

ORIGINAL
ARTICLE

Inflammation induces multinucleation of Microglia via PKC inhibition of cytokinesis, generating highly phagocytic multinucleated giant cells

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Abstract

Microglia are brain macrophages, which can undergo multinucleation to give rise to multinucleated giant cells that accumulate with ageing and some brain pathologies. However, the origin, regulation and function of multinucleate microglia remain unclear. We found that inflammatory stimuli, including lipopolysaccharide, amyloid β , α -synuclein, tumour necrosis factor- α and interferon γ , but not interleukin-4, induced multinucleation of cultured microglia: primary rat cortical microglia and the murine microglial cell line BV-2. Inflammation-induced multinucleation was prevented by a protein kinase C (PKC) inhibitor Gö6976 (100 nM) and replicated by a PKC activator phorbol myristate acetate (160 nM). Multinucleation was reversible and not because of

cell fusion or phagocytosis, but rather failure of cytokinesis. Time-lapse imaging revealed that some dividing cells failed to abscise, even after formation of long cytoplasmic bridges, followed by retraction of bridge and reversal of cleavage furrow to form multinucleate cells. Multinucleate microglia were larger and 2–4 fold more likely to phagocytose large beads and both dead and live PC12 cells. We conclude that multinucleate microglia are reversibly generated by inflammation via PKC inhibition of cytokinesis, and may have specialized functions/dysfunctions including the phagocytosis of other cells.

Keywords: abscission, Alzheimer's disease, HIV-associated dementia, microglia, neuroinflammation, phagocytosis. *J. Neurochem.* (2013) 10.1111/jnc.12477

Multinucleate cells form from mononucleate cells in a variety of physiological and pathological conditions depending on cell type. For example, skeletal muscle fibres and osteoclasts are normally multinucleate in adult mammals because of cell fusion into a syncytium. During chronic inflammation, as a result of persistent infection or foreign bodies, macrophages can form so-called multinucleated giant cells (MGCs). The function of such MGCs is unclear and they may even lack a function or be dysfunctional (Quinn and Schepetkin 2009; McNally and Anderson 2011). Multinucleate cells may in principle arise either from fusion (Zhou and Platt 2011), phagocytosis (McNally and Anderson 2005) or failure of cytokinesis (Normand and King 2010), but in general it is assumed that MGCs arise from fusion (Vignery 2008; Helming and Gordon 2009; Quinn and Schepetkin 2009; McNally and Anderson 2011).

Microglia are resident brain macrophages, which mediate innate immunity and phagocytosis of debris in the brain, but inflammatory activation of microglia is implicated in the pathology of multiple neurological diseases (Perry *et al.* 2010). Microglia can form MGCs when inflammatory activated in particular ways. In cultured rat microglia, MGC

formation was found to be stimulated by interleukin-3 (IL-3), IL-4, interferon gamma (IFN- γ), granulocyte macrophage-colony stimulating factor (GM-CSF) and phorbol myristate acetate (PMA), while IL-1, IL-6 and tumour necrosis factor alpha (TNF- α) had no effect (Lee *et al.* 1993). In cultured murine microglia, single cytokines failed to induce MGC, but IL-4 and IL-13 induced microglial MGC formation in the

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Abbreviations used: A β , amyloid beta; CFSE, 5(6)-carboxyfluorescein diacetate-*N*-succinimidyl ester; cRGDFV, cyclo (Arg-Gly-Asp-D-Phe-Val); DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; GCA, giant-cell arteritis; GM-CSF, granulocyte macrophage-colony stimulating factor; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; Hoechst, Hoechst 33342; IFN- γ , interferon gamma; IL, interleukin; LPS, lipopolysaccharide; MGC, multinucleated giant cell; PBS, phosphate buffered saline; PEG, polyethylene glycol; PKC, protein kinase C; PMA, phorbol myristate acetate; TAMRA, 5-(and-6)-carboxytetramethylrhodamine succinimidyl ester; TNF- α , tumour necrosis factor alpha; α -Syn, alpha-synuclein.

presence of colony stimulating factors (Suzumura *et al.* 1999). However, in cultured pig microglia MGC formation was stimulated by mycobacteria, TNF- α (Peterson *et al.* 1996) and IFN- γ and inhibited by GM-CSF (Tambuyzer and Nouwen 2005). Phagocytosis of cell debris has also been found to stimulate microglia to form MGCs (Beyer *et al.* 2000).

Multinucleated giant cells derived from microglia have been implicated in a variety of brain pathologies. In particular, HIV-associated dementia is mediated by HIV-infected microglia which become MGCs, but how these infected multinucleate microglia cause dementia is less clear (Ghorpade *et al.* 2005; Nardacci *et al.* 2005). Microglia were also reported to form MGCs in the spinal cord of rats expressing a mutant Cu/Zn superoxide dismutase gene, modelling amyotrophic lateral sclerosis in humans (Fendrick *et al.* 2007). Giant-cell arteritis (GCA) is an inflammatory disease of arteries of the head, characterised by the presence of MGCs in the vessel wall. Amyloid beta (A β)-related angiitis, a form of GCA, is associated with an accumulation of microglia and MGCs containing intracellular deposits of A β (Melzer *et al.* 2012). Whether A β can induce microglia to form MGCs is unknown. MGCs derived from microglia accumulate in the brain with age (Hart *et al.* 2012). Thus, it is important to understand the origin and function of multinucleate microglia.

In this work, we tested whether agents known to inflammatory activate microglia could induce MGC formation by microglia. We found that lipopolysaccharide (LPS), A β , alpha-synuclein (α -Syn), dead PC12 cells, IFN- γ and TNF- α , but not IL-4, induced microglial multinucleation, apparently via protein kinase C (PKC). We investigated whether multinucleation was because of fusion, phagocytosis or blocked cytokinesis. We found that microglial multinucleation was because of failure of abscission, followed by reversal of the earlier stages of cytokinesis. Microglial MGCs appear normal, apart from being multinucleate and large, and have an increased phagocytic capacity.

Materials and methods

Materials

Lipopolysaccharide (LPS; *Salmonella enterica* serotype typhimurium), MRS2578, 5(6)-carboxyfluorescein diacetate-*N*-succinimidyl ester (CFSE), tumour necrosis factor alpha (TNF- α) and phorbol myristate acetate (PMA) were purchased from Sigma, amyloid beta 1–42 (A β) from EZBioLab, interferon gamma (INF- γ) from R&D Systems, alpha-synuclein 1–140 (α -Syn) from rPeptide, interleukin-4 (IL-4) from ImmunoTools, cyclo(Arg-Gly-Asp-D-Phe-Val) (cRGDFV) from Bachem, Gö6976 from Calbiochem, Alexa 488-labelled *Griffonia simplicifolia* isolectin-B₄ and 1 μ m fluorescent carboxylate-modified microspheres from Invitrogen, 5 μ m fluorescent carboxyl particles were from Spherotech, 5-(and-6)-carboxy-tetramethylrhodamine succinimidyl ester (TAMRA) from Biotium Inc., anti-Ki-67 antibody from Millipore, anti-IL-6 antibody from Abcam and goat anti-rabbit-Cy3 and goat anti-mouse-Cy3 second-

ary antibodies from Jackson ImmunoResearch Laboratories. All tissue culture medium was supplemented with 100 units/mL penicillin G and 100 μ g/mL streptomycin sulphate (PAA Laboratories, Austria). Unless indicated otherwise, all other materials were purchased from Sigma.

