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Raft aggregation with specific receptor recruitment is required for microglial phagocytosis of A β ₄₂

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Abstract

Microglial phagocytosis contributes to the maintenance of brain homeostasis. Mechanisms involved however, remain unclear. Using A β ₄₂ solely as a stimulant, we provide novel insight into regulation of microglial phagocytosis by rafts. We demonstrate the existence of an A β ₄₂ threshold level of 250pg/ml, above which microglial phagocytic function is impaired. Low levels of A β ₄₂ facilitate fluorescent bead uptake, whereas phagocytosis is inhibited when A β ₄₂ accumulates. We also show that region-specific raft clustering occurs prior to microglial phagocytosis. Low A β ₄₂ levels stimulated this type of raft aggregation, but high A β ₄₂ levels inhibited it. Additionally, treatment with high A β ₄₂ concentrations caused a redistribution of the raft structural protein flotillin1 from low to higher density fractions along a sucrose gradient. This suggests a loss of raft structural integrity.

Certain non-steroidal anti-inflammatory drugs, e.g. the COX-2-specific NSAID, celecoxib, raise A β ₄₂ levels. We demonstrated that prolonged celecoxib exposure can disrupt rafts in a manner similar to that seen in an elevated A β ₄₂ environment: abnormal raft aggregation and Flot1 distribution. This resulted in aberrant receptor recruitment to rafts and impaired receptor-mediated phagocytosis by microglial cells. Specifically, recruitment of the scavenger receptor CD36 to rafts during active phagocytosis was affected. Thus, we propose that maintaining raft integrity is crucial to determining microglial phagocytic outcomes and disease progression.

Keywords

microglia; phagocytosis; rafts; CD36

Introduction

Rafts are liquid ordered microdomains consisting of specific proteins, cholesterol and sphingolipids in a defined stoichiometry (Simons and Ehehalt 2002). In their native state, raft size can vary from 50–100 nm (Simons and Ikonen 1997), although recent reports have documented smaller raft sizes (5–50 nm) (Hancock 2006; Sharma et al. 2004). Raft structure is based on flotillin/reggie protein oligomers interspersed with lipids, like GM1 ganglioside (Langhorst et al. 2005). Flotillin1 (Flot1) is a raft structural protein and is considered to be a major indicator of raft structural integrity. Flot1, however, may also be involved in signaling processes and has been visualized in a number of different subcellular locations including the Golgi, endosomes and phagosomes (Babuke and Tikkanen 2007).

As discreet structural entities, rafts function as platforms for spatio-temporal orchestration of cellular signaling cascades including apoptosis, cytokine signaling, endocytic recycling, and phagocytosis (Fullekrug and Simons 2004; Simons and Ikonen 1997). These reports also show that in response to stimuli, rafts aggregate to form activated clusters through recruitment of other rafts and crosslinking with associated cell-surface molecules. It is this organizational capability that makes them vital to the regulation of cellular processes. Further, rafts have been implicated in the pathogenesis of many neurodegenerative diseases. For instance, processing of the amyloid precursor protein (APP) by β - and γ -secretases occurs within rafts (Ehehalt et al. 2003), with the resulting $A\beta_{42}$ being neurotoxic (Korotzer et al. 1993). Thus, examining the role of rafts may provide some mechanistic insight into disease pathogenesis.

While raft activation plays a critical role in peripheral macrophage phagocytosis (Kay et al. 2006), this has yet to be demonstrated in the brain macrophage, the microglia. Here we examine whether rafts play a role in the regulation of microglial phagocytosis. Phagocytosis entails the recognition of material of interest, followed by molecular and morphological changes that enable tethering, signaling, engulfment and internalization of material through formation of a phagocytic cup (Gregory and Devitt 2004). Recognition mechanisms are initiated through specific cell surface recognition receptors, depending on the material being cleared. For instance, pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), use pathogen-associated molecular patterns (PAMPS) for clearing pathogens (Kaisho and Akira 2006). Morphological changes include assumption of an amoeboid shape, extension of pseudopodia for phagocytic cup formation, and phagosome closure with regulated actin rearrangement (Swanson et al. 1999). A defect in these mechanisms results in impaired phagocytosis (Paresce et al. 1996).

Phagocytosis is important for maintaining healthy cellular environments. Removal of debris can contribute to the modulation of immune responses by promoting survival and growth of surrounding cells (Reddy et al. 2002). Furthermore, reduced or defective clearance of debris or unwanted proteins can be associated with the initiation of autoimmune diseases such as type 1 diabetes and systemic lupus erythematosus, as well as upregulation of inflammatory processes during traumatic brain injury and neurodegeneration (Akiyama et al. 2000; Koshinaga et al. 2000; Krysko et al. 2006; O'Brien et al. 2002). Thus, disruption of the phagocytic process can severely impact the development of human disease through persistent inflammation and subsequent tissue damage (Krysko et al. 2006).

In the brain, defective clearance has been implicated in the development of proteinopathies and neurodegeneration (Bard et al. 2000). For instance, increased deposition of specific proteins in the brain with age, e.g. amyloid- β_{42} ($A\beta_{42}$), may be a causative factor in both age-related neurodegeneration and Alzheimer's Disease (AD) (Monaco et al. 2006; Selkoe 2001). Such deposition has been linked to increased microglia activation in aging mouse brain and age-related neurodegeneration that manifests as increased production of interleukin (IL)-1 and IL-6, astrocyte activation, APP and apolipoprotein E (APOE) transcription, and $A\beta_{42}$ production. In addition, anti-inflammatory, protective factors such as transforming growth factor- β_1 (TGF- β_1) and insulin-like growth factor 1 (IGF-1) decline with age-related neurodegeneration (Griffin 2006; Griffin et al. 2006; Puglielli 2007; Sierra et al. 2007). Despite the increased activation, microglial phagocytic function, however, decreases with aging and in neurodegenerative diseases (Streit 2006).

Defective clearance of $A\beta_{42}$ in the brains of Alzheimer's disease (AD) patients has been associated with decreased microglial, and possibly infiltrating macrophage, function in clearing the excess protein (Fiala et al. 2005). A similar phenomenon exists in the inability of activated microglia to clear $A\beta_{42}$ deposits in brains of mouse models of AD (Wyss-Coray et al. 2001). Areas of $A\beta_{42}$ deposition have also been linked to an increased activation of

microglia, inflammation, and altered enzyme activities including cyclooxygenase 2 (COX-2) (O'Banion et al. 1997; Rogers et al. 2002). Given that peripheral macrophages and microglia share a monocytic lineage, as well as phenotypic and functional characteristics (Chan et al. 2007), we propose that phagocytic activity in microglia will employ raft activation similar to that seen in peripheral macrophages.

There are numerous reports of the involvement of cell-surface receptors in the clearance of A β ₄₂ (Chung et al. 1999; Mitrasinovic and Murphy 2002; Paresce et al. 1996; Tahara et al. 2006; Wang et al. 2006b), including special types of receptors called scavenger receptors (SRs), such as SRA and SRB family members (Paresce et al. 1996), which may be present within rafts (Thorne et al. 2006; Triantafilou et al. 2006). However, a current model suggests an unconventional mechanism for microglial phagocytosis of fibrillar A β ₄₂ that uses a complex of CD36, a member of the SRB family, $\alpha_6\beta_1$ integrin complex at the cell surface, and the integrin-associated adhesion receptor, CD47. This mechanism does not involve the traditional FcR γ I or FcR γ III (type I phagocytosis) or complement receptors (type II phagocytosis) (Bamberger et al. 2003; Koenigsnecht and Landreth 2004). CD36 is a raft-resident, cell surface glycoprotein with expression limited to specific cellular subtypes including monocytes and macrophages (Thorne et al. 2000). CD36 is expressed in microglia, increases in AD (Coraci et al. 2002; Ricciarelli et al. 2004), and has been linked to microglial activation, migration and phagocytosis (Bottcher et al. 2006; El Khoury et al. 2003; Eto et al. 2003; Moore et al. 2002; Ren et al. 1995; Stuart et al. 2007). Thus, CD36 serves as a good candidate for investigating raft recruitment of receptors during phagocytosis.

