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Slow degradation in phagocytic astrocytes can be enhanced by lysosomal acidification

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Abstract

Inefficient lysosomal degradation is central in the development of various brain disorders, but the underlying mechanisms and the involvement of different cell types remains elusive. We have previously shown that astrocytes effectively engulf dead cells, but then store, rather than degrade the ingested material. In the present study we identify reasons for the slow digestion and ways to accelerate degradation in primary astrocytes. Our results show that actin-rings surround the phagosomes for long periods of time, which physically inhibit the phagolysosome fusion. Furthermore, astrocytes express high levels of Rab27a, a protein known to reduce the acidity of lysosomes by Nox2 recruitment, in order to preserve antigens for presentation. We found that Nox2 co-localizes with the ingested material, indicating that it may influence antigen processing also in astrocytes, as they express MHC class II. By inducing long-time acidification of astrocytic lysosomes using acidic nanoparticles, we could increase the digestion of astrocyte-ingested, dead cells. The degradation was, however, normalized over time, indicating that inhibitory pathways are up-regulated in response to the enhanced acidification.

Keywords

Phagocytosis; lysosome; digestion; glia; Rab27a; Nox2; pHrodo; nanoparticle

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Introduction

Compared to microglia, astrocytes' role in phagocytosis has been sparsely studied. Recent evidence however demonstrates an important role for astrocytes in clearance of dead cells, synapses and pathogenic protein aggregates, such as amyloid-beta (A β) and alpha-synuclein oligomers (Chang et al. 2000; Chung et al. 2013; Fellner et al. 2013; Jones et al. 2013; Loov et al. 2012; Magnus et al. 2002; Sokolowski et al. 2011). Some reports indicate that astrocytes are more efficient than microglia in taking up A β , particularly during the early stages of Alzheimer's disease (AD) and astrocytes has been shown to gradually accumulate A β throughout cortex in AD patients (Guenette 2003; Nagele et al. 2003; Nicoll and Weller 2003; Nielsen et al. 2010; Shafer 2001).

We have previously shown that astrocytes clear dead cells that would otherwise induce apoptosis in healthy neurons. By removing the dead cells from the immediate environment astrocytes can limit the progression of bystander cell death. Using a mouse model of brain trauma we showed that one week after traumatic brain injury, approximately half of the dead cells are closely associated with astrocytes, indicating a central role for astrocytes in cell corpse clearance (Loov et al. 2012). Astrocytes with multiple distinct nuclei have previously been described in several brain disorders (Sofroniew and Vinters 2010), but the underlying mechanisms for the occurrence of such astrocytes is unclear. Interestingly, our study show that cultured astrocytes (in contrast to macrophages) store the ingested cell corpses rather than degrade them (Loov et al. 2012), which may explain the appearance of multinuclear astrocytes in patient brain tissue.

Astrocytes' ineffective degradation is of great interest when it comes to the development and progression of neurodegeneration. For example, the majority of the patients with sporadic, late-onset AD do not have an increased A β production, indicating that the main cause of the disease may instead be the ineffective clearance by phagocytic cells. It has been shown that astrocytes derived from AD patients have a normal engulfment capacity, but altered degradation abilities (Nielsen et al. 2010; Nielsen et al. 2009). Moreover, it is known that patients with lysosomal storage disorders often develop neurodegenerative diseases including AD and Parkinson's disease (Appelqvist et al. 2013; Nixon et al. 2008).

It is well-described that there is an epidemiological association between traumatic brain injury (TBI) and the development of AD later in life (Fleminger et al. 2003; Shively et al. 2012). The cellular mechanism behind this link is still unclear, but it has been shown that A β plaques may be found following TBI (Johnson et al. 2010). At the time of impact, TBI results in death of neurons and glial cells and widespread axonal damage. The primary injury is followed by a complex secondary injury cascade that exacerbates the damage well outside the primary zone (Maas et al. 2008), a process that is going on for several years after the initial injury (Masel and DeWitt 2010).

Phagocytosis starts with the assembly of actin into the phagocytic cup that develops into the phagosome. The phagosome goes through a series of maturation steps by fusing with endosomes that changes the composition of the enclosing membrane, deliver digestive enzymes and acidify the lumen. Many of the proteins required for phago-lysosome fusion

have been identified, but the order in which they appear remains to be determined (Kinch and Ravichandran 2008). Two of the most well-known proteins involved in the maturation process are lysosome-associated membrane proteins 1 and 2 (Lamp1 and Lamp2) that are somewhat redundant, but expression of at least one is crucial for the final lysosomal maturation step (Huynh et al. 2007).