Cell culture

The murine microglial cell line BV-2 (Blasi *et al.* 1990; Bocchini *et al.* 1992) (passage < 30) was maintained in a glia medium consisting of Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% foetal bovine serum (FBS; PAA Laboratories) in 75 cm² flasks (Nunc) at 37°C, 5% CO₂. At confluence, cells were harvested using 0.5% trypsin (Invitrogen) in phosphate buffered saline pH 7.2 (PBS; Invitrogen) and seeded at 4×10^4 cells/well in DMEM supplemented with 0.5% FBS in 24-well plates (Nunc). Rat pheochromocytoma cells (PC12) (Greene and Tischler 1976) were maintained in Roswell Park Memorial Institute medium (Invitrogen) supplemented with 10% horse serum (PAA Laboratories) and 5% FBS, in flasks coated with 0.5 mg/mL collagen type IV.

Primary cell culture

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act (1986) and approved by the Cambridge University local ethical committee. Primary mixed glial cultures were prepared from the cortices of post-natal day 5–7 Wistar rat pups (both male and female, bred and housed locally) as described previously (Bal-Price and Brown 2001) then seeded in glia medium at 2×10^5 cells/well in 24-well plates coated with $5 \times 10^{-4}\%$ poly-L-lysine and allowed to mature *in vitro* for 6–8 days prior to treatment. Pure microglial cultures were prepared from mixed cortical glial cultures as described previously (Bal-Price and Brown 2001) then seeded in astrocyte conditioned medium mixed 1 : 2 with fresh glia medium at 7×10^4 cells/well in 24-well plates coated with $5 \times 10^{-4}\%$ poly-L-lysine.

Treatments

Cells were allowed to adhere for 24 h, then stimulated with LPS (100 ng/mL), IFN- γ (10 ng/mL; 0.6 nM), TNF- α (50 ng/mL; 2 nM), PMA (100 ng/mL; 160 nM), IL-4 (1–100 ng/mL; 0.07–7 nM), α -Syn (500 nM), A β (1 μ M) or cytochalasin D (0.5 μ M) for 24 h. Where indicated, cRGDFV (50 μ M), MRS2578 (1 μ M) or Gö6976 (100 nM) were added 30 min beforehand. PC12 were killed by treatment with staurosporine (10 μ M) for 24 h, washed and resuspended in PBS, then added at 1×10^4 /well for 24 h (the proportion of necrotic PC12 cells was $95 \pm 1\%$, as quantified using a 0.2% trypan blue solution).

Preparation of A β and α -Syn

1 mg of A β peptide was dissolved in 200 μ L of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) then HFIP removed by evaporation and aliquots kept at -80°C . A β was resuspended in dimethyl sulfoxide at a concentration of 2 mM and added to ice-cold DMEM at a concentration of 50 μ M. To prepare A β 'oligomers', the solution was incubated at 4°C for 24 h then centrifuged at 10 000 g for 5 min and the supernatant collected, as described previously (Stine *et al.* 2003). α -Syn peptide was resuspended in PBS at a concentration of 1 mg/mL and aliquots stored at -80°C . To prepare

aggregated α -Syn, aliquots were incubated at 37°C for 7 days then kept at –80°C.

Quantification of multinucleation

Cultures were incubated with the nuclear stains Hoechst 33342 (Hoechst; 4 μ g/mL; blue channel) and propidium iodide (4 μ g/mL; red channel), imaged using a Leica DMI6000 microscope (20 \times magnification; field size = $1.9 \times 10^5 \mu\text{m}^2$) and live cells counted and scored for multinuclearity using ImageJ software (National Institutes of Health, Bethesda, MD, USA) (two or more nuclei per cell, distinguished by Hoechst staining and bright field). Dead cells were identified by nuclear morphology (chromatin condensed – apoptotic) or permeable plasma membrane (staining with propidium iodide – necrotic) and were not included in live cell counts. In primary glial cultures isolectin-B₄ (1 μ g/mL; green channel) was used to identify microglia. Four microscopic fields (each $1.9 \times 10^5 \mu\text{m}^2$) per well in two wells per condition were quantified for a single experiment. For primary cells, experiments were performed with cultures from at least three independent culture preparations.

Fusion assay

BV-2 cells were harvested and stained for 10 min with 50 μ M TAMRA or 5 μ M CFSE in PBS, washed with PBS, combined at a 1 : 1 ratio and seeded at 4×10^4 cells/well in 24-well plates. After 24 h, cultures were treated as indicated, or incubated with polyethylene glycol (PEG) for 1 min, washed with PBS and then incubated with fresh medium. After 24 h, cells were stained with Hoechst (4 μ g/mL), imaged using a Leica DMI6000 microscope, and multinucleate cells scored for TAMRA, CFSE or TAMRA-and-CFSE staining (background (rolling radius 50 pixels) was subtracted from fluorescent images using ImageJ software). Four microscopic fields (each $1.9 \times 10^5 \mu\text{m}^2$) per well in two wells per condition were quantified for a single experiment.

Time-lapse imaging

Phorbol myristate acetate-treated BV-2 cells were imaged using a Leica DMI6000 microscope at 5 min intervals for 24 h. At least 100 multinucleation events over 15 experiments were analysed.

Immunostaining

Cells were fixed with 4% paraformaldehyde for 20 min at 22°C, permeabilised with 0.1% Triton X-100 for 5 min, blocked with 10% goat serum in PBS for 1 h and incubated for 2 h with anti-Ki-67 antibody (2 μ g/mL) or overnight at 4°C with anti-IL-6 antibody (2 μ g/mL). Goat anti-rabbit-Cy3 or goat anti-mouse-Cy3 were used as the secondary antibody (15 μ g/mL; 1 h), and nuclei counterstained with Hoechst (4 μ g/mL). Cells were imaged using a Leica DMI6000 microscope. Nuclei were scored for multinuclearity and the cellular Cy3 fluorescence intensity was calculated using ImageJ software; briefly, background was subtracted from the red channel image, individual cells were selected and the mean gray value was analysed. Four microscopic fields (each $1.9 \times 10^5 \mu\text{m}^2$) per well in two wells per condition were quantified for a single experiment.

Reversal of multinucleation

Experiments were performed as described above, except the medium was removed after 24 h of treatment and fresh DMEM, supplemented with 0.5% FBS, added for a further 24 h prior to imaging.

Bead phagocytosis assay

Phorbol myristate acetate pre-treated BV-2 cultures were incubated with a 0.002% w/v solution of 1 μ m carboxylate-modified microspheres for 1 h or a 0.025% w/v solution of 5 μ m carboxyl particles for 2 h. The medium was removed and cells were washed with ice-cold PBS to remove unattached beads. Cells were stained with Hoechst (4 μ g/mL) and imaged using a Leica DMI6000 microscope. BV-2 cells were scored for multinuclearity and the number of beads per cell counted. Four microscopic fields (each $1.9 \times 10^5 \mu\text{m}^2$) per well in at least two wells per condition were quantified for a single experiment.

PC12 phagocytosis assay

PC12 cells were harvested and stained for 10 min with 50 μ M TAMRA in PBS, then washed with PBS. PC12 were seeded at 2×10^5 cells/well on PMA pre-treated BV-2 cultures either immediately (live PC12; proportion necrotic = $0.7 \pm 0.06\%$; proportion apoptotic = $1.2 \pm 0.2\%$) or after 24-h treatment with 10 μ M staurosporine (dead PC12; proportion necrotic = $95 \pm 0.9\%$). After 3 h the medium was removed and cultures were washed twice with ice-cold PBS to remove the PC12. Cells were stained with Hoechst (4 μ g/mL) and isolectin-B₄ (1 μ g/mL) and imaged using a Leica DMI6000 microscope. BV-2 cells were scored for multinuclearity and TAMRA staining (indicating phagocytosis of PC12) using ImageJ software. Four microscopic fields (each $1.9 \times 10^5 \mu\text{m}^2$) per well in four wells per condition were quantified for a single experiment.

