aβ42 absorption, these chambers were blocked with 10% milk for 1hr. Microglial cells were then isolated from 1 month old mice, 15 months old mice and mixed glial cultures (MGC) were seeded on RS chambers at approximately 3×10^5 cells/chamber. The cultures were incubated overnight at 37°C with 5% CO_2 and saturated humidity. The next day, cultures were rinsed and exposed to Aβ42 diluted in DMEM with 10% FBS at 4μg/mL. The cells were allowed to internalize Aβ42 from the media for 3hrs in 37°C (1.5ml/well). They were rinsed and incubated at 37°C with 1.5mLs of culture media lacking Aβ42. This media and that of cells lysed immediately following rinsing were collected, as were the conditioned media and lysate from wells incubated for 3hrs and 16hrs. The lysis buffer (1.5ml/well) consisted of NP40 (Invitrogen, Carlsbard, CA) supplemented with protease inhibitor cocktail (1×, Sigma, St. Louis, MO)

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and PMSF (1mM). For each age group, lysate and media representing 5–7 adult mice or 14 neonatal pups (2 MGCs) were collected. To determine the fate of Aβ42, we employed a sandwich-style ELISA (Invitrogen, Carlsbard, CA), configured with two capture antibodies (recognizing epitopes on the N terminus and C terminus of human Aβ42) to first capture the N terminus of Aβ42 and then the C terminus (Schmidt, et al., 2005). Microglial degradative activity on Aβ42 causes N terminal truncations (Majumdar, et al., 2007), thus ELISA reactivity is limited to non-degraded Aβ42. We were therefore able to 1) follow the loss of Aβ42 and 2) the expulsion of Aβ42 from microglia. The ELISA was processed with duplicates of each sample and absorbance read at 450nm using a spectrophotometer (Bio-Tek, Winooski, VT). The detection limit of the assay was 10pg/mL.

2.12. Statistical analysis

Average cytokine data are presented as mean ± SEM. Statistical analysis was carried out using SPSS ver. 14.0 for Windows (SPSS, Chicago, IL). To analyze differences between groups, we used unpaired, two-tailed Student's t-test or ANOVA with a post hoc Bonferroni's test when appropriate. A p-value of <0.05 was considered statistically significant. Average Aβ data are presented as mean ± SEM. For statistical comparison of Aβ internalization between age groups, we used unpaired, two-tailed Student's t-test. For statistical comparison of Aβ fate, paired, two-tailed Student's t-test was used to compare Aβ levels immediately following internalization and 16hrs later. Data was normalized to mock-treated wells that were treated as described above but contained no cells. For comparison to mock-treated wells, raw data was used. A p-value of <0.05 was considered statistically significant.

3. Results

As reported previously (de Haas, et al., 2007,Frank, et al., 2006), centrifugation of dissociated whole brain within discontinuous percoll gradients can separate microglia from other brain cells. In our hands, the techniques described in the literature yielded insufficiently pure cultures for the pulse-chase experiments we performed (Fig 1B–C). Specifically, we observed debris fields which limited the adherence of microglia and may contain components that possibly sequester amyloid beta peptide (Li, et al., 2005). To address this, we utilized dispase II, an enzyme that has been described as particularly gentle, yet capable of tissue dissociation (Borchelt, et al., 1992,Gao, et al., 2004,McDermott, et al., 2003). Furthermore, we increased the density of percoll by 16% from that described in the literature (de Haas, et al., 2007). We observed that brains treated with this methodology, i) had greater separation of dissociated microglia from tissue chunks (Fig 1A–B), ii) yielded numerous adherent microglia, and iii) were largely devoid of debris (Fig 1C–D).

Altogether, the combination of these techniques resulted in the extraction of up to 3×10^6 microglia per brain. On average, 8.5×10^5 microglia per brain were extracted from young and aged mice (Fig 2B). 94% of DAPI counterstained cells were reactive to Iba1 (Fig 2A) as determined by 3 observers. The cells had a characteristic amoeboid, phase bright morphology similar to previous reports of adult microglia (Tham, et al., 2003). To further confirm that our isolated cells were indeed microglia, we isolated cells from transgenic mice

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where GFP expression is under the fractalkine receptor promoter. These mice are reported to have microglia as the only brain cell type to express GFP (Jung, et al., 2000). Upon isolation of cells, our cultures were reactive to antibodies specific to GFP (Supp Fig S3A). *Ex vivo* cultures of adult microglia that were allowed to adhere overnight were comparable to the HEK 293 cell line in viability (Fig 2C). Recent studies have raised the possibility that GM-CSF could push cultured microglia towards a dendritic cell fate (Esen and Kielian, 2007). In our cultures, microglia grown in 0.15μg/ml GM-CSF or in GM-CSF free conditions both maintained a rounded morphology (Supp Fig S4A) and had no immunoreactivity to the dendritic cell antigen, Cd11c (Supp Fig S4B, C). Interestingly, neonatal microglia derived from mixed glial cultures lacking GM-CSF exhibited a ramified phenotype when cultured overnight in GM-CSF containing media (Supp Fig S2). This morphology is similar to that observed in mixed glial cultures with prolonged exposure to GM-CSF (Esen and Kielian, 2007). It is possible that the conditions within mixed glial cultures prime microglia to adopt a dendritic-like morphological phenotype upon exposure to GM-CSF.