Whether astrocytes are able to elicit T cell responses remain controversial, but it is known that astrocytes express both major histocompatibility complex class II (MHC II) (Aloisi et al. 1998; Vardjan et al. 2012) (Dong and Benveniste 2001) and the co-stimulatory molecules B7-1 or B7-2 that are needed to activate T cells (Cornet et al. 2000; Nikcevic et al. 1997; Soos et al. 1999; Zeinstra et al. 2003). In order to preserve antigens for presentation via MHC II, antigen-presenting cells need to control the degradation process and protect the antigens from complete destruction (Zhou and Yu 2008). Hence, professional antigen-presenting cells, such as dendritic cells (DCs), delay acidification of lysosomes (Jancic et al. 2007; Savina et al. 2006) and have reduced levels of cathepsins, the lysosomal proteases (Delamarre et al. 2005). Rab27a is expressed by DCs in order to delay the degradation by [1] prolonging the stage of actin-coating around the phagosome (Yokoyama et al. 2011) and [2] reduce the acidity of lysosomes (Jancic et al. 2007; Kim et al. 2008). The actin coat works as a physical barrier that inhibits vesicular fusion, resulting in reduced acidity and a decreased activation of cathepsins that subsequently delays protein degradation. Rab27a reduces the acidity through the recruitment of Nox2 to the lysosomes (Jancic et al. 2007; Savina et al. 2006; Savina et al. 2009). Nox2 (also known as gp91^{phox}) is the subunit that make up the functional NADPH oxidase that produces superoxide anions in the lumen of the NADPH oxidase-containing vesicles (Cross and Segal 2004). The superoxide quickly reacts with hydrogen to produce reactive oxygen species in a proton consuming process, thereby rescue antigens from destruction (Jancic et al. 2007; Savina et al. 2006).

While delayed acidification and degradation may be beneficial for antigen-presenting cells, there is increasing evidence that insufficient lysosomal degradation is involved in the pathogenesis of different neurodegenerative diseases, including AD, Parkinson's disease and Huntington's disease (Appelqvist et al. 2013; Nixon et al. 2008). Decades of research have focused on the neuronal abnormalities in these brain pathologies, while much less attention has been given to astrocytes. Recent data, however, demonstrates that astrocytes are highly involved in various neurological disorders (Clarke and Barres 2013; Sofroniew 2009; Sofroniew and Vinters 2010) and understanding their role in lysosomal degradation is imperative.

Material and methods

Animals

All animal experiments followed the Swedish animal welfare legislation and the study was pre-approved by Uppsala Animal Ethics Committee, Uppsala, Sweden (Permit number: C 115/11). C57/BL6 mice were kept at 24°C in 12 h light/dark cycles with access to food and water *ad libitum*.

Astrocyte cell cultures

Dissected cortices from E14 mice were dissociated in GIBCO Hank's Balanced Salt Solution (x1) supplemented with 8 mM HEPES buffer solution and 50 units of penicillin and 50 mg of streptomycin per ml (Life Technologies), hereafter only referred to as HBSS. The cell suspension was centrifuged and resuspended in medium (GIBCO Dulbecco's Modified Eagle Medium (D-MEM/ F12) with GlutaMAX (x1) supplemented with 50 units $\times$ ml⁻¹ penicillin and 50 mg $\times$ ml⁻¹ streptomycin, 8 mM HEPES buffer, B-27 serum free supplement (x1)) with mitogens 10 ng $\times$ ml⁻¹ Fibroblast Growth Factor 2 (FGF2) (Life Technologies) and 20 ng $\times$ ml⁻¹ natural mouse Epidermal Growth Factor (EGF) (Becton Dickinson). The cells were grown into neurospheres in suspension and passaged every 2nd to 4th day by dissociation in HBSS and resuspension in new medium supplemented with mitogens at concentrations previously described. The neurospheres were dissociated in HBSS and plated as a monolayer at a concentration of 1.5×10^4 cells $\times$ ml⁻¹ on coverslips coated with poly-L-ornithine (Sigma-Aldrich Inc.) and laminin (Life Technologies). The first 24 h, the cells were maintained in EGF and FGF2 supplemented medium, before being replaced with mitogen-free medium to initiate cell differentiation. The medium was replaced in full every second to third day during the differentiation period of 8 days. Only neurospheres from passage 1–3 were used for experiments.

Macrophage cultures from spleen

Spleens from adult C57/BL6 female mice were harvested and mashed in 3 ml HBSS using a syringe plunger. The cell suspension was centrifuged, and the pellet was suspended in 1 ml of sterile red blood cell lysis buffer (2.075 g ammonium chloride, 0.25 g of potassium bicarbonate and 25 l of 0.5 M EDTA in 250 ml distilled water) and incubated in RT for 5 min. The cells were centrifuged and the supernatant carefully removed and the cells dissolved in DMEM with 10% fetal bovine serum (Life Technologies) and seeded on poly-L-ornithine coated glass coverslips (approximately 6 glasses/spleen). The plates were incubated in 37°C, 5 % CO₂, humidified air for 2 h followed by two washes to clear the non-adherent cells. The macrophages were cultured for 2 days prior to addition of dead cells for 1 d (Loov et al. 2012).

Preparation and addition of dead cells

Undifferentiated neural stem cells were dissociated as described above and transferred to a 60 mm in diameter untreated Petri dish. The dish was placed in a UV Chamber (GS Gene linker, BioRad) sans lid, and treated with first one burst of 240 mJ of UV-light, mixed well, and then a second burst of 240 mJ of UV-light, 480 mJ in total. The cells were collected and counted manually and added to the differentiated cell cultures at a concentration of 1.5×10^4 cells $\times$ ml⁻¹ for 3 d. At day 3, the cultures were washed twice in medium to clear any unattached, dead cells. The 3 d cultures were fixed at this time and the rest incubated for additional 2, 6, or 12 days in dead-cell-free medium before being fixed in 4 % paraformaldehyde or lysed.

pHrodo succinimidyl ester (pHrodo)

The pHrodo dye reacts with amine groups on the cell surface and is non-fluorescent at neutral pH, but emits red light at an increasing intensity as the pH is lowered. The dye has pKa of approximately 6.5. Dead, UV-treated neural stem cells were pre-stained with pHrodo dye as previously described (Loov et al. 2012) in medium for 45 min, in RT. The cells were washed once with HBSS and dissolved in medium before added to differentiated, neural cell cultures.