Statistical analysis

All data shown are expressed as the mean \pm SEM for at least 3 independent experiments. Normality of data was verified by Kolmogorov–Smirnov test. Means were compared by student's t-test or by one-way ANOVA and *post hoc* Bonferroni test (normally-distributed data) and by Kruskal–Wallis and Mann–Whitney *U* test (not normally-distributed data). The *p* values < 0.05 were considered significant.

Results

Microglia exhibit multinucleation

We observed a low incidence of multinucleate microglial cells, containing 2 or more distinct nuclei, in primary glial cultures in the absence of inflammatory stimuli (multinucleate cells were $2.0 \pm 0.4\%$ of total microglial cells; Fig. 1a) and a somewhat higher occurrence in the BV-2 murine microglial cell line (multinucleate cells were $4.7 \pm 0.5\%$ of total BV-2 cells; Fig. 1b). Only about 2% of the multinucleate BV-2 cells had 3 or more nuclei in these conditions. Multinucleate BV-2 cells were larger than mononucleate BV-2 cells (multinucleate BV-2 cross-sectional cell area was $164 \pm 21\%$ of mononucleate area), but otherwise appeared normal. The proportion of multinucleate BV-2 microglia was independent of the serum concentration used to culture the cells (BV-2 multinucleation in 10% serum was $101 \pm 19\%$ of that in 0.5% serum, despite the higher proliferation rate), so serum does not affect the mononucleate to multinucleate conversion rate.

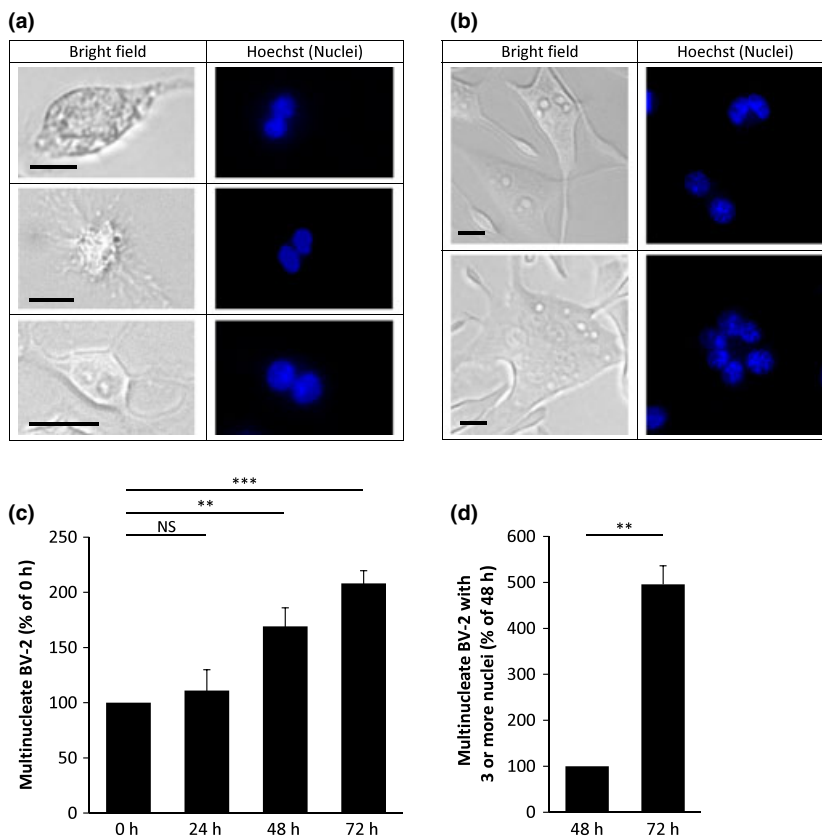


Fig. 1 Primary microglia and BV-2 cells exhibit multinucleation. Bright field and fluorescent images of multinucleation in primary rat cortical microglia (a) and BV-2 cells (b) stained with Hoechst 33342 (blue; nuclei). (c) Multinucleation in BV-2 increases with time. NS = not significant; **/** $p < 0.01/0.001$ versus 0 h. (d) The degree of BV-2 multinuclearity increases over 24 h, 48 h after seeding. ** $p < 0.01$ versus 48 h. Data shown are means \pm SEM for at least three independent experiments; representative images; scale bar = 20 μ m.

The number of multinucleate BV-2 increased 2 fold over a 72-h period after seeding (Fig. 1c), but the ratio of multinucleate to mononucleate BV-2 fell because the total number of cells increased 8 fold. This suggests either that multinucleate cells proliferate less than mononucleate cells, or that the conversion from mononucleate to multinucleate is slower than the conversion from multinucleate to mononucleate cells during exponential growth. In addition, we observed a 5-fold increase in the number of multinucleate BV-2 with 3 or more nuclei between 48 h and 72 h after seeding (Fig. 1d), which suggests that mitosis of multinucleate cells acts to increase the degree of multinuclearity, rather than the number of multinucleate cells. If multinucleate cells do not proliferate as such, the increase in absolute numbers observed suggests that multinucleate cells are continuously produced from mononucleate cells.

Lipopolysaccharide, interferon γ , amyloid β and α -synuclein induce multinucleation

We investigated whether inflammatory stimuli were able to induce microglial multinucleation, given that MGCs are formed in inflammatory conditions (McNally and Anderson 2011). To this end, we stimulated microglia for 24 h with a range of inflammatory mediators. In primary microglia lipopolysaccharide (LPS; 100 ng/mL), interferon gamma (INF- γ ; 10 ng/mL; 0.6 nM) and tumour necrosis factor alpha

(TNF- α ; 50 ng/mL; 2 nM) increased the number of multinucleate cells 4–5 fold compared to untreated cells (Fig. 2a), while the number of mononucleate cells was unaffected (data not shown). In BV-2 cells, LPS, IFN- γ and TNF- α increased the number of multinucleate cells 1.6, 1.5 and 2.8 fold, respectively, relative to untreated cells over 24 h (Fig. 2b), while the number of mononucleate cells decreased to 0.7, 0.8 and 0.8 fold, respectively, (data not shown) over the same period. Addition of dead PC12 cells (1×10^4 cells/well) increased multinucleate cells 2.1 fold, without affecting mononucleate proliferation. Over 48 h of LPS stimulation, the amount of multinucleation increased 2.2 fold compared to untreated cells (data not shown). However, treatment with interleukin-4 (IL-4; 0.07–7 nM) had no effect on multinucleation in primary microglia (multinucleate microglia per field 24 h after stimulation with IL-4 0 ng/mL = 2.9 ± 0.6 ; 1 ng/mL = 3.0 ± 0.6 ; 10 ng/mL = 3.3 ± 0.7 ; 100 ng/mL = 3.0 ± 0.9) or BV-2 (multinucleate BV-2 cells 24 h after stimulation with IL-4 0 ng/mL = 100%; IL-4 1 ng/mL = $103\% \pm 16\%$; IL-4 10 ng/mL = $110\% \pm 12\%$; IL-4 100 ng/mL = $97\% \pm 12\%$).