Previously, histological findings of dystrophic microglia in the aged and diseased brain have led our laboratory to suggest that microglial function may deteriorate with normal aging. Therefore, we sought to study elements of pathology that are mainly conferred by microglia *in vivo* and are known to change with aging and disease (Bolmont, et al., 2008,El Khoury, et al., 2007,Meyer-Luehmann, et al., 2008,Streit, et al., 2004,Ye and Johnson, 1999). Recent studies have shown that mRNA copies of inflammatory cytokines are increased in microglia from aged brains (Sierra, et al., 2007,Ye and Johnson, 1999). However, mRNA transcripts may not necessarily translate to secreted protein levels (Munger, et al., 1995,Storm van's Gravesande, et al., 2002), a more ultimate measure of functional change. To determine if microglia vary their secretion of cytokines with age, we obtained microglia from young and aged mice and measured their cytokine levels with and without exogenous immune stimulation.

The most striking observation in this respect was the dramatic increase in IL-6 release under basal conditions (young: 211.8±31.7 pg/ml vs. aged: 3735.9±1000.2 pg/ml, $p<0.001$). In both young and aged microglia, a significant dose-effect relation following either LPS or PAMCSK3 stimulation was observed (Supp Fig S5A). Moreover, maximal release of IL-6 was significantly enhanced in aged microglial cells following LPS (100ng/ml) or PAMCSK3 (1μg/ml) stimulation (Fig 3A).

As with IL-6, the amount of TNF-α produced by aged microglial cells was significantly higher under basal conditions when compared to young microglia. While microglia derived from young mice produced no TNF-α under basal conditions, the amount was significantly increased to 917.2 ± 91.9 pg/ml in supernatants of aged microglia cultured for 24h without any exogenous stimuli ($p<0.001$). This striking difference confirms age-related higher basal levels of cytokine production previously observed with mRNA transcript analysis (Sierra, et al., 2007) and shows that aged microglia produce more TNF-α than microglia from young mice. This high release under basal conditions may explain the lack of a significant dose-effect relation in TNF-α production following either LPS or PAMCSK3 stimulation (Supp. Fig S5B). In contrast, in young microglia a significant dose-effect relation was observed. Moreover, although the maximal amount of TNF-α released by aged microglia in response

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to 1μg/ml PAMCSK3 was slightly though significantly increased, responses to 100ng/ml LPS were not different between aged or young microglia (Fig 3B).

In addition to quantifying microglial cytokine production as a function of age, we were interested in whether the ability of microglia to serve as an oxidative sink and to internalize amyloid beta changes with age. Glutathione acts as antioxidant by neutralizing free radicals and peroxides and microglia are reported to be the primary glutathione containing cells in the brain (Hirrlinger, et al., 2000,Lindenau, et al., 1998). We found a trend indicating that microglia in aged brains have 21% less total glutathione (oxidized and reduced) compared to microglia from young brains (Supp Fig S6). This result suggests the reactive oxygen species (ROS) insult that can be caused by amyloid beta internalization (Milton, et al., 2008) maybe more injurious to microglia in aged brains.

Amyloid beta accumulation is a well recognized feature of AD, however, extensive amyloid deposits may be found in many aged, non-demented individuals (Bouras, et al., 1994). This pathology may result from AD-independent deterioration of clearance processes. Microglial scavenger activity on amyloid beta is proposed as a clearance process that contributes in maintaining amyloid beta at physiological levels by counterbalancing constitutive amyloid beta secretion by neurons. Our laboratory and others have published accounts of microglial degeneration associated with aging (Flanary, et al., 2007,Simmons, et al., 2007,Streit, et al., 2008,Streit, et al., 2004). If microglia represent a major amyloid beta clearance mechanism, their degeneration would result in progressively increasing amyloid beta levels with age and therefore would have significant implications to the occurrence of amyloidosis in AD and some aged individuals. We currently lack the means to isolate degenerating microglia for experimentation. However, *ex vivo* assessment of microglia isolated acutely from young and aged mice likely emulates *in vivo* processing of amyloid beta more so than neonatal or ‘microglial-like’ cell lines and may give insight to the degeneration of microglia with age. *Ex vivo* cultures of microglia were given media with 4μg/mL of Aβ42-FITC conjugate or non-conjugated Aβ42. Western blot analysis indicated that non-conjugated Aβ42 preparations contained Aβ42 monomers, oligomers and SDS resistant species larger than 220kDa that are likely fibrils thus reflecting *in vivo* amyloid burden (Fig 4A). Internalization of Aβ42 by living microglia was confirmed visually with cell-associated FITC fluorescence (Fig 4B). Aβ42 observed in fixed cells was colocalized with lysosomes (Fig 4C).

To quantify internalization, microglial lysates were measured using an Aβ42 sensitive ELISA. Our results indicate that microglia from aged (15 month old) mice internalize 0.279ng/ml of Aβ42 (Fig 5A). In similar experiments recently performed by Zaghi et al., monocytes from aged humans (mean age:77years) are reported to internalize 0.256 ng/ml of Aβ42, a value within 8% of our findings in aged mouse microglia (Zaghi, et al., 2009). Because circulating monocytes are under certain conditions able to infiltrate the parenchyma, differentiate into microglia (Mildner, et al., 2007,Simard and Rivest, 2004) and modify amyloidosis (Simard, et al., 2006), the similarity of our measurements to human monocyte measurements indicates a strong possibility that our experiments model human physiology.