Inhibition experiments

Latrunculin B from *Latruncula magnifica* (Sigma-Aldrich) dissolved in 99.5 % ethanol was used to inhibit filamentous actin. After the dead cells were removed at day 3, Latr was added to the medium at a concentration of either 0.1 μ M or 1 μ M. The medium was then changed to new medium containing Latr every 2–3 days for the duration of the experiment. Two independent cultures were used in controls and three were treated with inhibitor. All analyses were analyzed for normal distribution before choosing statistical analysis. One-way ANOVA was used for actin-rings and Mann Whitney was used for dead cell area analyses. To inhibit Nox2 activity, Apocynin (Sigma-Aldrich) dissolved in 99.5 % ethanol was added to the cultures after the dead cells were removed. Three concentrations of Apo was used, 25 μ M, 100 μ M and 400 μ M. The medium was exchanged every 2–3 days with medium containing Apo. Two independent cultures were used for all treatments. Equivalent amounts of 99.5 % ethanol were added to the controls.

Staining techniques and visualization

All stained cells were fixed for 10–15 min at room temperature in 4 % paraformaldehyde in PBS. Primary antibodies used in this study included: Rabbit anti-Glial Fibrillary Acidic Protein (GFAP, 1:400, DakoCytomation), mouse anti-GFAP (1:400, Sigma), rabbit anti-Lysosome-associated membrane protein (Lamp) 1 (1:200, Abcam), rat anti-Lamp2 (1:200, Abcam), rat anti-LEAFTM Purified mouse I-A/I-E (MHC II, BioLegend, 1:200), sheep anti-Rab27a (1:200, R & D Systems), goat anti- Nox2 (1:200, Santa Cruz biotechnology). Secondary antibodies (IgG) were used at dilutions of 1:400 and were from Molecular Probes: AlexaFluor 350 against mouse or rabbit, AlexaFluor 488 antibody against rabbit, rat or sheep. AlexaFluor 555 against mouse or rabbit, AlexaFluor 647 antibody against mouse or rabbit. Alternative staining techniques included filamentous actin staining by Phalloidin-FITC (5 μ M in PBS, Sigma-Aldrich) at RT for 30 min. Cell culture coverslips were permeabilized and blocked for 30 min in 0.1% Triton X-100 (vol/vol, Sigma) and 5% normal goat serum (NGS) (vol/vol) in PBS. Incubation of primary antibodies was performed at either RT for 1–4 h or overnight at 4°C. Coverslips were washed three times in PBS before incubation with secondary for 1 h in RT. Mounting was done with hard set Vectashield with DAPI (Vector Labs) or in EverBrite (Biotium) without DAPI. LysoTracker red DND-99 (LysoT, Life Technologies) was used at 0.5 μ M to study acidic lysosomes and LysoSensor DND-153 (pKa ~ 7.5, LysoS, Life Technologies) was used at 1 μ M to study changes in lysosome acidification. LysoT excitation/emission maximum of 577/590 nm and LysoS has an excitation/emission maximum of 442/505 nm. The slightly alkalic pKa of LysoS mean that it has a higher fluorescence in non-acidic lysosomes than in acidic ones.

The dyes were incubated in cell culture medium at their designated concentrations for 2 h prior to fixation quickly followed by mounting.

A wide-field microscope (Zeiss AxioImager.Z1) was used for direct quantifications of actin-rings per field in x63 magnification. Confocal micrographs were taken with Zeiss 510 confocal microscope with a x40 magnification objective.

Transmission Electron Microscopy

Cells were seeded directly on the plastic surface but otherwise cultured as described above. Dead cells were incubated with differentiated neural cultures for 3 days washed and incubated for additionally days as described above before being washed once in PBS prior to fixation in 2.5% glutaraldehyde in sodium cacodylate buffer (SCB). The cultures were put in 4°C overnight and then rinsed with SCB for 10 min. The dishes were incubated in 1% osmium tetroxide in SCB at RT for 1 h followed by 10 min in SCB. Dehydration was performed with 70% ethanol for 30 min, 95% ethanol for 30 min and 99.7% ethanol for 1 h. The dishes were then rinsed with a little plastic and a new, thin layer of plastic was added to the cells for 2–4 h to permit evaporation of the alcohol. A second plastic layer was poured and left overnight before a thicker, newly made plastic layer was added. The dishes were incubated in RT for 1 h before polymerization in an oven at 60°C for 48 h. Plastic capsules attached to the plastic were used as handles during sectioning. Following sectioning, the specimens were studied in a Hitachi H-7100 transmission electron microscope.