In addition, we examined the effects of amyloid beta ($A\beta$) and alpha-synuclein (α -Syn) on multinucleation, given their roles in Alzheimer's and Parkinson's disease, respectively, and their ability to activate microglia (Smith *et al.* 1998; Hirsch and Hunot 2009; Mandrekar-Colucci and Landreth

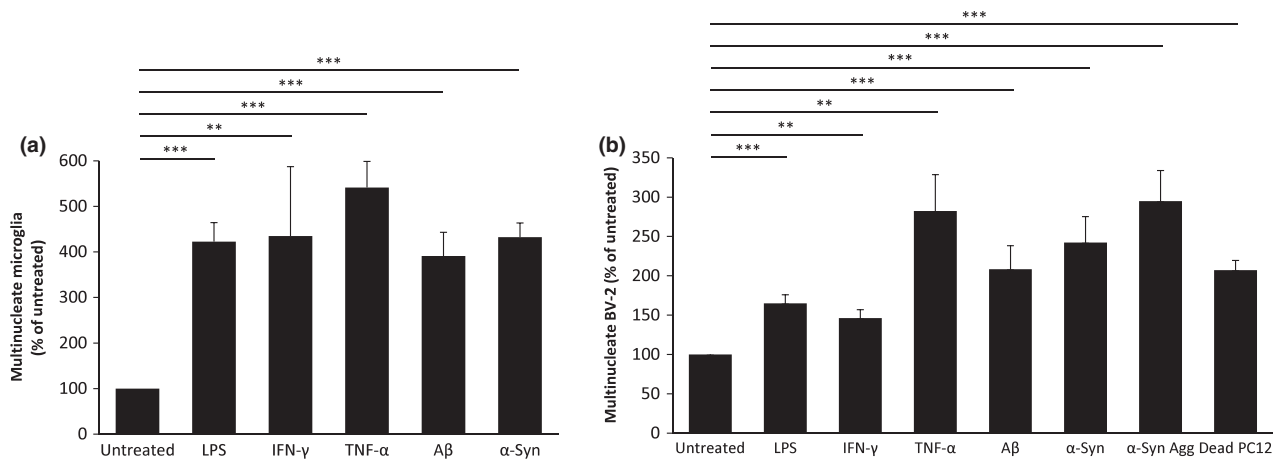


Fig. 2 Inflammatory stimuli induce microglial and BV-2 cell multinucleation. (a) Stimulation of microglia with lipopolysaccharide (LPS; 100 ng/mL), interferon gamma (IFN-γ; 10 ng/mL), tumour necrosis factor alpha (TNF-α; 50 ng/mL), amyloid beta (Aβ; 1 μM) or alpha-synuclein (α-Syn; 500 nM) for 24 h strongly induces multinucle-

ation. (b) Stimulation of BV-2 with LPS, IFN-γ, TNF-α, Aβ, α-Syn or aggregated α-synuclein (α-Syn Agg; 500 nM) or the addition of dead PC12 cells (1×10^4 cells/well) for 24 h induces multinucleation. Data shown are means \pm SEM for at least three independent experiments; **/*** $p < 0.01/0.001$ versus untreated.

2010). Aβ (1 μM) and α-Syn (500 nM) increased multinucleation in microglia 4 fold over 24 h compared to untreated cells (Fig. 2a), with no change in mononucleate cell numbers. In BV-2 the number of multinucleate cells was increased 2.1 fold by Aβ, whilst α-Syn increased multinucleation 2.4 fold when added as a monomer and 3 fold when added in its aggregated form (Fig. 2b), without affecting proliferation of mononucleate cells. Therefore, these data showed that a range of inflammatory stimuli induce multinucleation of microglia.

Multinucleation is mediated by protein kinase C (PKC)

The PKC activator phorbol myristate acetate (PMA) has been reported to stimulate multinucleation of rat microglia (Lee *et al.* 1993; Smith *et al.* 1998). We found that PMA (100 ng/mL; 160 nM) increased the number of multinucleate primary microglia 5 fold (Fig. 3a) and the number of multinucleate BV-2 cells 2.5 fold (Fig. 3b) over 24 h, despite inhibiting the proliferation of mononucleate cells (PMA-treated mononucleate microglia and BV-2 were $70\% \pm 7\%$ and $62\% \pm 4\%$, respectively, of the density of untreated mononucleate cells after 24 h), indicating that PKC activation can cause multinucleation.

Activation of microglia with LPS, INF-γ or Aβ is known to stimulate PKCs (Combs *et al.* 1999; Shen *et al.* 2005; Wen *et al.* 2011), and therefore it is possible that inflammation-induced multinucleation is mediated by a PKC. To test this, we treated microglia and BV-2 cells with Gö6976, a specific inhibitor of the α, β and μ isoforms of PKC, 30 min prior to induction of multinucleation. Treatment with Gö6976 (100 nM) completely prevented LPS- and Aβ-induced multinucleation in BV-2 (Fig. 3c), as well as blocking PMA-induced multinucleation in primary microglia (Fig. 3d) and in

BV-2 in a concentration-dependent manner (Sup Fig. S1a). Although Gö6976 alone had an anti-proliferative effect (Gö6976-treated BV-2 were $73 \pm 11\%$ of the density of untreated cells), viability was not significantly affected (Gö6976-treated BV-2 had a proportion of necrotic cells $120\% \pm 15\%$ of untreated) and there was no greater decrease in proliferation with PMA co-treatment (Sup Fig S1b). These results indicate that LPS-, Aβ- and PMA-induced multinucleation is mediated by PKC.

Multinucleation results from abscission failure, not fusion or phagocytosis

As multinucleate cells can be formed through fusion, phagocytosis or inhibition of cytokinesis, we investigated which of these mechanisms mediated inflammation-induced microglial multinucleation. Cell fusion and phagocytosis can be visualised by staining two populations of cells each with a different dye, and looking for cells stained by both dyes as a result of fusion or phagocytosis (Borges *et al.* 2003). We stained separate populations of BV-2 cells with the intracellular fluorescent dyes CFSE and TAMRA, mixed them in a 1 : 1 ratio, then induced multinucleation with LPS or PMA and analysed the staining of multinucleate cells. We observed a low incidence of phagocytosis in untreated and treated BV-2 mononucleate and multinucleate cells ($7.4\% \pm 0.7\%$ of cells), which was visible as punctate staining, presumably confined to lysosomes, and readily distinguishable from uniform whole-cell staining by TAMRA or CFSE (Fig. 4a I). Brief treatment with polyethylene glycol (PEG), an inducer of cell fusion, produced a population of BV-2 microglia homogeneously stained with both TAMRA and CFSE (37% of PEG-treated multinucleate cells; Fig. 4a II & 4b), indicating fusion between two distinct cells. In contrast, following LPS or PMA