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To determine whether Aβ42 processing by microglia from younger mice is reflective of microglia from aged mice, we next performed experiments with microglia from neonates (< 8 days old, mixed glial culture derived) and young (1 month old) mice (Supp Fig. S1). We found that young microglia internalized 53% more Aβ42 relative to aged microglia, while neonatal microglia internalized 45% more Aβ42 than young microglia (Fig 5A). Neonatal microglia internalized 74% more Aβ42 than aged microglia. This suggests that the neonatal microglia commonly used for research on Alzheimer's disease and aging, may not necessarily model adult microglial Aβ42 clearance activity.

Internalization of Aβ42 is a prerequisite step for intracellular clearance; however it is by no means a surrogate marker for biophysical Aβ42 degradation. To more comprehensively determine the fate of phagocytosed Aβ42, we bathed cells in fresh media and measured the levels of Aβ42 in this media and within cells over 16hrs. We observed that internalized Aβ42 is invariably expelled by younger microglial cells within 3hrs (Fig 5B). The amount of Aβ42 that was expelled by neonatal microglia stayed nearly constant after 3hrs and averages to 18% less than what was originally internalized. This result may mean neonatal microglia were able to degrade some internalized Aβ42. Young microglia appeared to continuously expel Aβ42 (41% increase between 3hrs and 16hrs). At 16hrs, young microglia expelled 27% more Aβ42 than neonatal microglia. We did not see significant evidence of Aβ42 expulsion by aged microglia though these microglia have reduced amounts of internalized Aβ42 over time. The amount of Aβ42 internalized by aged microglia is not significantly different from mock experiments. Therefore, we were unable to assess whether these cells have degradation capacities.

Interestingly, the amount of Aβ42 expelled by young microglia at 3hrs and 16hrs was 19% and 53% more than was initially internalized. In mock measurements performed alongside neonatal and young microglial experiments, we found that Aβ42 capture is enhanced by 31% in the media compared to lysis buffer. Lysates are composed of a complex mixture that includes chemicals for lysis, protease inhibitors, cell-derived proteins as well as lipids. These are not found in the media. Because a more limited set of soluble proteins compete for ELISA antibody binding in the media, one would expect enhanced capture of Aβ42 in this environment. Consequently, our assay does not yield stoichiometric data needed for affirmative determination of Aβ42 degradation. However our assay clearly detects significant increases in conditioned media Aβ42 content suggesting substantial movement of Aβ42 to the outside of cells following initial internalization. This effect is age-related as it is only observed in younger microglial cells.

4. Discussion

In this report, we aimed to reduce debris contamination which is a feature of microglial cultures derived from gradient centrifugation based methodology. The brains of mice that are designated by the National Institute on Aging as an aging model were treated successively to steps that significantly increased the purity of *ex vivo* microglial cultures. Analysis of such cultures, derived from mice of various aging categories, revealed that microglia from aged brains have markedly increased basal levels of IL-6 and TNF-α secretion, have reduced glutathione levels and have a limited capacity to ingest Aβ42. In

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contrast, microglia from younger mice are able to temporarily contain Aβ42. Together, these *ex vivo* findings provide evidence that microglia are subject to age-associated changes in biology.

Prior reports from our laboratory have shown that microglia have IL-6 and TNF-α mRNA (Streit, et al., 2000) and more recently, others have found age-related changes in microglial IL-6 and TNF-α mRNA (Sierra, et al., 2007). We extend on these results by measuring secreted IL-6 and TNF-α proteins. Our results indicate significantly more pronounced changes in basal cytokine production and responsiveness. It is difficult to make direct comparisons of mRNA and secreted protein measurements. However, it is of note that the margin of change we observe in the basal production of IL-6 between microglia from young and aged mice is approximately 4-fold higher than that observed by mRNA analysis (Δ17.6× vs. Δ5× respectively). We also did not observe detectable levels of basal TNF-α by microglia from young animals in our studies. These differences can perhaps be explained by varying sensitivities of the employed detection methodologies or by post-transcriptional effects. The half-life of mRNA can often be rate-limiting in translation (Ross, 1995). Secondarily, secretory pathway modulation of newly produced cytokines may also modulate the concentration of cytokines in the extracellular milieu independent of DNA transcription. As microRNAs are involved in regulation of gene expression at the post-transcriptional level, possible changes in this machinery can also be mentioned to explain the discrepancy between protein and mRNA levels. Our results, though in agreement with previous reports, indicate that microglia from brains of various aging groups have much greater differences in cytokine production, and responsiveness to immune stimulation than was previously thought.

What are the possible implications of age-related changes in microglial cytokine production? A number of authors commonly describe IL-6 and TNF-α as neurotoxic molecules involved in AD pathogenesis (Bruunsgaard, et al., 1999,Collins, et al., 2000,Culpan, et al., 2003,He, et al., 2002,Li, et al., 2007,Licastro, et al., 2000,McGeer and McGeer, 2001). However, experiments presenting alternative viewpoints have been published (Brunello, et al., 2000,Loddick, et al., 1998,Marz, et al., 1998,Streit, et al., 2000,Tarkowski, et al., 1999,Thier, et al., 1999,Wei, et al., 1992). IL-6 may have a role in regeneration of injured tissue in the brain (Loddick, et al., 1998,Streit, et al., 2000,Tarkowski, et al., 1999), has known anti-apoptotic properties (Wei, et al., 2001) and in mice that overexpress both IL-6 and its receptor, IL-6Rα, there is no evidence of neurotoxicity (Brunello, et al., 2000). Inflammation is a component of the healing process of acute CNS lesions (Klein, et al., 1997,Streit, et al., 2000). Recently, it has been reported that the more chronic lesions of tauopathy and amyloidosis can be found in 20–40% of non-diseased, aged adults at levels comparable to AD patients (Price, et al., 2009). Both TNF-α and IL-6 increase with aging (Bruunsgaard, et al., 1999,Wei, et al., 1992). Thus, the aging brain appears to exist in a constant state of injury. Inflammatory processes, such as microglial secretion of IL-6, maybe needed for persistent regeneration or neuroprotection. The viewpoints that cytokine release is exclusively neurotoxic or neuroprotective could be equally considered speculative. Further studies which consider region-specific and age-specific differences in cytokine

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response are needed, amongst others, to shed light onto the role of cytokines released by microglia on the nervous system.