The presence of an increased inflammatory component in aging and AD, in addition to the fact that rheumatoid arthritis patients generally did not develop AD, spurred the use of non steroidal anti-inflammatory drugs (NSAIDs) in clinical trials. These trials, however, have not yielded much success for reasons that may relate to type of NSAID used. Since recent studies reported that some COX-2-selective NSAIDs increased levels of A β ₄₂ *in vivo* and *in vitro*, shifting the A β ₄₂/A β ₄₀ ratio in the direction that facilitates deposition; we used celecoxib as a pharmacological inducer of A β ₄₂ (Kukar et al. 2005; Leuchtenberger et al. 2006) and compared the effects to direct treatment with A β ₄₂ on microglial phagocytosis, raft aggregation and receptor recruitment. We demonstrate that microglial raft clustering directly reflects phagocytic function, and that raft recruitment of specific receptors, such as CD36, is necessary for microglial phagocytosis.

Materials and Methods

Chemicals

A β ₄₂ (American Peptide, Sunnyvale Ca) was resuspended in sterile water at 0.5 mg/ml and fibrillized by incubation at 37°C for 5 days. Fresh dilutions of 100 pg/ml, 250 pg/ml, 500 pg/ml and 500 ng/ml were made in media [DMEM/5% horse serum/5% FBS/1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). Celecoxib, γ -secretase inhibitor MDL 28170, zymosan and filipin were obtained from Sigma-Aldrich (St. Louis, MO). Celecoxib stock: at 0.1M/DMSO. 20 μ M working solution was made in media. Quality control and stability assays were performed by Research Triangle Institute (RTP, NC). For all treatment endpoints, untreated controls were compared to 0.02% DMSO vehicle controls and no significant differences were found (data not shown).

Microglia cultures

Primary mixed glia cultures were prepared from CD1 mice or Long Evans hooded rats (Charles River Laboratories, Raleigh, NC) as previously described (McCarthy and de Vellis 1980) with slight modification. Cortices were removed from 10 PND 2 pups, freed of meninges, and placed

in saline 1 buffer (140 mM NaCl, 5.36 mM KCl, 1.01 mM Na₂HPO₄, 8.6 mM KH₂PO₄, 22 mM glucose, 0.002g/L phenol red) on ice. Tissue was minced and digested (1% trypsin) prior to sequential filtration through 165 and 42 μ m nylon mesh. The filtrate was incubated at 37°C for 15 min in a T175 flask (BD Biosciences-Falcon, Franklin Lakes, NJ) then cultured in media, at $6-7 \times 10^4$ cells/cm² at 37°C with 5% CO₂/ambient O₂ until confluence was reached. The overlaying microglia were shaken off at 35 rpm at 37°C (mouse: 6 hrs, rat: 7 hrs) and plated (7000–7500 cells/cm²) onto 12mm sterile, poly-D-lysine treated glass coverslips (BD Biosciences), placed in multi-well plates (Falcon), or into fresh T75 flasks (Falcon) and incubated for further treatment. All experiments were conducted according to an animal use protocol approved by NIEHS/NIH Animal Care and Use Committee.

Identification of raft clustering

Mouse microglia were grown on coverslips overnight then treated with normal media or media containing A β ₄₂ (250 pg/ml, 500 pg/ml, or 500 ng/ml) for 3, 6 and 24 hrs. Cells were washed and raft clustering was assessed by GM1 ganglioside distribution using the Lipid Raft Vybrant Assay kit (Promega, Madison WI). Following fixation in 4% paraformaldehyde/PBS for 15 min at 4°C, cells were stained with FITC-labeled *Griffonia simplicifolia* IB4-lectin (1:200; Sigma-Aldrich) in 1X Automation buffer (Fisher Scientific, Pittsburgh, PA) plus 1mM Mg²⁺ and Ca²⁺, and mounted with Prolong Gold Anti-Fade (Invitrogen). Positive controls were treated with 100 pg/ml A β ₄₂ for 3 hrs or 0.5 mg/ml zymosan for 1 hr. For each treatment, cells from 5 random fields (20 $\times$ mag) were imaged from duplicate wells and the average % of cells with phagocytic cup formations determined by morphological analysis. The area and perimeter of GM1 clusters in the phagocytic cup region and those outside of the cup region was determined using Metamorph™ software v6.0 (AutoQuant Imaging, Inc. Bethesda, MD). To disrupt rafts, cells were incubated with 0.02 mg/ml filipin at 37°C for 1 hr. Cell viability was also measured using Promega Cell Titer Glo Kit protocol.

Microscopy

Images were acquired with a Nikon Eclipse TE-2000E microscope (Melville, NY) and processed using Metamorph™ software. 0.2 μ m Z stack images were taken at 60 $\times$ and subjected to point spread function-based iterative 3-D blind deconvolution. For fluorescent quantification of raft aggregation, individual z-plane images were thresholded and regions of GM1 aggregation were expressed as number of thresholded pixels/ μ m². The area and perimeter of raft regions and co-localization of fluorophore overlap were measured.

Isolation of DRMs

Microglia cultures were pooled to obtain 10×10^6 cells, centrifuged at 10,000 $\times$ g for 1 min, the supernatant removed, the pellet frozen on dry ice, and stored at –80°C. Cell pellets were thawed on ice, resuspended in 225 μ l of lysis buffer [1 $\times$ tris buffered saline (TBS; pH 8), 1% Triton-x 100, 1% proteinase inhibitor cocktail, 1mM PMSF, 5mM NaF, 1mM Na Orthovanadate (Sigma-Aldrich)] in SW60 ultracentrifuge tubes, and incubated on ice for 30 min. While on ice, the suspension was mixed with 225 μ l of 85% sucrose/TBS. 3.0 mls of 35% sucrose/TBS was overlaid, followed by 675 μ l 5% sucrose/TBS. The tubes were centrifuged at 38,500 rpm (200,000 $\times$ g) for 18 hrs at 4°C. Fifteen fractions of 260 μ l each were gently removed from the top of the tube and individually aliquoted. The fractions were stored at –80°C.

Western blot analyses

DRM fractions were thawed on ice and protein concentrations measured (BCA Assay, Pierce, Rockford, IL). Equal sample volumes (15 μ l) (Arvanitis et al. 2005), were loaded onto 4–12% SDS polyacrylamide gels. High density fractions (fractions 13–15) were pooled. Protein was transferred onto PVDF membranes (Invitrogen). Membranes were incubated with an antibody

to flotillin 1 (1:1000, Flot1, cat# 610821, BD Biosciences). Invitrogen Western Breeze Chemiluminescent kits were used for protein detection. Blots were processed using the Kodak Digital Imaging system. Membranes were stripped and re-probed with an antibody to transferrin receptor (1:500, TfR; cat# 13-6800, Zymed, San Francisco, Ca). TfR, which is not associated with rafts, was used as a non-raft marker.