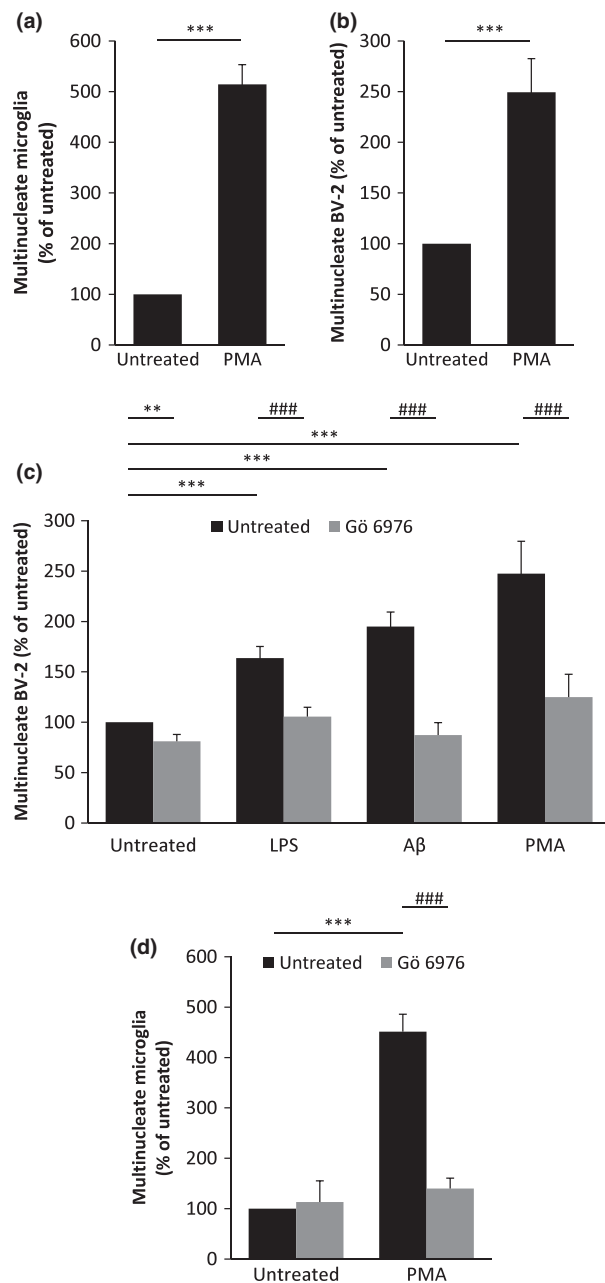


Fig. 3 Microglial inflammatory-induced multinucleation is mediated by protein kinase C (PKC). Stimulation of primary microglia (a) or BV-2 cells (b) with phorbol myristate acetate (PMA; 100 ng/mL) for 24 h strongly induces multinucleation. Treatment with the PKC inhibitor Gö6976 (100 nM) for 30 min prior to stimulation with lipopolysaccharide (LPS; 100 ng/mL), amyloid beta (Aβ; 1 μM) or PMA prevents inflammatory-induced multinucleation in BV-2 cells (c) and primary microglia (d). Data shown are means ± SEM for at least three independent experiments; **/*** $p < 0.01/0.001$ versus untreated; ### $p < 0.001$ versus stimulated.

treatment, all BV-2 microglia were evenly stained for either TAMRA or CFSE (Fig. 4a III & 4b), never both dyes, showing that microglial multinucleation was not because of fusion.

As we saw phagocytic events and activated microglia are able to phagocytose live cells (Brown and Neher 2012), we examined whether phagocytosed nuclei might be preserved in the phagocytic cell, thereby producing a multinucleate phenotype. We treated mixed cultures prior to induction of multinucleation with two different phagocytosis inhibitors, MRS2578 and cyclo (Arg-Gly-Asp-D-Phe-Val) (cRGDFV), which have previously been shown to block microglial phagocytosis (Koizumi *et al.* 2007; Neher *et al.* 2011), including BV-2 phagocytosis of other BV-2 cells (unpublished data). Addition of the inhibitors MRS2578 (1 μM) and cRGDFV (50 μM) did not affect multinucleation (multinucleate BV-2 cells untreated = 100%; LPS = 161% ± 13%; LPS + cRGDFV = 167% ± 21%; LPS + MRS2578 = 159% ± 19%), indicating that phagocytosis does not contribute to multinucleation.

Treatment of BV-2 cells with cytochalasin D (0.5 μM), an inhibitor of actin polymerisation and cytokinesis (and phagocytosis), caused most BV-2 cells to become multinucleate (Fig. 4c) showing that inhibition of cytokinesis was very effective at causing multinucleation in these cells.

To further test whether multinucleation resulted from fusion, phagocytosis or inhibition of cytokinesis, we used time-lapse imaging of PMA-treated BV-2 in culture, which clearly showed that multinucleation resulted from inhibition of cytokinesis. In all cases of multinucleation observed, cytokinesis started and cells underwent normal cleavage furrow ingression, but pseudo-daughter cells remained connected by cytoplasmic bridges, sometimes many cell widths in length, and then subsequently retracted to form a multinucleate cell (Fig. 5). This indicates that multinucleation was because of failure of abscission. We never observed the formation of multinucleate cells by cell fusion or phagocytosis of one cell by another.

Multinucleate cells are viable, but not committed to their fate

Binucleate cells might in principle either die, undergo mitosis to increase the degree of multinuclearity, or undergo cytokinesis to revert to the mononucleate state, so we investigated the viability and fate of multinucleate cells. The level of death in untreated BV-2 cells 48 h after plating was 2.4% ± 0.6%, increasing to 5.0 ± 0.8% at 72 h, as measured by nuclear morphology (chromatin condensed – apoptotic) or permeable plasma membrane (staining with propidium iodide – necrotic), with virtually all dead cells being necrotic. There was no difference in the proportion of dead multinucleate or mononucleate cells (multinucleate BV-2 cell death at 48 h = 2.6 ± 1.1%; mononucleate = 2.4 ± 0.6%; 72 h multinucleate death = 3.9 ± 1.1%; mononucleate = 5.1 ± 0.8%), implying multinucleate cells are as viable as mononucleate cells. Furthermore, although 24-h treatment with PMA increased the number of dead cells 2.8 fold, the