Microglial surveillance of the parenchyma involves scavenging of potentially hazardous materials. Proteins such as LPS and Aβ induce ROS and it is thought that the increased levels of antioxidant molecules such as glutathione found in microglia relative to other brain cells (Hirrlinger, et al., 2000,Lindenau, et al., 1998) protect microglia from ROS burden associated with scavenging activity (Dringen, 2005,Milton, et al., 2008,Qin, et al., 2004,Tchaikovskaya, et al., 2005). Our findings of reduced glutathione levels in microglia immediately analyzed after brain extraction may be indicative of broader microglial loss of function with age.

Our overall conclusion of age-related changes in microglial processing of Aβ is indirectly supported by published work demonstrating decreased protein turnover in adult rat microglia (Stolzing, et al., 2006). More recently, experiments by Zahgi et al. show monocytes from aged humans internalized Aβ42 at amounts comparable to what we observe in aged mouse microglia (Zaghi, et al., 2009). Similar to our findings, the Aβ internalized by monocytes was not detected in cell lysates within hours. In other work, media was measured for radio labeled soluble Aβ previously internalized by neonatal microglia. These investigators concluded that most Aβ is expelled in an interval of time similar to our findings (Chung, et al., 1999). Together, these data provide independent support for the amount of Aβ we measure in lysates, the kinetics of Aβ movement, and our conclusion that the principal consequence of Aβ internalization by younger microglia is expulsion.

Microglia are thought to participate in plaque burden regulation by sequestering and processing of Aβ. Our purpose in performing Aβ metabolism experiments with *ex vivo* cultures of microglia was to further understand the relationship between microglia and Aβ42 homeostasis. Individual microglia surrounding Aβ plaques become enlarged as plaques become smaller with time (Bolmont, et al., 2008). Despite this correlative evidence of microglial participation in Aβ plaque reduction, natural processes do not appear to reverse amyloidosis (Jankowsky, et al., 2005). Previously, we have suggested that microglial function may deteriorate with time. This was mainly inspired by histological findings, demonstrating the appearance of a dystrophic microglia phenotype with normal aging and around neuritic plaques in AD brains (Streit, et al., 2009). The finding that microglia from young brains internalize more Aβ42 than microglia from aged brains likely reflects a more global change in microglial functionality with aging.

In younger mice, the temporary sequestration of Aβ42 suggests that microglia are not directly involved in significant amounts of degradation. However there is clear potential for microglia, which are highly motile (Bolmont, et al., 2008), to be involved in the movement of Aβ in the nervous system. The cycling of Aβ42 through microglial endocytosis and exocytosis could result in redistribution that modulates peptide availability for amyloid formation. Our observations indicate that microglia remove Aβ peptide out of the extracellular milieu at ng/ml quantities, possibly creating a dynamic cellular compartment *in vivo*. A 2- to 4-fold age-related decrease in microglial Aβ internalization as observed in our experiments could result in less transfer of Aβ from the extracellular space, thereby making

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available more Aβ for plaque formation. It is plausible that the dynamic redistribution of Aβ, a protein that is particularly aggregation prone, is needed for homeostatic maintenance. Perturbation of this process due to age-related changes of microglial function such as that described here could contribute to unhinged accumulation of Aβ to toxic concentrations in the extracellular space. In 3 month old mice where *in vivo* microglia were selectively killed, soluble Aβ40 and Aβ42 levels increase by 3–4 fold (Grathwohl, et al., 2009). This suggests that the presence of microglia is needed to prevent the accumulation of soluble pools of Aβ in young mice. Such pools contain the building blocks of Aβ pathology according to the amyloid cascade theory (Tanzi and Bertram, 2005). Interestingly, amyloidosis has recently been described to occur in up to 40% of non-demented, aged adults (Price, et al., 2009). It is possible that changes in microglial processing of Aβ may contribute to occurrences of amyloidosis in the aged population. Suggestive evidence for this comes from studies of monocytes. In these studies, monocytes from Alzheimer's patients internalize half as much Aβ42 as those from aged adults (Zaghi, et al., 2009). Importantly, aged monocytes have a similar capacity to internalize Aβ42 as aged mouse microglia measured here. We infer from our data that monocytes and microglia from younger humans will have greater internalization of Aβ42. We propose that as microglia and possibly monocytes age, they lose important capacities to internalize and redistribute Aβ. A progressive lose of cellular function as described here may be an understudied mechanism that contributes to the development of amyloid pathology in aged, non-demented subjects and in patients with Alzheimer's disease.