Phagocytic bead uptake

Mouse microglia were treated with either vehicle or a single dose of 100, 250, 500 pg/ml, or 500 ng/ml A β ₄₂ and incubated for 0, 3, 6, 21, 24, or 27 hrs. For multiple dosing experiments, cells were dosed at 0, 6, 18, and 24 hrs with 250 pg/ml, 500 pg/ml, or 500 ng/ml of A β ₄₂, and incubated for 0, 3, 6, 2, 24, or 27 hrs. Cell media was changed at each addition. Cells were washed and fixed in 4% paraformaldehyde/4% sucrose/PBS for 20 min at 4°C, washed and stained with FITC-labeled IB4-lectin as previously described. For the 0 hr time point, the beads were added and immediately removed. As a positive control for induced phagocytosis, cells were exposed to 0.5 mg/ml zymosan/media with heat-inactivated serum for 1 hr. Phagocytic activity was determined by the uptake of beads [1ul/ml media of 1um Texas Red-labeled fluorescent tracker beads (Invitrogen)] over different time periods. For each time point, the number of bead-positive cells from 6 random fields/well was determined from duplicate wells. Data represents the total number of bead-positive cells as a percent of matched controls.

A β ₄₂ ELISA and γ -secretase activity

Mouse microglia were plated as described and treated with DMSO vehicle or 20 μ M celecoxib for 0, 6, 12, or 24 hrs. From each well, 100 μ l of media was collected. Plated cells were harvested by scraping to determine released and intracellular A β ₄₂ levels using an A β ₄₂-specific ELISA (IBL, Japan, #27711, cross-reacts with mouse protein, discontinued). To determine γ -secretase activity, plated microglia were pre-treated with 200 μ M MDL 28170 for 1 hr at 37°C, washed and treated with media or 20 μ M celecoxib for 0, 6, 12, or 24 hrs. The media was analyzed by ELISA for A β ₄₂ release and the cells harvested and assayed for γ -secretase activity (R&D Systems) at OD₄₅₀.

Quantitative Real Time PCR

Mouse microglia were plated into 24 well dishes as described above and RNA was isolated using the Qiagen RNeasy kit (Valencia, Ca). 1 μ g of total RNA was used to prepare cDNA according to the Superscript II RNase Reverse Transcriptase manufacturer's protocol (Invitrogen). qRT-PCR for IL-1 β , IL-1 α , IL-6, TGF β 1, IGF-1, and GAPDH were conducted using the ABI prism 7700 system™ Sybrgreen protocol (Applied Biosystems, Foster City, Ca). Primer sequences are listed in table 1.

CD36 and CD47 localization in microglia rafts

Cells were stained for rafts as above, fixed, placed in blocking solution (2% BSA/10% horse serum/PBS) for 1 hr at 25°C, followed by incubation with anti-mouse CD36 (cat# 100011, Cayman Chemical, Ann Arbor MI; 1:200) or anti-mouse CD47 (cat# AF1866, R&D systems, Minneapolis, MN; 1:100) for 1 hr. Cells were washed and incubated with an Alexafluor 350 secondary antibody (1:1000, Molecular Probes-Invitrogen) and IB₄ for 1 hr at 25°C and mounted. Cells were chosen randomly from 5 fields and the average co-localization between GM1 and CD36 or CD47 calculated. Data is representative of 3 independent experiments done in duplicate. Exposure time was constant. Controls included absence of primary antibodies or with IgG alone. Western blot confirmed specificity of the anti-CD36 antibody (88 kDa).

Statistical Analyses

Data analysis was conducted with GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com/) using a Chi Square for categorical data, a Mann Whitney U-test for comparison of non-parametric data, a one-way ANOVA followed by a Dunnett's t-test for comparisons back to control or 0 time point, and two-way ANOVA with treatment, time, and replicates as main factors followed by a Bonferroni post-hoc test for independent group means comparisons.

Results

Effects of different A β ₄₂ concentrations on microglial phagocytosis

Phagocytic stimulation was confirmed by bead uptake over a 27 hr period, in microglia treated with a low concentration (100 pg/ml) of fibrillized A β ₄₂ (Fig. 1a). We examined phagocytosis over this time period to investigate cumulative effects of A β ₄₂ on microglial phagocytosis. As a positive control for phagocytosis, we measured bead uptake induced by treatment with 0.5mg/ml zymosan in DMEM containing heat-inactivated serum. Zymosan can activate the complement pathway and stimulate inflammatory responses (Keystone et al. 1977) that include active phagocytosis and phagocytic cup formation (Tohyama and Yamamura 2006). Heat-inactivated serum was used instead of normal serum to prevent responses that could be due to complement factors present in serum. All changes observed in bead uptake were confirmed in cells maintained in macrophage defined serum-free media.

Next, we investigated the effects of higher concentrations of A β ₄₂. Cells received an acute dose of 250 pg/ml, 500 pg/ml, or 500 ng/ml fibrillar A β ₄₂ at the 0 hr time point. Treatment with A β ₄₂ at these levels generally inhibited microglial bead uptake as compared to controls, at all time points tested through 27 hrs; however, a slow, steady recovery was evident at 21, 24 and 27 hrs (Fig. 1b). To account for possible protein degradation of A β ₄₂ within the media, which could relieve the inhibitory effect on microglial phagocytosis during this late stage recovery phase, a multiple dosing regimen was used. Cells were initially dosed at 0 hrs and then again at 6, 18, and 24 hrs with 250 pg/ml, 500 pg/ml, or 500 ng/ml of fibrillar A β ₄₂ to produce a state of continually high A β ₄₂ levels in the media. This dosing regimen for A β ₄₂ maintained a steady inhibition of microglial bead uptake throughout the experimental time course (Fig. 1c).

Rodent microglia contain membrane rafts

Membrane rafts are very small entities that can only be visualized with light microscopy following their clustering at the cell surface (Langhorst et al. 2005). Raft clustering was visualized in primary mouse microglia cultures with an antibody to Flot1 or with the Promega Lipid Raft vibrant assay, which uses fluorescently-tagged Cholera Toxin B (CT-B) to identify aggregation of the raft lipid, GM1 ganglioside. In FITC-labeled *Griffonia simplicifolia* IB4-lectin positive microglia, Flot1 was present in a punctate distribution with minimal visibility at the cell surface (Fig. 2a). This pattern is consistent with previous work done in neurons (Persaud-Sawin et al. 2004) and other cells (Janich and Corbeil 2007), where Flot1 was not only present at the cell surface but within the cell body itself. Under normal culture conditions, some clustering occurs as cells respond to their immediate environment and surrounding cells. The average cluster size was 0.284 μm^2 in area and 1.36 μm in perimeter (Suppl. Table 1). The average number of clusters within an individual cell was 7.25. The variance in each of these measures was within a 10–15% range.

To corroborate raft presence in primary microglia, cell lysates were subjected to 1% Triton-X 100 detergent isolation at 4°C, followed by sucrose gradient density ultracentrifugation (Brown and London 2000). This method allows for the separation of detergent-resistant membranes (DRM) fractions that are cholesterol- and sphingolipid-rich and have associated

glycosylphosphatidylinositol-linked or acylated proteins that broadly define membrane rafts (Brown and Rose 1992; Lingwood and Simons 2007). A discontinuous sucrose density gradient of 5%/35%/85% was used to isolate rafts (Wang et al. 2006a). The requirement of approximately $10\text{--}15 \times 10^6$ cells per isolation necessitated the use of rat microglia. The total protein concentration in each fraction was consistently less in the upper, low density fractions 1–3 (0.05–.2 $\mu\text{g/ml}$) than in the middle (4–7) to high density fractions (8–15; 0.8–0.9 $\mu\text{g/ml}$). Since protein concentration did not provide a true baseline parameter for comparison across fractions, equal volume loading for the Western blot was used as previously described (Arvanitis et al. 2005). This method of loading accounts for the differential solubility of proteins in Triton-X 100 and the fact that proteins will be concentrated in specific fractions following sucrose density ultracentrifugation. DRM fractions were identified with an antibody to Flot1 and non-raft fractions were identified with an antibody to TfR by Western blot (Fig. 2a). Flot1 was consistently present in the low to medium density fractions only (1–5), consistent with published work (Macdonald and Pike 2005). TfR was absent from the first three fractions isolated from lysates by sucrose gradient. Hence, the first three fractions in our study are designated as the raft fractions.