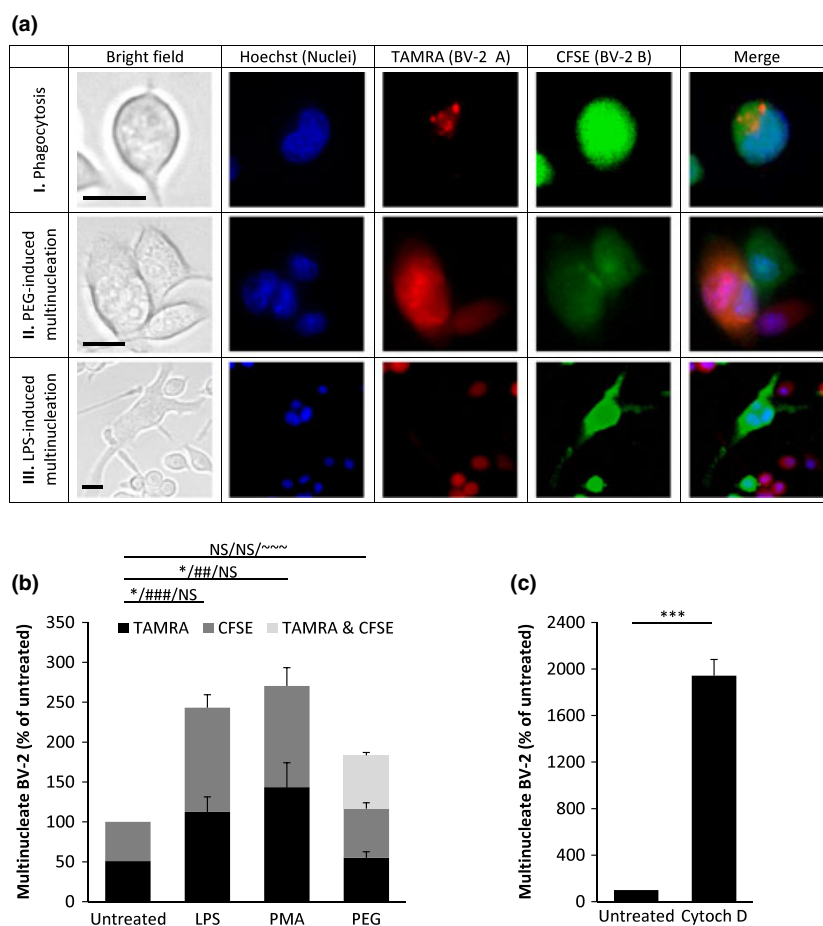


Fig. 4 Microglial inflammatory-induced multinucleation is not because of fusion or phagocytosis. (a) Bright field and fluorescent images of multinucleation in BV-2 stained with Hoechst (blue; nuclei) and one of two cytosolic stains, either 50 μ M 5-(and-6)-carboxytetramethylrhodamine succinimidyl ester (TAMRA; red; BV-2 A) or 5 μ M 5(6)-carboxyfluorescein diacetate-*N*-succinimidyl ester (CFSE; green; BV-2 B). I-Phagocytic events occur in mononucleate and multinucleate cells, visible as distinct punctate TAMRA or CFSE staining in cells of the other colour. II-Brief treatment with polyethylene glycol (PEG) induces multinucleation by fusion between differentially stained BV-2 A and BV-2 B cells, visible as homogenous overlapping TAMRA and CFSE staining. III-Stimulation with lipopolysaccharide (LPS; 100 ng/mL) for 24 h induces multinucleation without fusion or phagocytosis as

cells stain evenly for either TAMRA or CFSE and no hybrid multinucleate cells are formed. Representative images; scale bar = 20 μ m. (b) Stimulation of a mixed population of BV-2, stained either with TAMRA or CFSE, with LPS or phorbol myristate acetate (PMA; 100 ng/mL) for 24 h produces no hybrid cells, whilst PEG induces fusion and produces a new population of double positive TAMRA and CFSE stained cells. * $p < 0.05$ TAMRA versus TAMRA untreated; ##### $p < 0.01/0.001$ CFSE versus CFSE untreated; ~~~ $p < 0.001$ TAMRA & CFSE versus TAMRA & CFSE untreated. (c) Treatment with the actin polymerisation inhibitor cytochalasin D (Cytoch D; 0.5 μ M) for 24 h induces marked multinucleation. *** $p < 0.001$ versus untreated. Data shown are means \pm SEM for at least three independent experiments.

proportion of dead multinucleate cells remained at $2.6 \pm 1.5\%$, the same as in the untreated cultures, while the proportion of dead mononucleate cells rose to $9.1\% \pm 0.8\%$. Thus, multinucleate BV-2 microglia do not die, indeed they may be less prone to death than mononucleate cells.

We carried out immunocytochemistry for Ki-67, a marker of mitosis. All BV-2 exhibited varying degrees of Ki-67 staining, but there was no difference in the Ki-67 staining intensity of untreated mononucleate and multinucleate BV-2 (Fig. 6a), suggesting that they had similar levels of mitotic

activity. This suggests that the decrease over time of the proportion of multinucleate cells (noted previously) results from a slower conversion from mononucleate to multinucleate and/or an increased degree of multinuclearity, rather than less proliferation of multinucleate cells, although it is unclear whether mitotic activity of multinucleate cells results in more nuclei or more cells. BV-2 cells activated with PMA also had a similar level of Ki-67 immunostaining in mononucleate and multinucleate cells, although in both cases the intensity was lower than that of untreated cells (Fig. 6a), consistent with

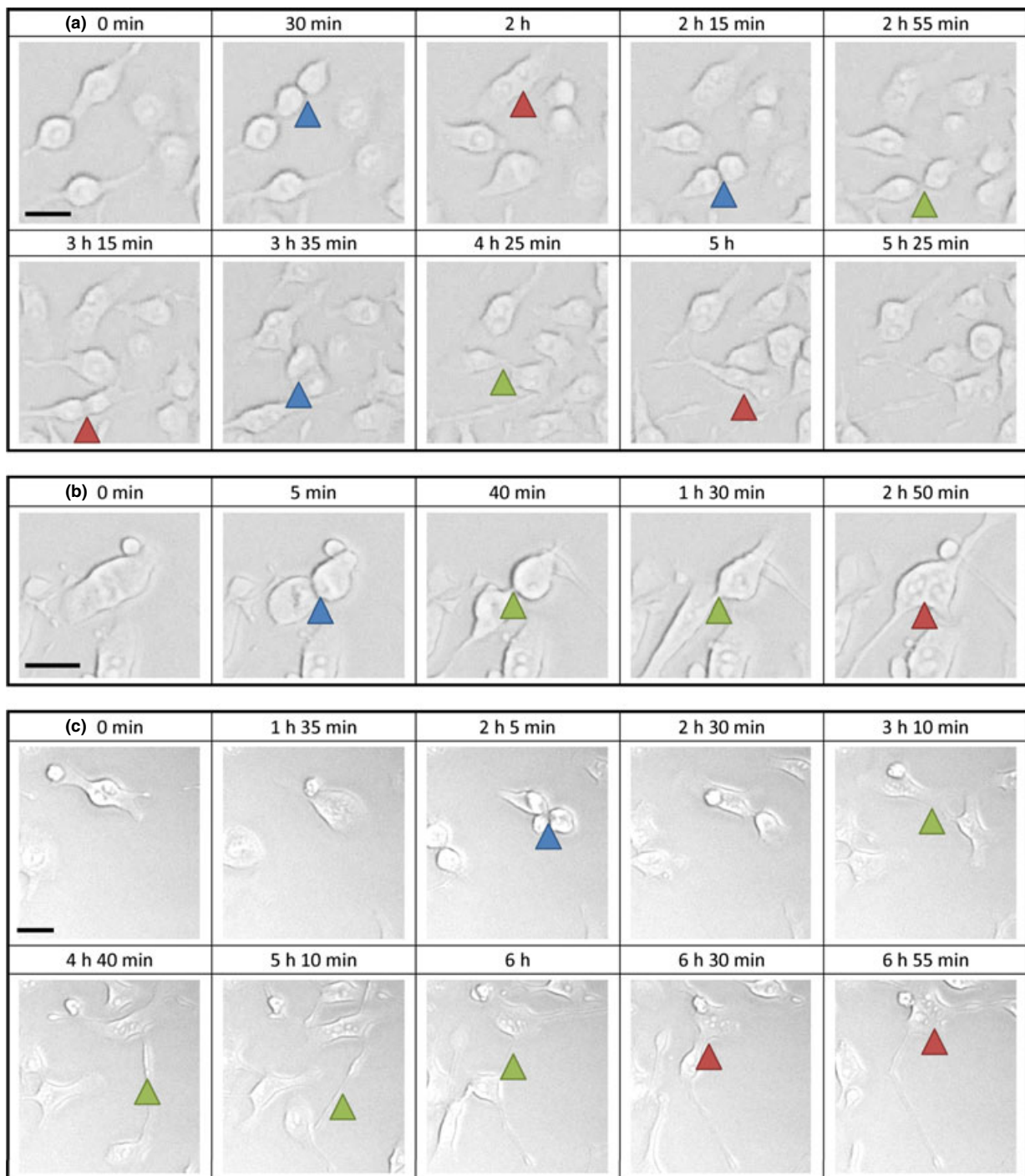


Fig. 5 Microglial inflammatory-induced multinucleation is caused by abscission failure. Live time-lapse imaging of phorbol myristate acetate (PMA; 100 ng/mL) stimulated BV-2 cells reveals cleavage furrow ingression (blue arrowheads), separation of daughter cells by a cytoplasmic bridge (green arrowheads), and abscission failure and retraction to form a multinucleate cell (red arrowheads). Cytokinesis failure events from three separate experiments are shown, including an extended cytoplasmic bridge and formation of a trinucleate cell (c). Representative images; scale bar = 20 μ m.

the anti-proliferative effect of PMA. However, it should be noted that Ki-67 staining is normally quantified as the percent of cells positive for Ki-67, whereas we found that in

these rapidly proliferating BV-2 almost all of the cells stained for Ki-67 to some degree, and thus we used the intensity of staining for comparison.