Western blot analysis

Protein lysates were prepared by incubating the cells in lysis buffer (20 mM Tris pH 7.5, 0.5% Triton-X-100, 0.5% Deoxycholic acid, 150 mM NaCl, 10 mM EDTA, 30 mM NaPyroP) supplemented with 15% protease inhibitor cocktail (Roche) and sodium orthovanadate (Na₃VO₄) (0.1 M, Sigma) on ice for 30 min prior to 10 min centrifugation at 10.000xg at 4°C. Twenty mg protein was loaded on a 4–12% Bis Tris gel (NuPAGE, Life Technologies) and the gel was run at 175 V for 90 min in NuPAGE MES buffer (Life Technologies). The proteins were blotted using a PVDF filter (0.2 mm, Life Technologies) pretreated in methanol for 5 min and for 10 min in transfer buffer (Life Technologies). Transfer was done at 30 V for 60–90 min. The filter was blocked in 5% BSA in 0.2% Tween in PBS (PBS-T) for 60 min, washed twice briefly in PBS-T prior to incubation with primary antibody in 0.5% BSA and 0.2% PBS-T at 4°C over night. After washing off any unattached primary antibody, the filter was incubated with peroxidase-conjugated secondary antibody for 1 h at RT, washed in PBS-T and developed using enhanced chemiluminescence (ECL) system (GE Healthcare). Before rehybridization the filter was dehybridized in 0.4 M NaOH at RT for 10 min and washed 4×5 min in PBS-T. Primary antibodies used: rabbit anti-Lamp1 (1:2000, Abcam), rat anti-Lamp2 (1:2000, Abcam), rat anti-LEAFTM Purified mouse I-A/I-E (MHC II, BioLegend, 1:1000), sheep anti-Rab27a (1:1000, R & D Systems), goat anti-Nox2 (1:500, Santa Cruz Biotechnology), rat-Cathepsin D (1:1000, R & D Systems), goat-Cathepsin S (1:1000, LifeSpan Biosciences), and rabbit anti-GAPDH (1:2000, Imgenex) as a loading control. Horseradish peroxidase-conjugated secondary antibodies used (all diluted 1:25.000): donkey anti-rabbit (GE Healthcare), goat anti-rat (GE Healthcare), rabbit anti-sheep (Life Technologies) and rabbit anti-goat (Life Technologies). Intensities of band were

measured from 3 independent experiments using ImageJ software and analyzed for relative changes in expression using one-way ANOVA in GraphPad Prism 5 software.

Nanoparticle experiments

Three different NPs with different acidic capacity were used: PLGA Resomere® RG 502H (NP1) has the greatest acid number at over 6 mg KOH×g⁻¹, PLGA Resomere® RG 503H (NP2) the second greatest at over 3 mg KOH×g⁻¹ and PLA Resomere® R 203S (NP3) has an acidification potential of around 1 mg KOH×g⁻¹ (Baltazar et al. 2012). One mg×ml⁻¹ of the different NPs were added to the medium after the dead cells were removed at day 3. After 2 days of incubation, the cells were thoroughly washed and fixed (3+2 d) or incubated without NPs for 4 additional days (3+6 d). Parallel cultures were incubated with Nile Red pre-stained NPs (rNP1, rNP2 and rNP3, respectively) to be able to follow the ingestion of the particles.

Area and intensity measures, cell counting and statistics

Images for quantifications were taken with x40 objective on a Zeiss AxioImager.Z1 with the same settings for all experiments. Analyses of dead cells over time were done in ImageJ. The images were analyzed manually, in a blinded fashion. A minimum of 10 images of 2 or 3 independent cultures were analyzed per experiment. The LysoS intensity and LysoT area were analyzed with the free and open-source software CellProfiler (www.cellprofiler.org) (Carpenter et al. 2006). All area and intensity measurements of LysoS and LysoT were set manually in CellProfiler. The total pixel area of LysoT per image was analyzed in 15 fields in 2 independent experiments and plotted as median ± interquartile range to display the distribution. LysoS' total intensity (integrated intensity) was measured per field in 15 fields in 2 independent experiments. None of the data was normally distributed when analyzed with Shapiro-Wilks normality test and therefore plotted as medians ± interquartile range. Nonparametric Kruskal-Wallis was used to study changes over time and non-parametric Spearman correlation analyses were performed between the area of dead cells and the intensity of LysoS. Actin-rings were counted manually in 10 randomly chosen fields in three independent experiments and plotted as the mean ± SD. The actin-rings were tested for normal distribution (Shapiro-Wilks) before being analyzed for differences over time by oneway ANOVA.

Results

Degradation of ingested dead cells in astrocytes is extremely slow.

The discrepancy between the degradation speed of dead cells in astrocytes and microglia made it important to exclude microglia from the culture system in this study. Hence, we used a cell culture model based on E14 mouse neural stem cells that give rise to astrocytes, oligodendrocytes and neurons, but not microglia (Ravin et al. 2008). The stem cells were differentiated for 8 days, resulting in a culture with predominantly astrocytes that were treated with apoptotic cells for 3 days, as we previously found that astrocyte engulfment then reaches a plateau (Loov et al. 2012).

In order to investigate the degradation capacity of the astrocytes, the cultures were incubated for additional 2, 6 or 12 days in medium only (3+2 d, 3+6 d, and 3+12 d, respectively). After fixation and DAPI incorporation, the cell cultures were micrographed (10 fields in three independent cultures per time point) and the area analyzed with ImageJ. Apoptotic cells have smaller and condensed nuclei that stain intensively with DAPI compared to the nuclei of live cells (Fig. S1 A). By using area rather than the number of dead cells, the partly digested, fragmented, dead cells get a lower value than whole, non-digested cells which is a more accurate way to calculate digestion in cases where the dead cells divide into several compartments or are only partly degraded. Importantly, the fluorescence of DAPI is insensitive to pH over the range of 3–8 (Szabo et al. 1986). The area comprised by dead cell nuclei decreases over time, which demonstrates that although astrocytes are extremely slow, degradation of ingested cells occurs with enough time (Fig. 1A). At 3 d, the highly significant increase ($p<0.001$) in dead cells show that astrocytes have ingested many of the added dead cells (Fig. 1A). At 3+12 d, the area of dead cells is back to background (0 d) and show that the engulfed, dead cells have been degraded after 12 days. There was a slight increase in live cell nuclei area by 3+12 d compared to day 3 ($p<0.05$, Fig. S1 B), which indicate that the addition of dead cells may have a modest proliferative effect on the live cells.