Together, our *ex vivo* quantification of microglial functions of cytokine production, glutathione levels and Aβ42 scavenging activity paint a complex picture of endogenous activity. Microglia from young mice produce less cytokines, while microglia derived from aged mice have higher basal levels of cytokine secretion than was previously thought and have reduced glutathione levels. Microglia derived from aged mice have diminished Aβ42 internalization capacity relative to their younger counterparts. However, these younger microglia expel significant amounts of internalized Aβ42. These direct assessments of microglial function in *ex vivo* experiments, free of confounding contributions of other brain cells and debris, demonstrate a nuanced view of microglial function and suggest that microglial biology may change with aging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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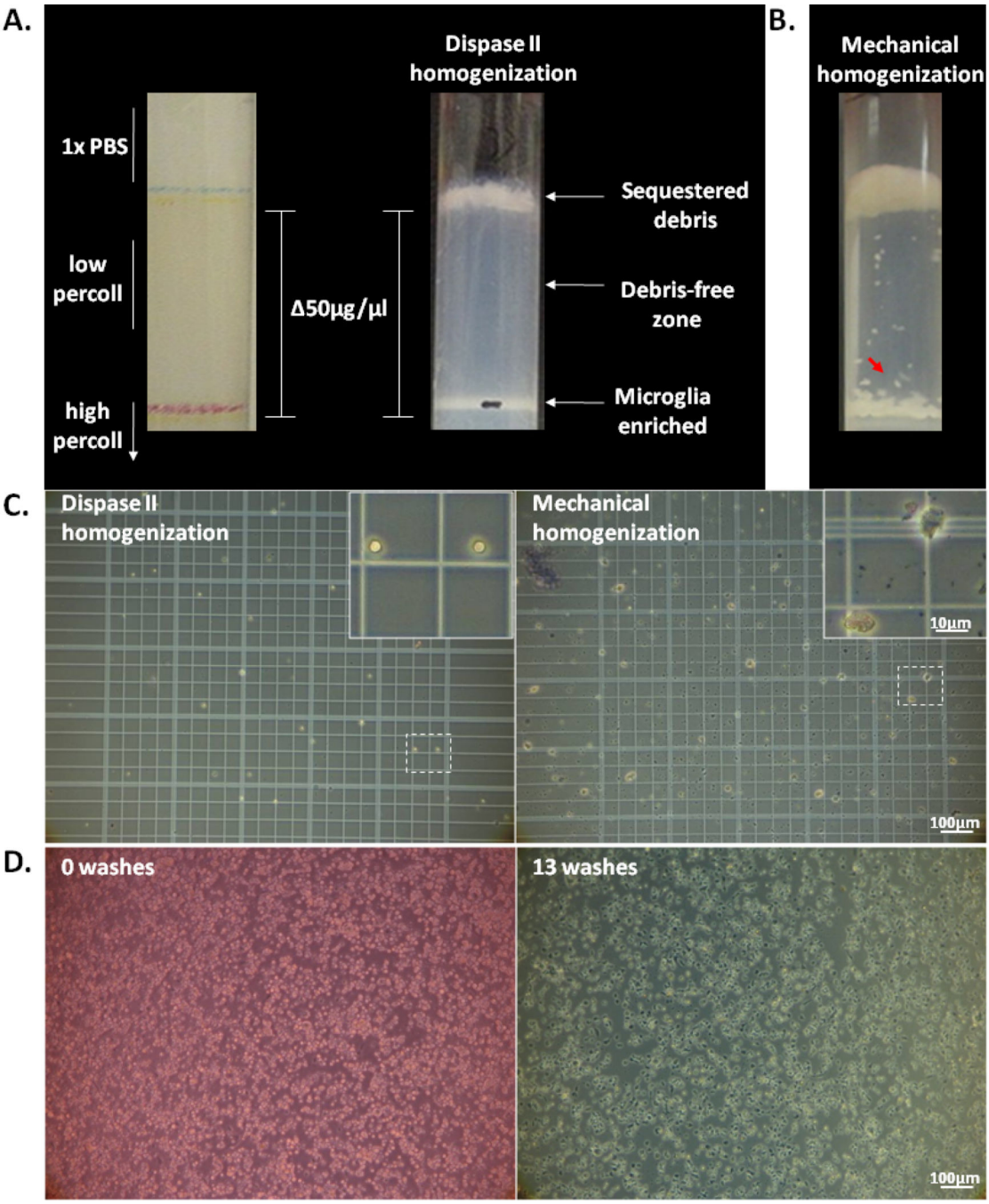

Fig. 1.

Dispase II density centrifugation methodology: (A) Brain hemispheres that were homogenized with dispase II and loaded onto discontinuous gradients composed of 35% 'low' percoll and 75% 'high' percoll sequestered microglia to an isopynic density of 1.077mg/µl as determined by beads with known densities. This configuration spatially separated unwanted brain matter to a density 50µg/µl more buoyant. (B) Density centrifugation methodology as described in the literature involved mechanical homogenization and reduced percoll densities. In our hands, such methodology failed to

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channel unwanted brain matter (tissue chunks, red arrowhead) to an isopynic position distal to the microglia enriched band. (C) Phase contrast images of freshly prepared cells under a hemacytometer demonstrate reduced particulate matter from dispase II homogenized brains and viable phase-bright cells that exclude trypan blue. Following 24hrs of culture, these cells remain adherent after multiple washes (D) and are thus compatible with experiments that involve media exchanges.