Effects of different A β_{42} concentrations on microglial raft aggregation

To determine whether rafts were involved in the phagocytic process, we investigated whether raft clustering occurred following stimulation with 100 pg/ml A β_{42} . As this is the first report quantifying raft aggregation, we define raft aggregation as the presence of concentrated accumulations of GM1 in a region-specific manner, e.g. possible clustering within the phagocytic cup formation. Since rafts increase in size as they cluster, the area and perimeter of GM1 clusters in the phagocytic cup area were quantified and compared to those occurring in the rest of the cell. We tested this following 3 hrs of 100 pg/ml A β_{42} or 1 hr with 0.5 mg/ml zymosan treatment; times during which phagocytosis is activated by low A β_{42} or zymosan, respectively. Both treatments induced phagocytic cup formation (% of cells with phagocytic cups: 79.5 with A β_{42} ; 89.5 with zymosan) with raft recruitment and clustering (Fig. 2, Table 1). Each dosing regimen created two different raft populations, one with larger clusters inside the cup region and the other with significantly smaller clusters outside of the cup region ($P < 0.001$) (Suppl. Table 1).

We then examined the effect of higher A β_{42} concentrations on raft clustering and phagocytic cup formation following 6 hrs of treatment. We chose the 6 hr time point since this was the time at which maximum phagocytic inhibition occurred. Phagocytic cup formation was observed in 3% or less of the cells. High concentrations of A β_{42} stimulated raft aggregation; however, GM1 clusters were relegated to areas that lacked a phagocytic cup formation (Fig. 2, Suppl. Table 1). These clusters were mainly present at the leading edge or uropod of the cell, indicating the initiation of directional movement (Fig. 6a) (Seveau et al. 2001). Our results suggest that a high A β_{42} environment impairs both phagocytic cup formation and associated raft clustering. Next, we tested whether A β_{42} affects raft integrity as assessed by Flot1 and TfR protein distributions in DRMs. At an A β_{42} concentration of 100 pg/ml (Fig. 2c), Flot1 was normally distributed in the low density fractions. At higher concentrations (250 pg/ml) of A β_{42} , Flot1 distribution not only shifted by one fraction into the higher density fractions, but there was also less of it, as indicated by the decreased band intensity, compared to treatment with 100 pg/ml A β_{42} . This indicates some disruption of raft integrity.

Disrupting rafts inhibits raft aggregation and phagocytosis

If rafts are required for phagocytosis, then their disruption should inhibit raft clustering and phagocytosis. Cells were pre-treated with 0.02 mg/ml of the cholesterol sequestering agent, filipin, allowing raft disruption without decreased cell viability and raft clustering compared to untreated controls (Fig. 3a & b). Following filipin treatment, the sizes of GM1 aggregates

were, on average, $0.195 \mu\text{m}^2$ in area with a $1.31 \mu\text{m}$ perimeter (Suppl. Table 1). Filipin pre-treatment prevented phagocytic cup formation. Filipin also inhibited bead uptake and $\text{A}\beta_{42}$ production (Fig. 3c; Suppl. Table 2), suggesting that maintaining intact raft structures is necessary for phagocytosis. We next examined the effect of filipin on Flot1 distribution in DRMs. In control microglia, Flot1 was distributed in the low density raft fractions 1–3. Filipin treatment induced loss of Flot1 in fractions 1 and 2, and a shifted distribution to fraction 3 and the higher density fractions, 4–7. Flot1 band intensity was also lower than controls, suggesting decreased protein consistent with disruption of raft integrity (Fig. 3d). TfR protein distribution shifted in the opposite direction, where it was now present in the low density raft fractions 2 and 3. This also supports a decrease in the stability of the raft structure.

Celecoxib increases $\text{A}\beta_{42}$ in primary mouse microglia cultures

As celecoxib was shown to increase levels of $\text{A}\beta_{42}$ *in vivo* and *in vitro*, we used it as a pharmacological inducer of $\text{A}\beta_{42}$ (Kukar et al. 2005; Leuchtenberger et al. 2006). The effect of different concentrations of celecoxib (5, 10, 20 and $50 \mu\text{M}$) on cell viability and the production of $\text{A}\beta_{42}$ from primary mouse microglia was tested *in vitro* by cell proliferation assays and $\text{A}\beta_{42}$ -specific ELISA. $20 \mu\text{M}$ celecoxib treatment provided a consistent increase in $\text{A}\beta_{42}$ ($\sim 600 \text{ pg/ml}$) with no effect on cell viability through 24 hrs (Fig. 4a). Compared to untreated and vehicle controls, celecoxib treatment significantly increased $\text{A}\beta_{42}$ release at 3, 6, 12 and 24 hrs (Fig. 4b). We tested a time point at 1 hr and found no significant increase in $\text{A}\beta_{42}$ levels over control (data not shown).

Microglia cells treated with celecoxib showed higher γ -secretase activity at 6 and 12 hrs, compared to untreated controls (Fig. 4c). Pre-treatment with the γ -secretase inhibitor, MDL 28170 ($200 \mu\text{M}$) followed by celecoxib, resulted in significantly lower $\text{A}\beta_{42}$ levels, confirming that celecoxib treatment affects γ -secretase activity. We then measured intracellular levels of $\text{A}\beta_{42}$ in cells treated with celecoxib. A cyclical increase in intracellular $\text{A}\beta_{42}$ at 0 and 12 hrs of celecoxib treatment was observed (Fig. 4d). The very early increase in intracellular $\text{A}\beta_{42}$ supports a rapid increase in γ -secretase activity with celecoxib treatment.

Effects of celecoxib on microglial phagocytosis

Cells were treated with $20 \mu\text{M}$ celecoxib for 0 – 27 hrs, producing an elevated level of $\text{A}\beta_{42}$ in the media and phagocytosis was assessed *via* uptake of $1 \mu\text{m}$ Texas Red-labeled beads. For each time point, bead uptake after $20 \mu\text{M}$ celecoxib treatment was normalized to vehicle controls, accounting for any stimulation due to beads alone. We found no significant differences between untreated and vehicle-treated cells (data not shown). The period for examination was chosen to incorporate times at which $\text{A}\beta_{42}$ was produced in culture (3 – 24 hrs) and to allow phagocytosis to occur (Swanson et al. 1999). Maximum bead uptake occurred at 3 hrs after treatment (Fig. 5). Between 6 – 21 hrs of treatment with celecoxib, no significant difference in bead uptake was detected in the treated cells as compared to the controls. However, with additional time, between 24 and 27 hrs, a significant reduction in bead uptake was observed in celecoxib-treated cells. This decrease in bead uptake occurred in the absence of any changes in cell viability or proliferation (data not shown).