To elucidate how the phagosomes of the engulfing astrocytes mature over time, dead cells were pre-labeled with the pH-dependent dye pHrodo before being added to the differentiated cultures. The low intensity of the dye indicated that the phagosome pH was relatively alkaline throughout the experiment (Fig. 1B, Fig. S1 C and D). To study whether the low intensity of pHrodo was dependent on impaired phago-lysosome fusion or if the lysosomes were alkaline all together, the cultures were incubated with LysoTracker Red DND-99 (LysoT) and LysoSensor Green DND-153 (LysoS). LysoT dye require protonation and labels all acidic organelles, whereas LysoS measure relative changes in pH, in which a higher pH increases the fluorescence and a low pH decreases it. The Lyso S experiments verified that the astrocytic lysosomes are indeed rather alkaline (Fig. 1C and Fig. S2). Interestingly, there is a positive correlation between the area of the dead cell nuclei and the intensity of LysoS ($p<0.0001$, $r=0.270$) (Fig. 1D), indicating that the acidification is reduced in astrocytes with a high load of phagocytosed, dead cells. In line with the LysoS results, LysoT was very low throughout the experiment (Fig. 1E and Fig. S2).

Astrocytes have a high expression of Lamp1 and Lamp2 which localizes to the ingested, dead cells.

Western blot analysis (WB) of Lamp1 and Lamp2 expression show that both proteins are expressed at high levels in the cell cultures at all time points (Fig. 2A and B). Immunocytochemical staining demonstrates that the Lamp-proteins change location over time to co-localize more with dead cells. The co-localization is most apparent at 3+6 d, but found around some vesicular compartments also at the other time points (Fig. 2B). Astrocytes cultured without addition of dead cells show a wide, vesicular distribution of Lamp2 (Fig. S3).

Filamentous actin surrounds engulfed dead cells but do not delay the degradation.

Transmission electron microscopy (TEM) shows that filamentous actin surrounds the astrocyte-ingested dead cells (green arrows in Fig. 3A and A'). To determine if prolonged actin coating around the phagocytosed, dead cells delayed the degradation, 0.1 μM or 1 μM Latrunculin B (Latr), an actin severing mushroom toxin, was added to the cultures. The number of dead-cell-containing actin rings was counted in ten randomly chosen fields in three independent cultures at 3+2 d, 3+6 d, and 3+12 d (Fig. 3B). One-way ANOVA analyses showed that the number of rings remained stable over time in the control cultures (with medium only). The lower concentration of 0.1 μM of Latr significantly reduced the actin rings at 3+2 d ($p < 0.01$), but not at later time points. The ratios of actin rings per live cell were significantly reduced at all time points with 1 μM of Latr ($p < 0.001$) (Fig. 3C). Although Latr reduced the number of actin-rings, the treatments had only a slight effect on the degradation of dead cells at the latest time point ($p < 0.05$, Fig. 3D).

Rab27a is highly expressed in the cultures and may delay the acidification of lysosomes

We next sought to investigate if the actin-rings and the low acidification were the result of Rab27a expression. WB analyses of cell lysates show a high expression of the Rab27a protein throughout the experiments (Fig. 4A). Similarly to the Lamp proteins, immunocytochemical stainings of Rab27a show that the location of the expressed protein changes over time. The protein is located around the astrocyte-ingested, dead cells, in vesicular structures that are noticeable at days 3+2 to 3+12 (Fig. 4B). Rab27a plays an important role in vesicular transport and exocytosis (Seabra et al. 2002) including vesicular translocation to and from the phago-lysosomes and the high expression in the cultures indicates a possible role of Rab27a in the maintenance of the high pH in astrocytic lysosomes.

Co-localization of Nox2 and engulfed cells indicate a possible NADPH oxidase driven alkalization.

WB analyses were performed to determine the expression of Nox2 in cultures treated with dead cells (Fig. 5A). Similar to Rab27a, the localization of Nox2 changes over time. At day 3, it localizes to the nuclei of the cells, but over time Nox2 become more and more concentrated to vesicular compartments that partly co-localize to the membrane surrounding the dead cells (Fig. 5B). To block NADPH oxidase activity, the Nox2 inhibitor apocynin (Apo) was added to the cell cultures. Apo is commonly used and has previously been shown to successfully inhibit oxidative stress in primary astrocytes induced by A β or toxins (Abramov and Duchon 2005; Abramov et al. 2005; Zhu et al. 2009). Apocinin has also been shown to block IL-1 β -induced ERK1/2 phosphorylation (Jensen et al. 2009) and neural cell death induced by toll like receptors in a neuron-astrocytes co-culture (Ma et al. 2013). In the present study, we used three different concentrations of Apo (25, 100, or 400 μM), but none of the concentrations had an impact dead-cell degradation (Fig. 5C).

Nanoparticle treatment greatly enhances the acidification of lysosomes and promotes the degradation of dead cells.