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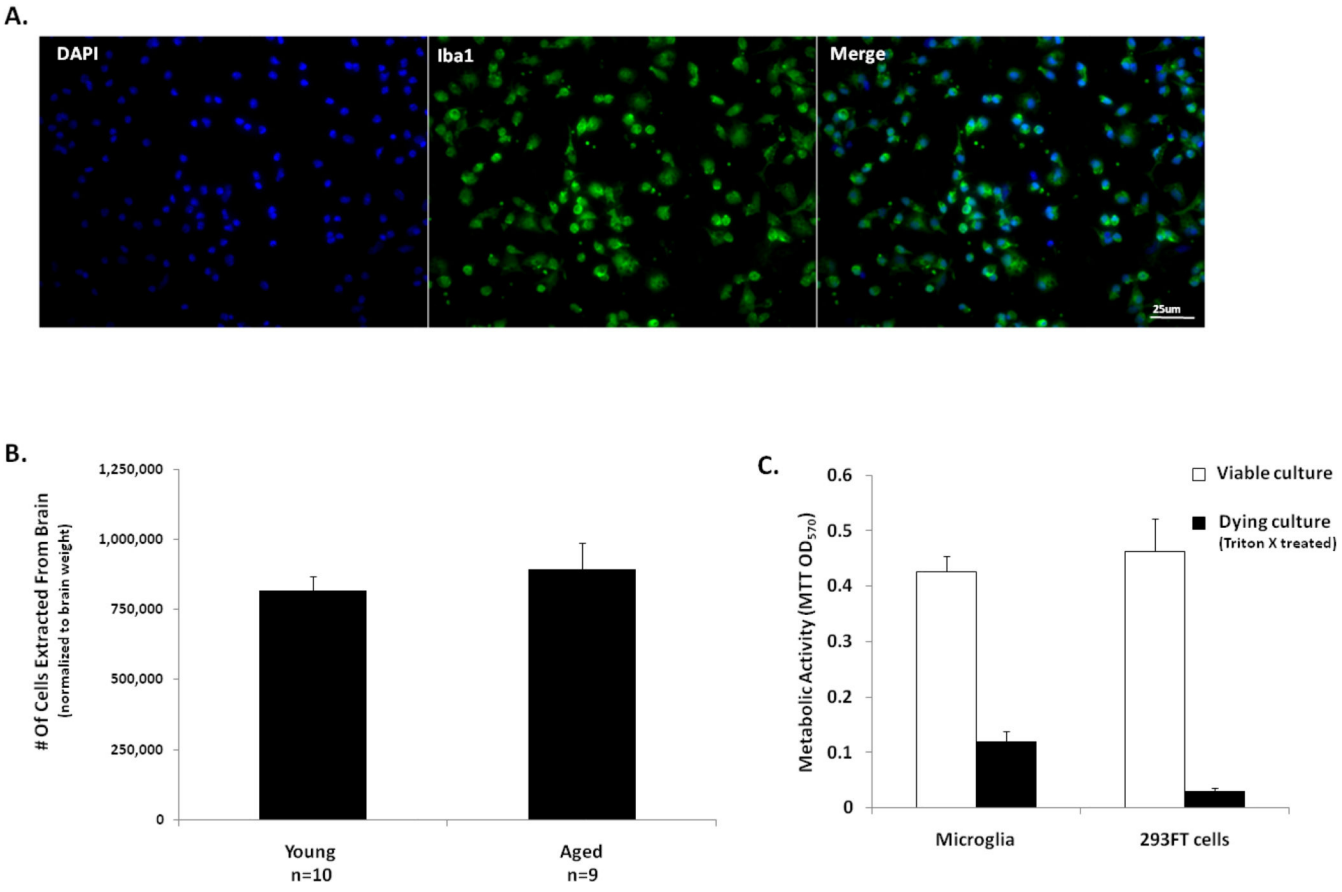

Fig. 2.

Purity, yield and viability of microglia: (A) Microglia isolated with dispase II density centrifugation methodology express Iba1, a marker commonly used to identify microglia *in vivo*. (B) Yields of microglia from 1 month old and 15 month old mice typically obtained using dispase II based density centrifugation methodology show little variability with age. (C) Measurement of mitochondrial respiratory activity indicated that isolated microglia form cultures comparable in viability to HEK 293 cells, an immortal cell line.

