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Microglia repetitively isolated from *in vitro* mixed glial cultures retain their initial phenotype

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Abstract

In vitro culture of rodent microglia is a common system used to model proinflammatory changes in the brain. However, typical postnatal brain isolation protocols are time consuming and cell numbers acquired are often a rate-limiting factor for experimental progress. Large studies that rely on the use of primary microglia can, therefore, require excessive numbers of animals at considerable expense, additional technical support and culture incubator space. Although the addition of mitogens such as macrophage colony-stimulating factor, granulocyte macrophage-colony stimulating factor, and epidermal growth factor to the cultures can facilitate a higher yield, this adds additional expense and likely alters the microglial phenotype. We have defined a simple, inexpensive modification of our standard culture protocol that allows us to repetitively isolate microglia. In order to define a method for improving microglia yield, we utilized our standard mixed glial culture preparation derived from postnatal day 1 mouse brains. After isolating microglia from mixed cultures at 14 days *in vitro*, we added fresh media to the cultures for an additional 7 and 14 days to monitor microglial proliferation. We acquired a constant number of cells at each successive time point although the numbers were reduced from the first isolation. More importantly, in order to determine if our successive microglia isolates differed phenotypically we characterized several parameters of function. We compared their ability to secrete the proinflammatory cytokines interleukin-6 and tumor necrosis factor alpha after LPS stimulation. We also contrasted the phagocytic ability, morphology, and specific immunoreactivity (CD11b, CD68, CD45 and MHC II) of the culture ages. Our data demonstrate that microglia can be obtained from extended-time cultures provided periodic isolation is performed. Moreover, the cells retain a comparable *in vitro* phenotype. This demonstrates that cells from all ages can be combined for any given study. These findings are a viable and inexpensive way to increase and extend the microglial yield without increasing the number of animals used or adding costly mitogens. This method will be particularly useful for the preparation of microglia cultures from limited transgenic colonies.

Keywords

Microglia; Lipopolysaccharide (LPS); phagocytic; TNF α , IL-6; CD68; CD11b; CD45; MHC II

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1. Introduction

Microglia are the resident immune cells in the CNS and play a critical role in the brain inflammatory response. Microglia respond to a plethora of stimuli leading to a cascade of inflammatory cytokine products such as TNF α and IL-1 (Sondag and Combs, 2006; Nakamura et al., 1999; Kawanokuchi et al., 2004; Xie et al., 2003). For this reason, studies determining the role of microglia in propagating or attenuating proinflammatory events in the brain are applicable to understanding the mechanism of many pathophysiologies in the central nervous system. Not surprisingly, *in vitro* culturing of primary microglia from rodents, in particular, has provided a reproducible means of studying this cell type in culture. However, murine primary microglial cultures are time consuming to prepare and yield relatively low numbers of cells, thus limiting the studies that can be conducted.

Isolation of the amoeboid phenotype microglia in particular, from postnatal rodent brain mixed glial cultures is a reliable and well-characterized method. Early culture protocols have verified that distinct populations of microglial phenotypes exist in a mixed glial culture. An amoeboid phenotype cell displays properties similar to macrophage including the ability to phagocytose, secrete cytokines, and reactive oxygen species with the clear ability to proliferate *in vitro* (Giulian and Baker, 1986). On the other hand, a ramified population has decreased phagocytic ability and proliferation rate (Giulian and Baker, 1986). The amoeboid cells typically rest on top of the astrocyte monolayer and can appear as clusters or colonies suggesting a clonal expansion from some existing precursor or individual cell. The ramified phenotype is invested within or underneath the astrocyte layer and the numbers of ramified phenotype cells increase with time in culture as the microglia integrate into the astrocyte layer (Tanaka et al., 1999; Kalla et al., 2003). Importantly, this process is reversible by, for instance, elevating cAMP levels or increasing intracellular calcium converting the cells back into an amoeboid phenotype (Kalla et al., 2003). This demonstrates that although two distinct phenotypes may exist, the cells can readily convert from one to another in the culture paradigm. Importantly, these two phenotypes correspond roughly with morphologic phenotypes that have been defined *in situ*. That is, the amoeboid cells are abundant in late embryogenesis decreasing postnatally and then again following injury (Murabe and Sano, 1983; Tseng et al., 1983). Although the amoeboid phenotype cells have been likened to tissue macrophage, clear differences have been reported contrasting the histologic phenotypes of primary amoeboid microglia cultures and tissue macrophage (Giulian et al., 1995). For example, GM-CSF only stimulates ramification of brain derived microglia cultures and not tissue macrophage or bone marrow precursor monocytes (Giulian et al., 1995).