Three different nanoparticles (NPs) with different acidic capacity were added in order to enhance the acidification of astrocytic lysosomes (Baltazar et al. 2012). One $\text{mg} \times \text{ml}^{-1}$ was added to the medium after the dead cells were removed at day 3 and incubated with the cells for 2 d. Parallel cultures were incubated with NPs labeled with Nile Red stain (rNPs). Immunocytochemical stainings of Lamp2 show that the rNPs were indeed engulfed by the astrocytes as Nile Red co-localizes with the Lamp2 at 3+2 d (Fig. 6A).

LysoT was used to study the effects of the NPs on lysosome acidification and subsequent degradation. There is a drastic upregulation in acidification in NP1 and NP2 treated cultures compared to controls that made quantifications of the area or intensity impossible due to saturation in the color channel. However, NP3 show no apparent effect on the protonation of LysoT compared to controls (Fig. 6B). The effect of NP1 and NP2 on lysosomal acidification remained significantly higher than controls also at 3+6 d. Interestingly, only the most acidic NP, NP1, has a significantly positive effect on degradation and shows a greater degradation of dead cells compared to control cultures at both 3+2 d and 3+6 d ($p < 0.001$) (Fig. 6C).

Cathepsin D (Cat D) activation is dependent on a low pH whereas Cathepsin S (Cat S) is optimal at neutral or slightly alkaline pH (Savina and Amigorena 2007). WBs of CatD and Cat S show that astrocytes have lower amount of Cat D than control MØs. Interestingly, all the Cat D was activated in astrocytes independently of the addition of NP1, in contrast to the MØs that contained unprocessed, pro-Cat D in addition to the higher levels of active Cat D. The total expression did not change over time or with increased acidity, indicating that Cat D was expressed to a maximum in astrocytes (Fig. 6D). Cat S was only expressed at very low levels in astrocytes compared to the MØs, and the expression was not significantly changed in response to the NP1-induced decrease in pH or over time (Fig. 6D).

The normalization over time of the degradation of dead cells raised the question whether Nox2 was upregulated by the astrocytes in order to suppress the enhanced acidity. To test this, WBs of cultures treated with NP1, the most acidic NP, were performed (Fig. 6E). The result shows that the treatment of NP1 did not significantly impact on the expression of Nox2 compared to controls (Fig. 6E).

Preservation of antigens for MHC II presentation may explain the alkalinized lysosomes in astrocytes.

Astrocytes' ability to cross-present antigens and elicit T cell responses via MHC II receptors remains controversial (Dong and Benveniste 2001), but could be a reason why the acidification of lysosomes are so tightly controlled in astrocytes. Dead cells, labeled with pHrodo were added to differentiated cultures for 3+12 d and stained with antibodies against MHC II (Fig. 7A). The MHC II is particularly located to the pHrodo-positive vesicles, but also show a scattered expression throughout the astrocytes, which may point to surface exposure (Fig. 7A). WBs show that the relative expression of MHC II in the cultures remains stable over time (Fig. 7B) and did not change by NP1-increased acidification (Fig.

7C), although the peptides loaded on the MHC II may have changed. Immunostainings of primary mouse MØs demonstrate a vesicular expression of MHC II similar to the pattern seen in the astrocytes. WB analysis shows that although astrocytes clearly express MHC II, the expression level of the protein is much lower than in MØs (Fig. S4).

Discussion

Phagocytosis by astrocytes has been described in several studies (Chang et al. 2000; Chung et al. 2013; Fellner et al. 2013; Jones et al. 2013; Loov et al. 2012; Magnus et al. 2002; Sokolowski et al. 2011), but the molecular mechanisms of astrocyte degradation remain elusive. Persons with genetic mutations in the degradative pathway often present with early onset neurodegeneration (Appelqvist et al. 2013; Nixon et al. 2008; Platt et al. 2012) and astrocytes derived from AD patients have been shown to have normal engulfment capacity, but reduced degradation abilities (Nielsen et al. 2010; Nielsen et al. 2009). These results highlight the importance of fully understanding, not only what cells are phagocytic, but also what happens to the material after engulfment.

In our previous study we compared phagocytosis by astrocytes and macrophages (which are indistinguishable from activated microglia in the brain after TBI). We found that astrocytes, effectively engulf dead cells, but then accumulate the ingested material as degradation is delayed or impaired compared to macrophages. By using the pHrodo-labeling technique we demonstrated that cell corpses ingested by macrophages fuse with lysosomes within 5 hours, whereas dead cells engulfed by astrocytes had still not fused with the lysosomes after 3 days (Loov et al. 2012). Hence this study was conducted to elucidate whether there was a delay in the phagosome maturation or if the astrocytes failed to acidify altogether.

We found that astrocytes do degrade the ingested material, but at an extremely slow rate. Since the astrocytes tend to accumulate an abundance of material, they may have problems supporting the neurons, which in turn would have detrimental consequences (Di Malta et al. 2012). Compared to MØs, that start to digest phagocytosed cell corpses directly (Loov et al. 2012), it takes almost two weeks for astrocytes to degrade the ingested, dead cells, which was hypothesized to depend on their relatively alkalic lysosomes shown by pHrodo, LysoS and LysoT stainings. Interestingly, LysoS intensity correlates positively with dead-cell area, indicating that the delay in degradation could be an inhibitor in itself, as a higher load of dead cells leads to less acidic lysosomes. A large phagocytic burden has previously been shown to inhibit degradation in macrophages by prolonging the time actin surrounds the phagosome (Liebl and Griffiths 2009). We show that actin-rings remain around the dead cells for long periods of time in the astrocytes, but severing actin with Latr did not speed up the degradation of dead cells. In contrast to the macrophages studied by Liebl and Griffiths (2009), the astrocyte-ingested cells co-localize with Lamp2, further invalidating actin in the slow degradation.