Prolonged treatment with celecoxib inhibits raft aggregation and microglial phagocytosis

We determined whether celecoxib treatment stimulated phagocytic cup formation and regional raft aggregation. Treatment with celecoxib for 3 hrs induced phagocytic cup formation in $60\% \pm 3.4$ (SD) of the entire cultured cell population. Concomitant raft clustering occurred in two separate pools, one in the region of cup formation and the other outside of this defined region (Fig. 6a). The average area of GM1 clusters outside of the cup region was $0.17 \mu\text{m}^2$ with a $1.18 \mu\text{m}$ perimeter. Within the cup area, the size of GM1 clusters was significantly increased ($P < 0.0001$), with an average area of $1.75 \mu\text{m}^2$ and perimeter of $5.97 \mu\text{m}$ (Suppl. Table 1)

demonstrating raft recruitment. The percent distribution of GM1 clusters in the phagocytic cup also increased significantly ($P<0.001$) as compared to outside of the cup region, suggesting that raft recruitment to this area is a prerequisite for phagocytosis.

At 6 and 24 hrs after celecoxib treatment, raft aggregation significantly decreased with $\leq 3\%$ of cells showing evidence of phagocytic cup formation, as compared to the 3 hr time point ($P<0.001$). In addition, raft clusters were, on average, smaller ($0.3\% \times 0.6 \mu\text{m}^2$), fewer in number ($P<0.0001$) and present along the leading edge of the cell (Fig. 6a), similar to that seen with higher $\text{A}\beta_{42}$. In cells displaying a phagocytic cup, there were no visible GM1 clusters within the cup, suggesting a lack of raft recruitment there at 6–24 hrs. These results were consistent with the inhibition of bead uptake by celecoxib at time points greater than 3 hrs. We demonstrated that celecoxib interferes with GM1 localization. Filipin inhibited the organized raft clustering that celecoxib induces (Fig. 6a). The pattern observed was similar to the effect of filipin alone.

Celecoxib affects raft integrity

As a measure of raft integrity, we investigated the effect of celecoxib on Flot1 distribution in raft fractions by Western blot (Fig. 6b). At 0 hrs treatment with $20 \mu\text{M}$ celecoxib, Flot1 was present in the low density raft fractions, 1–3, representing a normal distribution pattern. At 3 hrs after treatment, Flot1 was still present in low density fractions 2 and 3, but absent from fraction 1; similar to 100 pg/ml $\text{A}\beta_{42}$ treatment. At 6 hrs, further redistribution of Flot1 to higher density fractions was observed. Interestingly, at this time point, there was a shift in Flot1 distribution out of raft fractions 1 and 2. Although still present in raft fraction 3, Flot1 could now be detected in higher density fractions 4–7. At 12 (not shown) and 24 hrs, the redistribution of Flot1 to the higher density fractions became more evident. A progressive transition of TfR to the lower density fractions 2 and 3, at 6 through 24 hrs was observed.

Celecoxib increases IL-1 α and IL-6, but not TNF α

Microglia were treated with $20 \mu\text{M}$ celecoxib for 0, 6, 12, 24 and 48 hrs and mRNA levels of the tumor necrosis factor- α (TNF α), interleukin (IL)-1 α , IL-1 β , transforming growth factor- β 1 (TGF β 1) and insulin-like growth factor 1 (IGF-1) were measured. Basal TNF α mRNA levels were just within the detectable range and no increase occurred with celecoxib treatment (relative fold change fell within the range of $0.5 < \text{fold induction over vehicle control} < 0.8$; $P>0.05$ using ANOVA followed by the Dunnett's t-test), corroborating previously reported results that celecoxib does not increase TNF α expression (Cuzzocrea et al. 2002). IL-6 mRNA levels in treated cells progressively increased over controls through 48 hrs of treatment (Fig. 7). A significant increase was seen in IL-1 α , but not IL-1 β mRNA levels, following 24 hrs exposure. mRNA levels of the anti-inflammatory cytokines, TGF- β 1 and IGF-1, decreased.

Raft localization of CD36 is disturbed by high $\text{A}\beta_{42}$

We confirmed results by Landreth and colleagues (Bamberger et al. 2003) showing that clearance of $\text{A}\beta_{42}$ by microglia involves the SRB member, CD36. We investigated whether prolonged celecoxib treatment would affect CD36 localization within microglia membrane rafts. In untreated cells, CD36 co-localized with GM1 ganglioside in rafts, with a $42.37 \pm 2.3\%$ degree of co-localization (Fig. 8a). In cells treated with $20 \mu\text{M}$ celecoxib for 3 hrs there was a significant increase in CD36 within rafts where the percent co-localization with GM1 was $66.35 \pm 4.0\%$ ($P<0.05$). This suggests recruitment into rafts in the region of the phagocytic cup (Fig. 8b). The presence of CD36 in some, but not all, rafts points to at least two discreet raft pools. At 6 hrs of celecoxib treatment, a time coinciding with decreased phagocytic activity, CD36 co-localization with GM1 decreased to $16.98 \pm 0.01\%$ ($P<0.05$), indicating its redistribution out of rafts (Fig. 8c). Where present, rafts and CD36 were no longer visible in the phagocytic cup.

To ascertain whether receptor recruitment to rafts is specific, we examined CD47 since it was speculated that it may be involved in A β ₄₂ clearance (Bamberger et al. 2003). Microglia were treated with celecoxib for 3 and 6 hrs prior to measuring the degree of co-localization of CD47 to rafts (Fig. 9). In untreated cells, CD47 co-localization with rafts was 32 $\pm$ 2%. Treatment with celecoxib for 3 hrs induced movement of CD47 out of rafts, decreasing the amount of co-localization to 4 $\pm$ 0.07% ($P < 0.01$). At 6 hrs, CD47 presence in rafts significantly increased to 14.5 $\pm$ 1.2%, as compared to the 3 hr time point ($P < 0.05$), suggesting some re-entry. Together these data suggest that CD47 may not be involved in microglial phagocytosis of A β ₄₂, or it may serve as a negative regulator of phagocytosis.

Discussion

Increasingly, membrane rafts are being recognized as important components required for different vital cellular processes, including peripheral macrophage phagocytosis (Bottcher et al. 2006). Their importance as a potential pivot point in microglial phagocytic function, however, has not been previously investigated. As a novel approach to understanding mechanisms involved in the clearance of A β ₄₂, we investigated the importance of membrane rafts in regulating microglial phagocytosis. By examining raft aggregation through cell morphology and GM1 staining during phagocytic stimulation with direct A β ₄₂ addition or pharmacological treatment, we identified two inter-dependent processes that are required for efficient phagocytosis by microglia: (1) phagocytic cup formation and (2) localized raft aggregation in the phagocytic cup region. Moreover, raft aggregation occurred in two distinct pools, suggesting that the raft population is heterogeneous, with different pools varying in their respective lipid and/or protein components.