Similar to events occurring *in vivo* in which microglial mitogens are present embryonically during periods of developmental proliferation and the presence of amoeboid cells *in situ*, it has been known over a decade that astrocytes in mixed glial cultures secrete mitogens that also promote proliferation of amoeboid microglia (Giulian et al., 1991). Importantly, the mitogenic and ramification response of the amoeboid cells can be somewhat separated in culture. Over time rat microglia plated onto an astrocyte layer will increase in number as well as ramification while microglia separated from direct contact with astrocytes by a porous membrane will proliferate but not ramify (Giulian et al., 1995). Indeed, microglial ramification but not proliferation occurs even when rat microglia are plated onto fixed astrocyte cultures (Tanaka and Maeda, 1996). Although the nature of the mitogen may be varied, several reports from both rodent and human glial preparations have identified macrophage colony stimulating factor (M-CSF) and granulocyte/macrophage colony stimulating factor (GM-CSF) as potent astrocytesecreted microglial mitogens and trophic factors (Tomozawa et al., 1996; Gehrman, 1995; Lee et al., 1994; Giulian and Ingeman, 1988). Importantly, these same factors have also had varying reports of ability to induce microglial ramification (Schilling et al., 2001; Fujita et al., 1996; Liu et al., 1994). They likely affect microglial phenotype as well including increasing

phagocytic ability, altering cytokine production phenotype, and altering antigen presenting ability (Aloisi et al., 2000; Lee et al., 1993; Giulian and Ingeman, 1988; Flanary and Streit, 2006).

In order to minimize the possible change in microglial phenotype induced by adding exogenous mitogens yet utilize the endogenous proliferative capacity of our murine mixed glial cultures, we devised a simple modification of our existing protocol and now describe a method to repetitively isolate amoeboid microglia from mixed glial cultures derived from postnatal mouse brains with no apparent change in the phenotype of these cells during successive isolations. This technique demonstrates a useful way to increase and extend the overall microglial yield from individual mixed glial cultures without having to add exogenous mitogens to the cultures.

2. Methods

2.1 Tissue culture

Microglia were derived from postnatal day 1–3 (P1-P3) mouse brains (C57BL/6). Cortices were isolated and trypsinized. Three brains were triturated and plated onto tissue culture T-75 flasks in DMEM/F12 with L-glutamine (Invitrogen, Carlsbad, CA) containing 20% heat-inactivated FBS. After 24 hours all media and tissue was removed and fresh media was replaced. After 7 days one half of the media was replaced and cells were maintained at a mixed glia culture until day 14. At 14 days *in vitro*, microglia were removed from the mixed glial culture via a rotating shaker at 200rpm for 45 min. and the total number of microglia isolated per flask was calculated from yield 1. Fresh media was added to the flasks of remaining mixed glia for 1 week before microglia were collected again (yield 2). This was repeated a third time for yield 3. The composition of the mixed glial layer was not altered from brain isolation by addition of exogenous factors or cell types. The mixed cell culture reportedly contains a homogeneous mixture of primarily type 1 and type 2 astrocytes, microglia, oligodendrocytes, and O2A progenitor cells (McCarthy and deVellis, 1980). By dividing the average yield of microglia from yield 1 by the number of brains used per culture we derive an average microglia number of 297,231 cells/brain for yield 1. Although yields 2 and 3 are derived from intact cultures and not newly plated brain homogenates like yield 1, we can divide their average yields by the number of initial brains used to obtain a meaningful comparison to yield 1. For example, yield 2 averaged 42,271 cells/brain (14.28% of yield 1) and yield 3 averaged 81,658 cells/brain (31.5% of yield 1).

2.2 Cell stimulation

Microglia were plated onto 96 well tissue culture plates (20,000 cells/well, 75µL serum free DMEM/F12) for 6 or 24 hours. Cultures were stimulated with or without 25ng/mL LPS (Sigma, St. Louis, MO). Experiments were performed with 8 replicates per condition, 3–4 independent times. Data are presented as mean $\pm$ standard deviation. Values statistically different from controls were determined using one-way ANOVA. The Tukey-Kramer multiple comparisons post test was used to determine p values.