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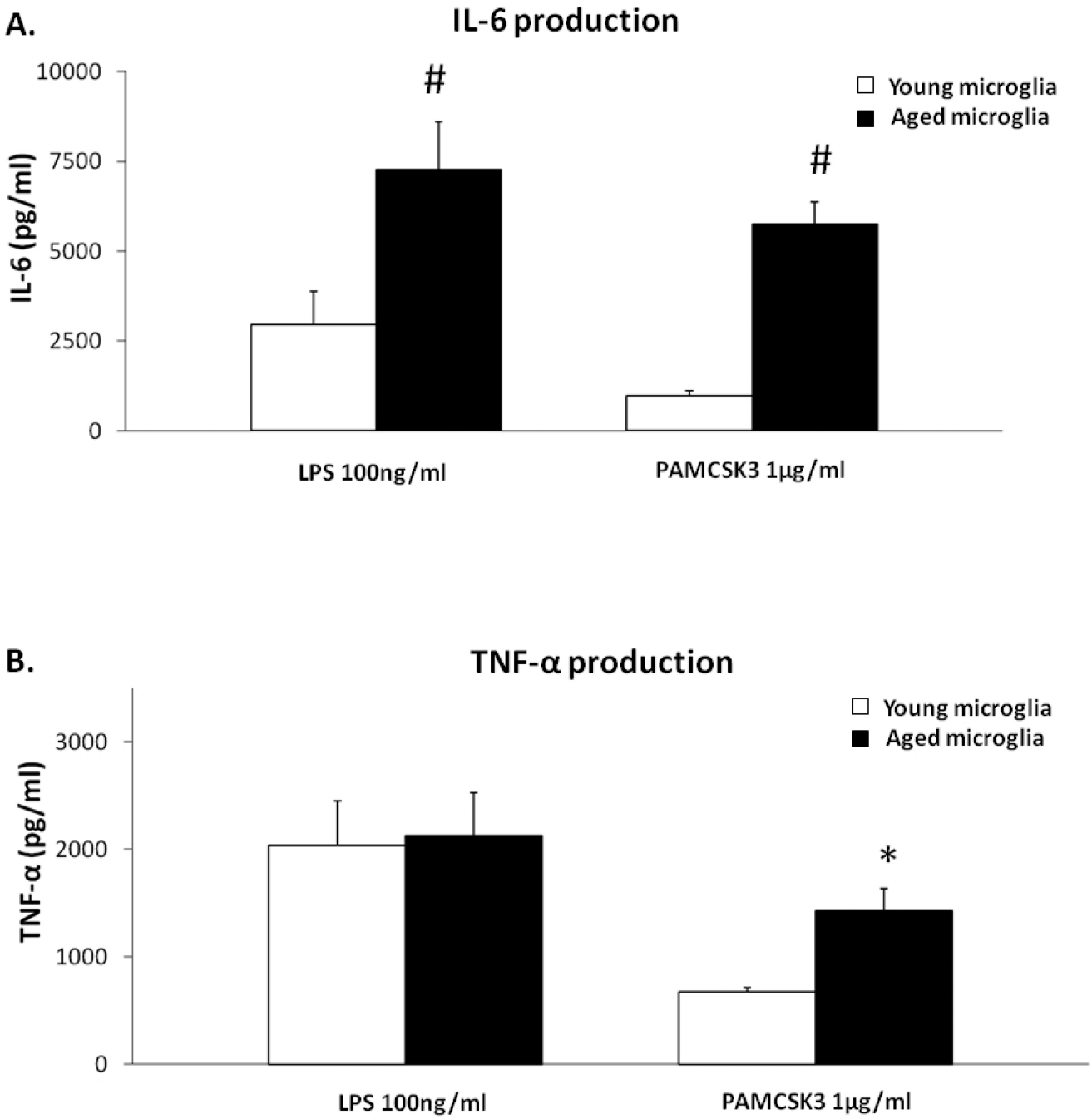

Fig. 3.

Cytokine secretion of young and aged microglia: (A) Upon stimulation with the biological immunostimulatory agent LPS (100ng/ml), or Pam3CSK4, a synthetic agonist of toll-like receptor 2 (1μg/ml), IL-6 production by aged microglia was markedly increased when compared to young microglia. (B) LPS (100ng/ml) stimulated similar TNF-α production in microglia derived from young and aged mice. Yet, TNF-α production was significantly increased in aged microglia following Pam3CSK4 (1μg/ml) exposure. *, $p<0.05$; #, $p<0.001$.

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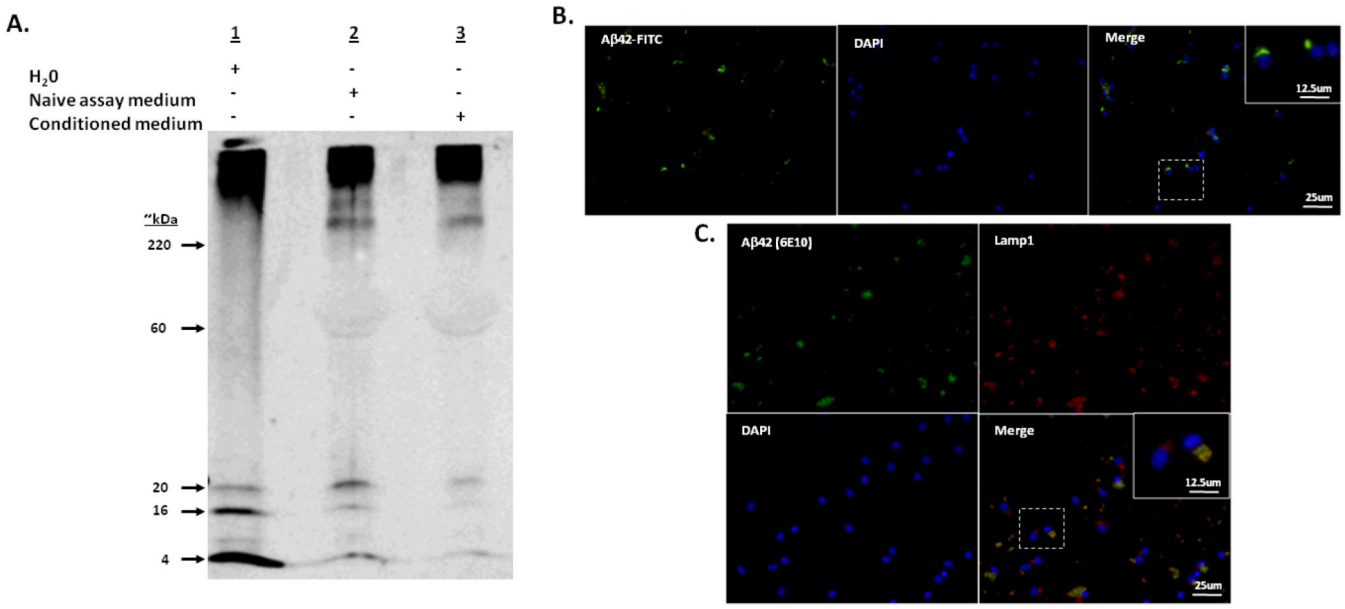

Fig. 4.