Existence of an A β ₄₂ threshold

In this study we investigated microglial responses to A β ₄₂, the most pathogenic form of A β . Whether rafts play a similar role in the phagocytosis of A β ₄₀ is not yet known. We demonstrate the existence of an A β ₄₂ threshold level of 250 pg/ml, above which microglial phagocytosis of fibrillar A β ₄₂ is severely inhibited. This value is comparable to the A β ₄₂ burden levels observed in the cerebrospinal fluid of patients suffering from early to mid-stage AD (500 nM) (Liu et al. 2005). One explanation for this may be that, at or above 250 pg/ml of fibrillar A β ₄₂, microglial receptors may become saturated with the peptide, rendering them incapable of efficient phagocytosis. An alternative may be that the increase in A β ₄₂ leads to cholesterol depletion, raft instability and subsequent cell death by autophagy (Cheng et al. 2006; Nixon 2007). With respect to the latter, we identified a shift in the Flot1 distribution into non-raft fractions following incubation with high A β ₄₂ concentrations, while TfR moved into raft fractions. This shift was also observed following treatment with filipin, which disrupts rafts through cholesterol sequestration. Our results demonstrate, for the first time, that levels of fibrillar A β ₄₂ above 250 pg/ml can produce definite disturbances in raft homeostasis and structural integrity that can lead to dysregulated microglia functions, including phagocytosis. Although a shift in Flot1 of one fraction was observed with our 100 pg/ml A β ₄₂ treatment, there was no loss of microglial phagocytic function. Various studies on raft isolation demonstrate that DRM fractions are generally seen from fractions 1 through 5, depending on isolation procedure and cell or tissue type (Brown and London 2000; Macdonald and Pike 2005; Wang et al. 2006a), suggesting that a shift to fraction 2 may not be detrimental to cellular function. This is supported by the observation that phagocytosis is not inhibited with 100 pg/ml A β ₄₂. Further shifts into fractions 3 and above, as seen at higher A β ₄₂ concentrations (250 pg/ml), however, do affect phagocytic function.

Celecoxib interferes with raft integrity and microglial phagocytosis

We confirmed a previous report that celecoxib raises A β ₄₂ levels (Kukar et al. 2005), in a cyclical manner. Maxfield and colleagues suggested the possibility that microglia may become so overloaded with A β ₄₂ in AD that they are rendered unable to efficiently degrade the protein (Chung et al. 1999). The observed cycling suggests that, in our system, A β ₄₂ does not accumulate intracellularly but may be degraded internally or secreted, over the time period tested. The lack of internal accumulation of fluorescent A β ₄₂ in microglia supported this (data not shown). This prompted us to examine possible effects of celecoxib on rafts. One study demonstrated that, at clinically relevant dose levels, celecoxib had no effect on membrane fluidity or activity of raft resident proteins such as alkaline phosphatase (Nair et al., 2006). Behl and colleagues (Gamerding et al. 2007), however, provide evidence that selective COX-2 inhibitors cause cholesterol-like decreases in membrane fluidity and increased association of APP and the β -secretase, BACE 1 in rafts, leading to substantial generation of A β peptide. Celecoxib can also affect sarco-endoplasmic calcium ATPases, similar to cholesterol (Johnson et al. 2002). Celecoxib may, therefore, exert raft-altering properties and, since raft integrity is cholesterol-dependent (Pike 2004), APP processing could also be affected (Simons et al. 1998). Our results support the Behl study (Gamerding et al. 2007) and demonstrate that celecoxib increased A β ₄₂ production *via* rafts, as filipin treatment prevented celecoxib-induced A β ₄₂. Further, since amyloidogenic processing *via* γ -secretase occurs within rafts (Ehehalt et al. 2003; Urano et al. 2005), and celecoxib increases γ -secretase activity (Kukar et al. 2005; Leuchtenberger et al. 2006), disrupting rafts will prevent celecoxib-induced A β ₄₂. Although celecoxib increased γ -secretase activity, this declined after 12 hrs of treatment. The leveling off of secreted A β ₄₂ shown in figure 1 supports this.

Additionally, celecoxib treatment appeared to structurally disrupt rafts, in a manner similar to a high A β ₄₂ environment; shifting Flot1 distribution toward the non-raft fractions. The trend observed with 3 and 6 hr celecoxib treatment was similar to 100 and 250 pg/ml A β ₄₂, respectively. We concluded from our data that microglial function was not adversely affected until Flot1 shifted out of fractions 1 and 2. With continued celecoxib treatment, Flot1 re-appeared in both raft and heavier, non-raft fractions. This could be due to the changing properties of the raft fractions, as they assume dynamics more similar to the non-raft fractions with prolonged celecoxib treatment. Of note is the significant decrease in Flot1 protein level following treatment with celecoxib, filipin or A β ₄₂. Since Flot1 is an integral part of rafts and has recently been shown to be involved in a number of signaling cascades (Babuke and Tikkanen 2007), disrupting rafts could have adverse effects on phagocytic signaling and the associated proteins. Therefore, Flot1 protein levels would decline following raft disruption, as observed following treatment with filipin or 250 pg/ml A β ₄₂ (Fig 2 & Fig 3).

Inhibition of COX-2 and prostaglandin-E₂ synthesis should stimulate phagocytosis (Aronoff et al. 2004). It may, therefore, seem counter-intuitive that celecoxib treatment initially stimulated phagocytosis but later inhibited it, whereas direct A β ₄₂ treatments, known to increase COX-2 expression (Hull et al. 2006), led to inhibition at all time points (Fig. 3). This suggests that some other property of the drug is affecting microglial phagocytosis. It could be that the production of monomeric A β ₄₂ by celecoxib (Kukar et al. 2005), which does not inhibit uptake, is responsible for the initial phagocytic spike at 3 hrs, where celecoxib induced A β ₄₂ levels of approximately 400 pg/ml. Continued accumulation at 37°C, however, facilitates fibrillization and, in this form, A β ₄₂ is difficult to phagocytize (Sadowski et al. 2006). This may explain our observations at 24–27 hrs of treatment. Also, in figure 1 we demonstrated that exogenously added fibrillar A β ₄₂ inhibits phagocytosis at concentrations above 250 pg/ml. This amount of A β ₄₂ is in addition to that which is normally produced by the cell. This is supported by the fact that continued accumulation of secreted A β ₄₂ in the media inhibited

phagocytosis (Fig 4 & Fig 5). Similar properties are exhibited by monomeric and aggregated α -synuclein (Park et al. 2008).

Since one of our questions was whether high $A\beta_{42}$ concentrations over an extended period of time would interfere with microglial phagocytosis, we did not examine acute effects of direct $A\beta_{42}$ treatment over the short term. Furthermore, celecoxib did not significantly increase $A\beta_{42}$ levels before 3 hrs. In our hands, we found that prolonged celecoxib treatment inhibited phagocytosis, despite the fact that some COX inhibitors can suppress phagocytic inhibition (Koenigsknecht-Talboo and Landreth 2005). Possible reasons for this contradiction may be that those investigators used immortalized BV-2 cells whereas we used primary microglia cells, the former tend to have a higher baseline activation state than primary microglia. Additionally, the aforementioned study used ibuprofen, which unlike celecoxib, does not cause increased $A\beta_{42}$ (Gasparini et al. 2004). Although contributing to an increased microglial activation state, these factors combined, could send the cell into senescence, thereby decreasing the overall phagocytic capability (Streit 2006; Streit et al. 2005). Despite evidence to support a potential therapeutic use for some NSAIDs, conclusive effects are still disputable; the effectiveness being related to many factors including type of drug, concentration reaching the brain and varied vulnerabilities of different neuronal populations (Leuchtenberger et al. 2006).