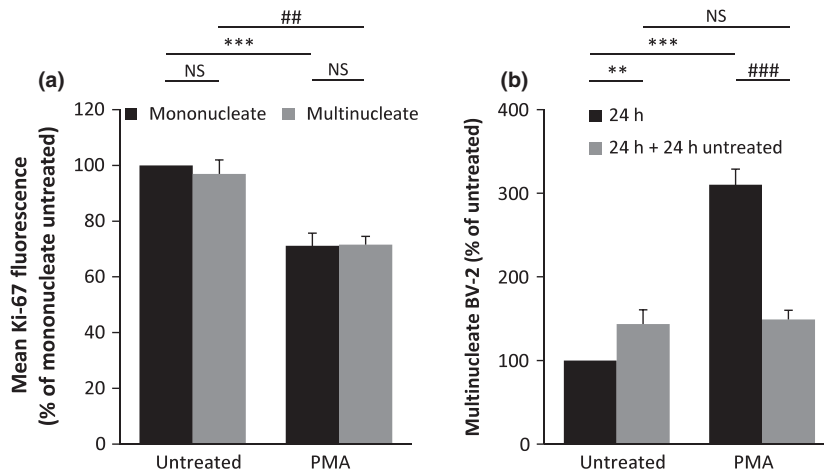


Fig. 6 Multinucleate microglia are viable and multinucleation is reversible. (a) Multinucleate BV-2 cells stain for the mitosis marker Ki-67 with the same intensity as mononucleate cells, while stimulation with phorbol myristate acetate (PMA) (100 ng/mL) for 24 h decreases staining intensity. NS = not significant; *** $p < 0.001$ versus mononucleate untreated; ## $p < 0.01$ versus multinucleate untreated. (b).

We next examined whether multinucleation could be reversed, or whether cells remained committed to being multinucleate. To test this, 24 h after plating, we stimulated BV-2 cells with PMA for 24 h, causing a 3-fold increase in the number of multinucleate cells relative to untreated cells, then washed the cells to remove PMA and incubated with fresh medium for a further 24 h. During this time the number of multinucleate cells fell by half, returning at 72 h to a level similar to untreated BV-2 at 48 h (Fig. 6b). This loss of multinucleation was not because of cell death, as although 24 h PMA pre-treatment increased multinucleate BV-2 death at 72 h 3 fold, this was less than the corresponding 5-fold increase in mononucleate BV-2 death (PMA pre-treated multinucleate BV-2 cell death = $13.0\% \pm 1.4\%$; mononucleate = $21.4\% \pm 0.6\%$). Thus, induced multinucleate cells appear to convert back to mononucleate cells once the inflammatory stimulus is removed, suggesting multinucleation is a fully reversible process.

Multinucleate cells show increased phagocytosis

Lastly, we tested whether multinucleation affected microglial function, as it has been suggested that MGCs are either dysfunctional or gain new functions (Quinn and Schepetkin 2009; McNally and Anderson 2011). To investigate the effect on phagocytosis, we incubated PMA-stimulated BV-2 with carboxylate-modified fluorescent beads (mimicking the negatively-charged surface of cells exposing phosphatidylserine). Multinucleate BV-2 cells ingested 80% more 1 μ m beads than mononucleate cells (Fig. 7a) as a result of an increased number of beads ingested per cell. This increased phagocytic capacity might partly arise from their larger size (the cross-sectional

area of multinucleate BV-2 cells was $64\% \pm 21\%$ larger than that of mononucleate cells). Phagocytosis of 5 μ m beads was 250% greater in multinucleate cells than mononucleate cells (Fig. 7b), mainly because of an increase in the proportion of cells phagocytosing a bead (proportion of mononucleate BV-2 cells phagocytosing = $12\% \pm 1\%$; multinucleate = $36\% \pm 3\%$). Thus, multinucleate BV-2 cells have a substantially greater ability to phagocytose large cell-sized objects.

To test whether multinucleate microglia were more or less capable of phagocytosing cells, we incubated PMA-stimulated BV-2 with either dead or live PC12 cells, a neuron-like cell line (Greene and Tischler 1976). The proportion of multinucleate cells phagocytosing dead or live PC12 was 2.4 or 3.2 fold greater than mononucleate cells, respectively (Fig. 7c and d). Therefore, multinucleate cells have a substantially increased phagocytic ability to phagocytose cells, particularly live cells.

Finally, we tested whether multinucleate microglia were more or less 'activated', as measured by the expression level of the pro-inflammatory cytokine IL-6 in PMA-activated BV-2. All BV-2 exhibited varying degrees of IL-6 immunocytochemistry staining, but there was no difference in the IL-6 staining intensity of mononucleate and multinucleate BV-2 (multinucleate BV-2 IL-6 staining was $103\% \pm 2.7\%$ that of mononucleate), suggesting multinucleate cells have a similar level of activation.

Incubation in fresh medium for 24 h (24 h + 24 h untreated) reverses BV-2 multinucleation induced by 24 h pre-treatment with PMA (24 h). NS = not significant; **/*** $p < 0.01/0.001$ versus untreated 24 h; ### $p < 0.001$ versus PMA 24 h. Data shown are means \pm SEM for at least three independent experiments.

Discussion

The proportion of multinucleate microglia varied considerably in different conditions, but was generally low ($< 20\%$),

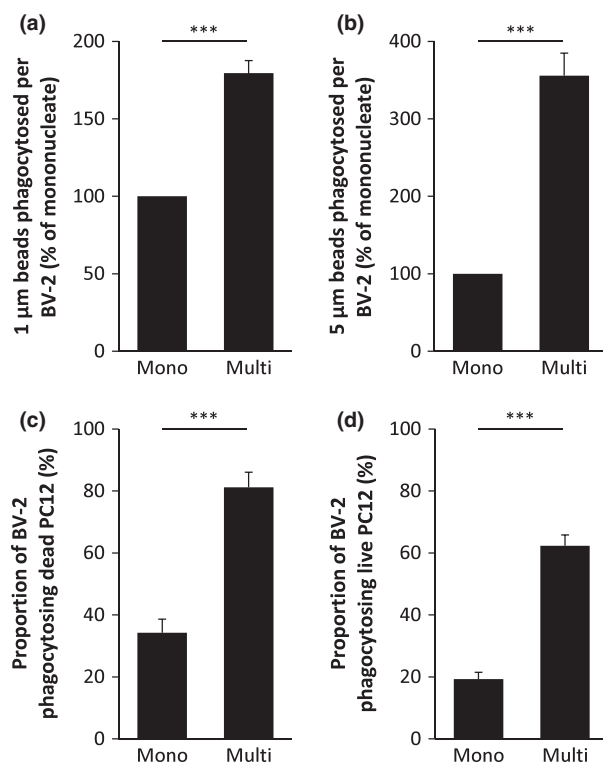


Fig. 7 BV-2 multinucleate cells have increased phagocytic capacity. Multinucleate BV-2 cells (Multi) phagocytose more 1 μ m carboxylate-modified microspheres (a) or 5 μ m carboxyl particles (b) than mononucleate BV-2 cells (Mono) in phorbol myristate acetate (PMA) pre-treated cultures. The proportion of multinucleate BV-2 phagocytosing dead PC12 cells (c) or live PC12 cells by phagoptosis (d) during 3 h of co-incubation is large compared to mononucleate cells. Data shown are means \pm SEM for at least three independent experiments; *** p < 0.001 versus mononucleate.