The neutral or even alkalic pH in the lysosomes of DCs is dependent on Nox2 (Jancic et al. 2007; Savina et al. 2006; Savina et al. 2009). Nox2's translocation to the lysosomes is in turn dependent on Rab27a expression and either Nox2 or Rab27a deficiency leads to an increased acidification in DCs (Jancic et al. 2007). The tightly controlled acidification in

DCs is connected to their role as antigen-presenting cells (APCs) where the material needs to be processed into smaller peptides that can be loaded on MHC II (Bryant and Ploegh 2004; Hiltbold and Roche 2002). Moreover, Rab27a may negatively regulate degradation by prolongation of the actin-coating stage around phagosomes. It has been demonstrated that actin-related processes, including F-actin coating and F-actin degradation, proceed more rapidly in Rab27a knockdown cells than in control cells. It is also known that Rab27a mutations cause type 2 Griscelli syndrome, which is characterized by uncontrolled macrophage activation and immunodeficiency (Yokoyama et al. 2011). Recently it has been shown that Nox2-dependent antigen preservation is cell type specific and that a certain subset of phagosomes are responsible for the antigen processing (Allan et al. 2014; Romao et al. 2013).

Our results suggest that similar to DCs, Rab27a and Nox2 alkalize the lysosomes in astrocytes. However, Apo did not influence the digestion or the pH of lysosomes, indicating that additional factors are involved, for example low concentrations of degradative enzymes.

By lowering the pH in the astrocytic lysosomes with NPs we predicted an increase in the degradation, since acidification is known to activate the lysosomal cathepsins (Savina and Amigorena 2007). Moreover, acidification may also break the feed-back loop, in which the elevated pH induces redistribution of cathepsins out of the cells rather than to fuse with the lysosomes (Claus et al. 1998). Interestingly, only the most acidic of the particles had a positive effect on the degradation of dead cells. This indicates that degradation in astrocytes is controlled by several inhibitory mechanisms that only the most acidic of the NPs were able to overcome. However, the inhibition may also lie in other factors besides the activity of pH-dependent cathepsins since the activity of Cat D remained unaffected by the lowered pH.

Hence, the question remains; why are astrocytes such good phagocytes but such bad degraders? One reason could be that, similarly to DCs, astrocytes need to preserve antigens. We show here that the astrocytes express MHC II which is particularly located to the lysosomes where the receptors are loaded. Addition of dead cells had no impact on the MHC II expression, but Hoffmann *et al.* (2012) showed that the levels of presentation remain constant even though the concentrations of antigen change. Another reason is that astrocytes are not equipped to handle an overload of dead cells compared to the professional phagocytes. The next question would be; are phagocytic astrocytes good or bad? Previously, we have shown that astrocytes can save neurons from contact induced apoptosis *in vitro* by removing the dead cells from the immediate environment (Lööv et al. 2012). Since the professional phagocytes are faster at degrading the ingested material, according to our *in vitro* data, but are fewer than the astrocytes, it is hard to accurately estimate the respective cell types' significance in the clearing of dead cells *in vivo*. However, one can speculate that the astrocytes situated in the injured tissue might be important for cell survival directly after an insult, before recruitment of professional phagocytes has taken place. The astrocytes may become phagocytic if there is an overload of dead cells that the professional phagocytes cannot handle or they may be responsible for the clearance later, when the inflammatory response has declined. It is known that the removal of dead cells from the tissues induce a release of chemoattractants that can recruit professional phagocytes to the area of need (Poon et al. 2010), and that engulfment of apoptotic cells also promote survival and cell

growth (Krysko et al. 2006). However, long-term storage by the astrocytes has been shown to induce neuronal degeneration (Di Malta et al. 2012) and lysosomal storage disorders are known to cause early onset neurological deficits (Platt et al. 2012). A growing body of research suggests that astrocytes contribute to the pathophysiology of AD (Avila-Munoz and Arias 2014) and epidemiological studies have found that prior neurotrauma increase the risk of the development of dementias (Shively et al. 2012). One can hypothesize that the long time storage in astrocytes can induce or alter neuronal physiology which can lead to degeneration and brain atrophy. In order to understand the underlying causes of neurodegenerative diseases, future research needs to be focused on uptake and degradation of cell corpses and toxic protein aggregates by different types of cells in the brain, including astrocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Main points:

- Astrocytes have several restraints in place to limit the digestion of engulfed material, particular in respect to the alkaline nature of their lysosomes.
- Lysosomal acidification by nanoparticles transiently increases the degradation.

Figure 1.