Inflammatory environments affect phagocytosis

Microglial clearance of $A\beta_{42}$ appears to be regulated by the inflammatory environment (Koenigsknecht-Talboo and Landreth 2005). Further, aggregated and fibrillar $A\beta_{42}$ activate microglia which can initiate signaling cascades *via* a number of chemokines and cytokines (Griffin 2006). There is ample evidence demonstrating that $A\beta_{42}$ stimulates phagocytosis and activates microglia (Butovsky et al. 2005; Chung et al. 1999; Koenigsknecht-Talboo and Landreth 2005; Koenigsknecht and Landreth 2004). Our results using isolated microglia were consistent with these observations and demonstrated that increased $A\beta_{42}$ exposure, resulting from direct application or pharmacological induction, resulted in morphological activation, the elevation of mRNA levels of pro-inflammatory cytokines IL-1 α and IL-6, and decreased mRNA levels for both TGF- β 1 and IGF-1 (Fig. 7). Since IL-6 can have anti-inflammatory effects (Quintana et al. 2008), it is possible that its upregulation may be an attempt at protection against $A\beta_{42}$ -induced toxicity. The decrease in levels of TGF- β 1 may be related to the effect of COX-2 inhibition (Liu et al. 2007). The oscillations seen for IGF-1 and TGF- β 1 may, therefore, be the combined result of anti-inflammatory aspect of celecoxib and the pro-inflammatory effect of $A\beta_{42}$. Increased IL-1 α and IL-6, coupled to decreased TGF- β 1 and IGF1, could impact cellular functions including processing of APP (Griffin 2006), early lymphocyte recruitment, astrocyte signaling (Stipursky and Gomes 2007), cell migration (Coras et al. 2007), synaptic plasticity and phagocytosis (Koenigsknecht-Talboo and Landreth 2005).

Receptor recruitment to rafts during phagocytosis

Specific recruitment of proteins to rafts is important for the regulation of different signaling cascades (Kim et al. 2006). Therefore, there is a strong possibility that raft recruitment of protein receptors is also involved in phagocytic regulation. This is supported by observations that receptor recognition occurs at the membrane surface and that receptors involved in phagocytosis, such as CD36, are present in rafts (Thorne et al. 2000). The specificity of different receptors participating in phagocytosis depends on both cell type and the material being cleared (Gregory and Devitt 2004; Koizumi et al. 2007; Lucas et al. 2006; Makranz et al. 2006; Ren et al. 1995; Seitz et al. 2007). Specifically, $A\beta_{42}$ clearance is facilitated by a number of different receptors including the TLRs, CD14, Fc, complement, integrin-associated proteins like CD47, and scavenger receptors such as CD36 (Bamberger et al. 2003; Koenigsknecht and Landreth 2004; Liu et al. 2005; Tahara et al. 2006). Based on their involvement in various types of

phagocytosis, we investigated whether CD36 and CD47 were actively recruited to rafts during phagocytosis. In this process, we hypothesized that structurally intact rafts are required for the juxtaposition of different receptor combinations involved in phagocytosis. We concluded that specific recruitment of CD36, but not CD47, to rafts was necessary for phagocytosis of A β ₄₂, supporting a pivotal role for rafts in this process.

Displacement of CD36 from rafts may interfere with the tethering or signaling phase of phagocytosis (Gregory and Devitt 2004). Redistribution of CD36 in microglia under raft-impairing, high A β ₄₂ conditions demonstrates that its presence in rafts is dependent upon maintenance of raft structural integrity. Engagement of CD36 in phagocytosis suppresses pro-inflammatory cytokine signaling (Bottcher et al. 2006). Thus, the observed destabilization of CD36-containing rafts in microglia could repress phagocytosis and increase the expression of the observed pro-inflammatory cytokines. This work supports studies from the Moore lab, where CD36 was one of the primary receptors affected by A β ₄₂, creating a pro-inflammatory environment through the inability of microglia to clear oxidized phospholipid-containing ligands (Kunjathoor et al. 2004).

Regarding CD47 involvement in microglia phagocytosis, it is likely that its actions are primarily those of a negative regulator. This supports previous work characterizing “don’t eat me signals” on apoptotic red blood cells (Oldenborg et al. 2000). That CD47 re-enters rafts slowly during phagocytic inhibition further indicates a regulatory role for rafts and suggests a selective compromise in the integrity of a defined subset of rafts, e.g. CD36-containing rafts, while others remain intact. Importantly, this data further suggests that CD36 and CD47 are not present within the same raft compartment.

Raft populations consist of ‘reserve rafts’, as well as, ‘stabilized receptor-clustered rafts’ (Kusumi et al. 2004). This implies that the different raft populations may be activated independently. Our identification of two distinct groups of raft clusters during phagocytosis supports this. Thus, under phagocytic conditions, only specific raft populations, i.e. CD36-containing rafts, are mobilized to the phagocytic cup. The subset of rafts outside of the cup may be part of the reserve population, or may be platforms for signaling processes other than phagocytic cup formation. In this study, we demonstrated that raft disruption, either by cholesterol sequestration or high A β ₄₂ conditions, interfered with raft aggregation and phagocytic cup formation. Disruption of raft integrity may affect raft homeostasis, resulting in aberrant aggregation and altered downstream signaling. This attests to the highly dynamic, yet fragile nature of rafts.

Recent work suggests that discrete membrane organizations may represent a basic cellular design required for signal transduction (Tian et al. 2007). Precedence for this comes from studies which report that alteration of raft integrity can lead to trafficking defects, as seen in Niemann-Pick (Simons and Gruenberg 2000) and Batten diseases (Persaud-Sawin et al. 2004). Additionally, prion diseases such as Creutzfeld-Jacob disease (CJD) and bovine spongiform encephalopathy require rafts for protein processing (Fantini et al. 2002), and the over-expression of α -synuclein in Parkinson’s Disease can upregulate the raft-related caveolar protein, caveolin 1 (Hashimoto et al. 2003) and activate microglia (Zhang et al. 2005).

Conclusions

The data presented here demonstrates that in an environment where there is a constant, but low level of A β ₄₂, microglia might be more responsive to phagocytic stimulation. Speculatively, this may be one reason why treatment of neurodegenerative diseases with NSAIDs has not been successful. Our observations suggest that current dosing regimes with selective NSAIDs may increase γ -secretase activity over time, leading to A β ₄₂ build-up, downstream IL-1 and

IL-6 increases and decreased microglial phagocytic capacity. The anti-inflammatory component provided by COX-2 inhibition may be over-ridden by the production of A β ₄₂, such that the subsequent inflammatory environment probably prevents microglia from adapting successfully. The data presented here provides novel insight into the importance of rafts in the microglial phagocytic machinery. We provide the first report that membrane raft aggregation in the phagocytic cup area is a prerequisite for efficient microglial phagocytosis. Deficiencies in microglial function as well as raft disruption appear to be central themes in many degenerative diseases including AD, Parkinson's Disease and prion disease (Ciesielski-Treska et al. 2004; Fiala et al. 2005; Salman et al. 1999). Therefore, rafts may be a necessary factor that, when disrupted, significantly contribute to the overall degenerative process. Understanding these mechanisms can allow certain raft components e.g., CD36 or Flot1, to be manipulated or used as targets for therapeutic intervention in the treatment of neurodegeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

AD, Alzheimer's Disease
 APOE, Apolipoprotein E
 APP, Amyloid precursor protein
 A β ₄₂, amyloid A β ₄₂
 COX-2, cyclooxygenase-2
 CJD, Creutzfeld-Jacob disease
 DRM, detergent-resistance membrane
 Fc γ I or Fc γ III, constant fragment of receptor γ subtype I or III
 Flot1, Flotillin1
 IL, interleukin
 IGF-1, insulin-like growth factor 1
 NSAID, non-steroidal anti-inflammatory drug
 TGF- β 1, transforming growth factor- β
 IPD, Parkinson's Disease
 PAMPS, pathogen-associated molecular patterns
 PRRs, pattern recognition receptors
 PND2, postnatal day 2
 SRA/B, scavenger receptor family A/B
 TfR, transferrin receptor
 TLRs, Toll-like receptors

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Fig. 1. A high Aβ₄₂ environment inhibits phagocytosis in microglia

Uptake of 1μM fluorescent beads was measured at 0, 6, 21, 24 or 27 hrs following a single dose of (a) 100pg/ml Aβ₄₂ or 0.5 mg/ml zymosan or (b) 250pg/ml, 500pg/ml or 500ng/ml Aβ₄₂. (c) Chronic exposure to 250pg/ml, 500pg/ml or 500ng/ml Aβ₄₂ for 6, 18 or 24 hrs. Data is represented as % number of bead-positive cells relative to vehicle controls at each time point. *P<0.05, **P<0.01, ***P<0.001 compared to controls using two-way ANOVA followed by the Bonferroni post-hoc test (n=4).