(A) Western blot analysis using 6E10, an antibody specific to the first 16 amino acids of Aβ42, indicates monomeric (4kDa), oligomeric (16kDa, 20kDa) and higher-order conformations larger than 220kDa in stock preparations as well as preparations that have been exposed to microglia. Higher-order conformations larger than 220kDa persist following the exposure of samples to buffer containing 2% SDS suggesting the presence of fibrillar species. 10% serum in media overloads gel at 60kDa and may block visibility of some Aβ42 species. 75ng Aβ42/well. (B) Internalization of Aβ42 by microglia from adult mice was directly observed in living microglia with FITC conjugated Aβ42 peptide and with 6E10 immunocytochemistry. In both cases, Aβ42 had a perinuclear localization. Lamp1 colocalization with 6E10 immunoreactivity suggests that some Aβ42 reached microglial lysosomal compartments (C).

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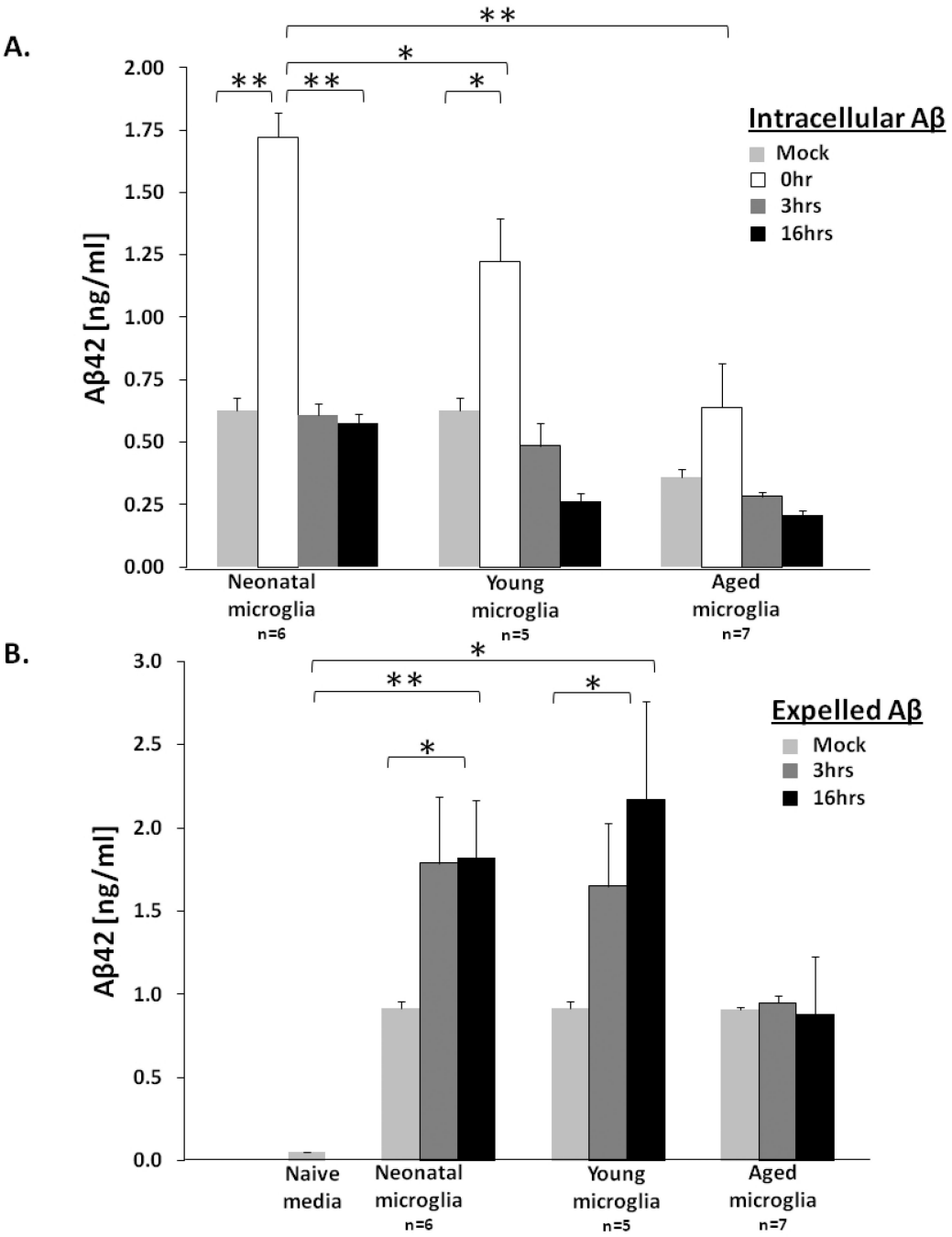

Fig. 5.

Fate of Aβ internalized by microglia: Microglia extracted from mice of various ages were exposed to Aβ42 preparations containing monomeric, oligomeric and SDS-resistant fibrillar species (reflecting *in vivo* amyloid diversity) in pulse-chase experiments. (A) Neonatal and young microglia respectively internalized 74% and 53% more Aβ42 relative to aged microglia. (B) Invariably, internalized Aβ42 was expelled by neonatal and young microglia within 3hrs of ingestion, suggesting disengagement from biophysical degradation following phagocytosis. Mock data (gray) represents experiments without the presence of cells to

control for non-specific adherence of Aβ to culture wells. Detection of Aβ42 requires the presence of both NH_2 and COOH terminals of Aβ42, thus only intact Aβ42 peptides are quantified in the above experiments. *, $p<0.05$; **, $p<0.01$.

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