Astrocyte-ingested dead cells are degraded extremely slowly. (A) The total area of dead cells per field was quantified in three independent experiments. By 3+6 d, the degradation had started, but the number of dead cells was still significantly higher compared to day 0. Twelve days after the removal of the dead cells (3+12 d), the degradation of the ingested cells is complete and back to the background levels seen at 0 d. (B) pHrodo-labeled, apoptotic cells were added to the differentiated cell cultures. During the initial 3 d and 3+2 d the acidification of the added, pHrodo-labeled dead cells is minimal. By 3+6 d some of the dead cells are acidified, but it is not until 3+12 d that the pHrodo-staining is more pronounced. (C) The total intensity (integrated intensity) of LysoS per field shows that the lysosomes in astrocytes are rather alkalic. (D) A strong correlation of ingested dead cells and LysoS intensity ($p = 0.001$, $r=0.270$) demonstrates that a higher load of dead cells reduces the acidity of lysosomes. (E) The total area of LysoT per field shows that the acidic lysosomes were few and small. Fifteen fields were analyzed in two independent experiments (C-E). The lines depict the median $\pm$ interquartile range (A and C-E), $*=p < 0.05$, $**=p < 0.01$, $***=p < 0.001$.

Figure 2.

Lamp-proteins are highly expressed around the dead cells in astrocytes. (A) WB analyses of cultures that have received dead cells show that both Lamp1 and Lamp2 are highly expressed throughout the experiment. Measures of three independent experiments were normalized against GAPDH and relative expressions were calculated and plotted as mean $\pm$ SD. (B) Confocal micrographs show that both Lamp1 and Lamp2 are located around the dead cells in astrocytes (blue arrow heads) and are especially visible at 3+6 d.

Figure 3.

Filamentous actin remains around the dead cells for a long time after engulfment. (A) TEM shows an astrocyte with a newly engulfed dead cell surrounded by actin (green arrowheads). The astrocytes' nucleus is denoted with Nu. (A') A higher magnification of the area marked by the white rectangle in (A), show the filaments around the phagocytosed dead cell. (B) Stainings with phalloidin-FITC (green) show the actin-rings that surround the dead cells (condensed, white nuclei) in astrocytes. (C) Quantification of the actin rings in 10 fields of 3 independent cultures show that the rings remain for long times in control cultures, and that continuous treatment with 1 μM Latr significantly reduces the actin-rings throughout the experiment. The lower concentration, 0.1 μM, only had an effect early after addition (3+2 d). (D) Quantification of the area of dead cells shows that inhibition of actin with 1 μM Latr has only a minor effect on the degradation rate at the last time point. A minimum of 10 fields were analyzed in three independent experiments. The lines depict the median±interquartile range, **= $p < 0.01$, ***= $p < 0.001$.

Figure 4.

Rab27a is highly expressed in the cultures and partly co-localize with dead cells. (A) WB analysis of Rab27a expression after addition of dead cells was measured in three independent cultures and normalized against GAPDH. (B) Confocal images show that the expression pattern of Rab27a changes over time. At day 3, the protein is localized to the nuclei and only very little is distributed to vesicle-like compartments. At 3+2 d, 3+6 d and 3+12 d, Rab27a is located to larger vesicles partly co-localized to dead cells (blue arrow heads).

Figure 5.

Nox2 partly localizes to dead cells and may contribute to the lysosomal alkalization. (A) WB analysis of Nox2 expression after addition of dead cells was measured in three independent cultures and normalized against GAPDH. (B) Nox2 stainings show a nuclear expression at day 3, but localizes to vesicular compartments that partly overlaps with engulfed, dead cells by 3+2 d. These compartments are most apparent at day 3+6, before becoming more scattered by 3+12 d. Blue arrow heads indicates vesicular Nox2 staining that are co-localized with the membranes surrounding the engulfed dead cells. (C) Quantification of the area of dead cells shows that inhibition of Nox2 activity with Apo does not change the rate of degradation. Fifteen fields were analyzed in two independent experiments and plotted as the median $\pm$ interquartile range.

Figure 6.

Acidic NPs are taken up by the astrocytes, acidify the lysosomes and enhance the degradation. (A) NPs with three different acid capacities were added to the cell cultures for two days directly after the removal of the dead cells. At 3+2 d, the Nile Red labeled NP (rNP1, rNP2 and rNP3) can be detected in the astrocyte lysosomes with Lamp2 stainings. Confocal micrographs show that the rNPs are positive for Lamp2 and partly overlap with dead cells. (B) LysoT and LysoS was incubated with the cultures initially treated with dead cells for 3 days before adding the NPs. NP1 and NP2, the two NPs with the highest acid number, showed an intense increase in LysoT, indicating an elevated acidification of astrocytic lysosomes, but NP3 was indistinguishable from the controls. (C) Only the most acidic NP, NP1 has a significantly positive effect on degradation and shows a greater degradation of dead cells compared to control cultures at both 3+2 d and 3+6 d. Fifteen fields were analyzed in two independent experiments and plotted as the median $\pm$ interquartile range, **= p 0.01, ***= p 0.001. (D) WB analyses of pro-Cat D, active Cat D and Cat S expression in controls (Ctrl) and NP1 treated cultures. (E) WB analyses of Nox2 expression in ctrl and NP1 treated cultures. Measures of three independent experiments were normalized against GAPDH and relative expressions were calculated and plotted as mean $\pm$ SD (E-D).

Figure 7.

MHC II is expressed by the astrocytes. (A) The pHrodo-positive vesicles in the astrocytes also stain for MHC II, but the scattered expression of MHC II throughout the cells may indicate surface exposure of loaded receptors. (B) WB analyses from three independent experiments were measured and normalized against GAPDH. (C) The expression of MHC II was measured after addition of NP1 and compared to Ctrl in three independent experiments after GAPDH normalization.