Fig. 2. Membrane rafts are present in rodent microglia

(a) Untreated mouse microglia stained for IB₄ (green) and Flot1 (red). White arrow shows raft clustering. Magnification: 60 $\times$. Western blot of rat microglia fractions: Top panel: Flot1 protein identified in low density fractions 1–6. Bottom panel: TfR is absent from fraction 1–3, indicating that these are true raft fractions. Data is representative of 3 individual experiments. (b) Representative color aligned images of GM1 (red) and IB₄ lectin (green) in mouse microglia treated with 0.5mg/ml zymosan for 1 hr, 100 pg/ml, 250 pg/ml, 500 pg/ml and 500 ng/ml $A\beta_{42}$ for 3 hrs. Arrows indicate areas of raft clustering. White boxes show phagocytic cup formations. Magnification: 60 $\times$; Scale bar=10 μ m (c) Western blots of rat microglia fractions following treatment with 100 pg/ml and 250 pg/ml $A\beta_{42}$ for 3 hrs. Flot1 bands shift by 1 fraction into fraction 3 with 250 pg/ml $A\beta_{42}$ treatment.

Fig. 3. Filipin disrupts microglial raft structural integrity

Representative color aligned images of GM1 (red) and IB₄ l (green) in mouse microglia: (a) controls, (b) treated with 1mg/ml filipin. Data is representative of 2 independent experiments of 50 cells each. Magnification: 60×; Scale bar = 10 μm. (c) Uptake of 1μM fluorescent beads was inhibited by a single dose of 1 mg/ml filipin. ***P<0.001 compared to controls using two-way ANOVA followed by the Bonferroni post-hoc test (n=4). (d) Western blots of rat microglia fractions following 1mg/ml filipin treatment. Top panel: Flot1 protein at 48–50 kDa is decreased by treatment, Bottom panel: TfR protein at 130 kDa. Blots are representative of 3 individual experiments.

Fig. 4. Celecoxib increases A β ₄₂ levels in microglia

(a) Cell proliferation assay following treatment with 20 μ M celecoxib. Average OD_{490nm} is plotted against treatment time (hrs). $P > 0.05$ using one way AVOVA followed by the Dunnett's t-test ($n=4$). (b) Secreted A β ₄₂ measured by A β ₄₂-specific ELISA over 24 hrs following 20 μ M celecoxib treatment. (c) Relative γ -secretase activity in PND2 microglia after 20 μ M celecoxib treatment for 0, 3, 6, 12 or 24hrs. ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA followed by the Dunnett's t-test ($n=3$). (d) Intracellular A β ₄₂ measured by A β ₄₂-specific ELISA over 24 hrs following treatment with 20 μ M celecoxib. * $P < 0.05$, ** $P < 0.01$ for celecoxib-treated vs untreated, two-way ANOVA followed by Bonferroni post-hoc test ($n=3$). Data represents mean $\pm$ SD.

Fig. 5. Celecoxib treatment inhibits phagocytosis in microglia

Uptake of 1 μ M fluorescent beads was measured at 0, 3, 6, 21, 24 or 27 hrs following a single dose of 20 μ M celecoxib. Data is represented as % number of bead-positive cells relative to controls at each time point. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two-way ANOVA followed by the Bonferroni post-hoc test, $n=4$). Data represents mean + SD.

Fig. 6. Celecoxib disrupts microglial raft structural integrity

(a) Representative images of mouse microglia stained for IB₄ lectin (green) and GM1 ganglioside (red). Panels are color aligned and show stained cells treated with 20 μ M celecoxib for 0, 3, 6, 24 hrs and filipin +celecoxib treatment. Arrows indicate raft clustering; white boxes outline phagocytic cup formation. Magnification: 60 $\times$, Scale bar = 10 μ m. Size of clusters decrease with increasing celecoxib treatment. Filipin/celecoxib treatment was similar to filipin alone. (b) Western blots of rat microglia fractions following treatment with 20 μ M celecoxib for 0, 3, 6, or 24 hrs. Panels on right show blots for Flot1 protein, Left panels show blots for TfR protein. Blots are representative of 3 individual experiments. Flot1 protein shifts out of the raft fractions and TfR shifts into raft fractions. At 24 hrs raft properties appear disrupted as Flot1 distribution is widespread.

a **b**

Fig. 7. Celecoxib stimulates pro-inflammatory cytokine mRNA expression in mouse microglia (a) IL-1 α and IL-6 or (b) TGF- β and IGF-1 mRNA levels determined by qRT-PCR following 20 μ M celecoxib treatment for 0, 6, 12, 24 or 48 hrs. Data is plotted as fold induction over 0 hr control as a function of time (n=3 in duplicate). *P<0.05, ***P<0.001 using one-way ANOVA followed by the Dunnett's t-test.

Fig. 8. Celecoxib affects raft distribution of CD36 in mouse microglia

(a) Immunofluorescent staining of IB₄ (green), CD36 (blue) and GM1 ganglioside (red). Right panel shows color alignment between CD36 and GM1. (a) Untreated control cells. (b) Cells treated with 20μM celecoxib for 3 hrs. (c) Cells treated with 20μM celecoxib for 6hrs. Degrees of co-localization percentages are average measurements (n=4). White box indicates phagocytic cup area with CD36 accumulation in raft clusters (white arrows). Magnification: 60×. Scale bar = 10 μm.

Fig. 9. CD47 presence in rafts is not required for microglial phagocytosis

(a) Immunofluorescent staining CD47 (blue) and GM1 ganglioside (red). Right panel shows color alignment between CD47 and GM1. Images were thresholded and the degree of co-localization was measured by Metamorph software v6.0. (a) Untreated control cells. (b) Cells treated with 20μM celecoxib for 3 hrs. (c) Cells treated with 20μM celecoxib for 6hrs. There is a noticeable absence of phagocytic cups. Degrees of co-localization percentages are average measurements from n=4. White box indicates phagocytic cup formation. White arrows indicate raft clustering. Magnification: 60×. Scale bar = 10 μm.

Table 1

qRTPCR Primer Sequences

Primer name	5'-3' sequence
GAPDH forward	TCACCACCATGGAGAAGGC
GAPDH reverse	CATAAGCAGTTGGTGGTGCA
IL-1 β forward	CAACCAACAAGTGATATTCTCCATG
IL-1 β reverse	ATCCACACTCTCCAGCTGCA
IL-1 α forward	GCAACGTCAAGCAACGGGAAGATT
IL-1 α reverse	AGGTGTAAGGTGCTGATCTGGTT
IL-6 forward	GAGGATACCACTCCCACAGACC
IL-6 reverse	AAGTGCATCATCGTTGTCATACA
TGF β 1 forward	TGACGTCACCTGGAGTTGTACGG
TGF β 1 reverse	GGTTCATGTCATGGATGGTGC
IGF1 forward	CCCACTGACATGCCCAGA
IGF1 reverse	GAGGAAACGTCGAAGCAAAAGA