except in the presence of an inhibitor of cytokinesis, cytochalasin D (61%). Factors that increased the proportion of multinucleate microglia included LPS, A β , α -Syn, IFN- γ , TNF- α , dead PC12 cells and PMA. In general these conditions inhibited the proliferation of the mononucleate cells, and the increase in multinucleation in these conditions is consistent with multinucleation being caused by an inhibition of cytokinesis, which would inhibit proliferation and cause mitotic cells to accumulate multiple nuclei. However, our results are not consistent with multinucleation being a terminally differentiated state, as multinucleation was fully reversed after withdrawal of PMA (Fig. 6b). This suggests that mononucleate and multinucleate microglia are in rapid equilibrium, and changes in the ratio may be caused by stimulations or inhibitions of the interconversion in either direction, as a result of inhibition or stimulation of cytokinesis (replication of the cell) or karyokinesis (replication of the nucleus by mitosis).

The origin of MGCs (i.e. large cells of monocyte/macrophage origin with multiple nuclei that appear under

inflammatory conditions) has almost invariably been attributed to fusion of cells. There is extensive evidence for such fusion occurring, particularly in the presence of IL-4 or foreign bodies (large structures such as catheters that cannot be phagocytosed) (Anderson *et al.* 2008; Helming and Gordon 2009; McNally and Anderson 2011). However, there is also extensive evidence that multinucleation can alternatively arise from inhibition of cytokinesis, i.e. inhibition of cell division after nuclear division has been completed, and cytokinesis can stop at various stages and then reverse to form a binucleate cell (Normand and King 2010). Known causes of multinucleation by this means include: non-disjunction of chromosomes (i.e. failure to separate) during meiosis (Shi and King 2005) and endomitosis during megakaryocyte differentiation (Geddis *et al.* 2007). We observed no fusion between red and green stained microglia, and the multinucleate cells induced by LPS or A β were never a homogenous mixture of red and green, despite the fact that we could easily observe such cells when fusion was induced by PEG. We also observed no cases of multinucleation occurring by fusion when examined by time-lapse microscopy. In contrast, all cases of multinucleation observed by video microscopy arose from a single cell undergoing mitosis (nuclear division), starting cytokinesis, forming a cleavage furrow and in some cases a long cytoplasmic bridge, but then reversing cytokinesis to form a multinucleate cell. We were surprised to find that these cytoplasmic bridges could be quite long (several cell widths), with the two pseudo-daughter cells behaving independently for some time before retraction of the cytoplasmic bridge. This raises the intriguing possibility that clonal microglia (and other cells) may be linked by cytoplasmic bridges *in vivo*, but we know of no evidence that this is the case. Spermatids and some other cells are known to be connected by cytoplasmic bridges as a result of failure of abscission (Normand and King 2010).

Various PKCs are known to be activated in microglia by LPS (Shen *et al.* 2005; Wen *et al.* 2011), A β (Combs *et al.* 1999; Nakai *et al.* 2001) and INF- γ (Shen *et al.* 2005), and foreign-body induced multinucleation of macrophages is known to be mediated by PKCs (McNally *et al.* 2008). Thus, inflammatory agents might cause multinucleation of microglia via stimulation of PKCs. Consistent with this we found that the PKC activator PMA increased microglial multinucleation, and that a PKC inhibitor Gö6976 prevented LPS and A β -induced multinucleation. Gö6976 specifically inhibits PKCs α , β and μ (PKC μ is also known as PKD), suggesting that one or more of these PKC/PKD isoforms are involved in microglial multinucleation. PKC α and PKC δ may inhibit cytokinesis (Akakura *et al.* 2010), while PKC ϵ may be required for completion of cytokinesis (Saurin *et al.* 2008). However, such effects may be indirect as PKCs regulate multiple processes, and multiple processes regulate cytokinesis and its last stage, abscission (Normand and King

2010; Schiel and Prekeris 2010). PKCs can activate NADPH oxidases, which are implicated in multinucleation, although via increasing fusion (Quinn and Schepetkin 2009). The PKC activator PMA has been shown to induce multinucleation of mesenchymal stem cells because of inhibition of cytokinesis rather than induction of fusion (Yoshida *et al.* 2007), consistent with our results.

The function, dysfunction or lack of function of MGCs is unclear (McNally and Anderson 2011). We found that multinucleate microglia behaved similarly to mononucleate microglia as far as we could discern (similar levels of cell death, Ki-67 expression and IL-6 expression), but were larger and had a higher capacity to phagocytose large beads and cells. It has previously been suggested that osteoclasts and MGCs are large to enable phagocytosis of larger structures (McNally and Anderson 2011), and this appears to be true of multinucleate microglia, enabling them to phagocytose cell-sized beads and cells that are at least as large as a mononucleate microglia. The mononucleate microglia phagocytosed 5 μm beads (0.11 ± 0.01 beads/cell/hour) at about 1/90 of the rate for 1 μm beads (9.9 ± 3.0 beads/cell/hour), presumably reflecting the difficulty of phagocytosing something approaching the size of the phagocyte. While the multinucleate microglia had a marginally higher capacity to phagocytose the smaller beads (proportionate to their larger size), these larger cells had a disproportionately higher capacity to phagocytose the larger beads, suggesting that the larger microglia could phagocytose larger structures. However, it could be that these multinucleate cells have changes (not measured by us), other than their size, that enable them to phagocytose larger structures. Large, multinucleate, cells might be less mobile, but if so this did not prevent them from having a higher phagocytic capacity.

The multinucleate microglia had a substantially increased ability to phagocytose dead and live PC12 cells (relative to mononucleate microglia), which may reflect their increased ability to phagocytose large structures, possibly as a result of their larger size. Both mononucleate and multinucleate microglia preferred to phagocytose dead rather than live PC12 cells, but the multinucleate microglia were less discriminating between dead and live cells (Fig. 7c and d). We have previously shown that activated microglia can phagocytose live neurons and thereby kill them – a form of cell death we have called ‘phagoptosis’ (Neher *et al.* 2011; Brown and Neher 2012). It would appear from the research reported here that a small subset of microglia, the multinucleate, is particularly active in the phagocytosis of cells, and this multinucleate subset is increased by inflammation. Stimulating multinucleation could be beneficial during neurodegenerative disease, facilitating clearance of dead and dying cells without increasing microglial activation and inflammation, or perhaps during viral infection by promoting removal of live virus-infected cells. However, in the healthy brain, multinucleation would appear to be detri-

mental and might result in additional neuronal loss through phagoptosis.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TCH performed the experiments and drafted the manuscript. UN performed some initial experiments on multinucleation. GCB conceived of the study, and participated in its design and coordination and drafted the manuscript.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. BV-2 PMA-induced multinucleation is mediated by PKC. Treatment with the PKC inhibitor G66976 for 30 min prior to BV-2 stimulation with PMA (100 ng/mL) for 24 h (a) prevents PMA-induced multinucleation in a concentration dependent manner and (b) is anti-proliferative. Data shown are means \pm SEM for at least three independent experiments; NS = not significant; **/** $p < 0.01/0.001$ versus untreated; ##### $p < 0.01/0.001$ versus PMA-stimulated.

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