2.3 Quantitation of secreted TNF α and IL-6

Following 24 hour stimulation, 70µL and 10µL of media were removed from the individual culture wells for quantitation of TNF α and IL-6, respectively. Concentrations of secreted cytokines were then determined using commercially available mouse colorimetric sandwich ELISA plates according to the manufacturer's protocol (R & D Systems, Minneapolis, MN).

2.4 Phagocytosis assay

Phagocytosis was quantitated by measuring the uptake of FITC-labeled *Escherichia coli* (K-12 strain) Bioparticles (Molecular Probes, Eugene, OR). Briefly, 0.25mg/mL FITC-E. coli Bioparticles were incubated with the microglia in 96 well plates for 6 hours. To quench the signal from the extracellular peptide, media was removed and the cells rinsed with 0.25mg/mL trypan blue in PBS. Application of trypan serves to quench any remaining peptide on the plate as well as any bound to the external leaflet of the plasmalemma. Intracellular fluorescence was read (480nm excitation and 520nm emission) via fluorescent plate reader (Bio-Tek, Winooski, Vermont).

2.5 Cell viability assay

To determine cell viability following 24 hour stimulation, cellular release of lactate dehydrogenase (LDH) was measured from culture media using a commercial nonradioactive assay (Promega, Madison, WI). Absorbance measurements were taken at 490 nm.

2.6 Immunocytochemistry

To perform culture immunocytochemistry, microglia were plated on glass chamberslides for 24 hours then fixed in 4% paraformaldehyde (37°C, 30 min) and immunostained using anti-CD68, anti-CD11b, anti-MHC II, (Serotec, Raleigh, NC) and anti-CD45 (BD Biosciences, San Diego, CA) antibodies using texas red or FITC conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Each experiment was performed in quadruplicate 3–4 times. Images were captured using a Leica DM4000 upright microscope (Bannockburn, IL).

2.7 Immunostaining quantitation

Microglia from the first and third harvest were plated onto black 96 well clear bottomed plates in serum free DMEM/F12 for 24 hours then fixed in 4% paraformaldehyde (37°C, 30 min) for immunostaining as above using anti-CD68, CD11b, MHC2 and CD45 using VIP as the chromogen (Vector Laboratories, Burlingame, CA). Cells were counterstained with DAPI. Cellular fluorescence from DAPI was read at 346nm excitation and 460 emission using a SpectraMax Gemini EM fluorescent plate reader (Molecular Devices, Sunnyvale, CA). VIP absorbance was read at 560nm excitation and 650nm emission using a plate reader (Bio-Tek, Winooski, Vermont). Immunostaining absorbance values were normalized against their respective nuclear DAPI relative fluorescence units and averaged $\pm$ SD. 5 replicates per antibody were immunostained and the experiment was repeated 3 independent times.

2.8 Cell Counting

Microglia were harvested and collected by shaking each flask on a reciprocal shaker for 45 min at 200rpms. Astrocyte layers remained intact and all media and loosely adhered microglia were removed, centrifuged and counted on a hemacytometer to determine each individual flask yield.

2.9 Animal Care and Use

All procedures were reviewed and approved by the University of North Dakota Institutional Animal Care and Use Committee (protocol #0012–3). Mice were housed at the Center for Biomedical Research on a 12 hour light/dark cycle and were allowed food and water *ad libitum*. Postnatal pups were euthanized via decapitation.

3. Results

3.1. Primary microglial cell culture

Our prior observations suggested that murine mixed cortical glial cultures retained some small amount of amoeboid microglia on the astrocyte monolayer in spite of vigorous agitation. We speculated that these remaining cells would serve as starter microglia that could proliferate atop the astrocyte layer to provide a second population of cells for collection at a later date. To determine whether amoeboid microglia could be repetitively isolated from our cultures we first isolated microglia according to our standard protocol at 14 days *in vitro*. We added fresh media onto the bed of mixed glia for subsequent microglial isolation at 21 and 28 days *in vitro*. Although the number of cells collected per culture certainly decreased in collection 2 or 3 versus the first collection there was no significant difference in the yield number between the second and third yields suggesting that some basal amount of cells could be repetitively harvested on a weekly basis (Fig. 1). Yields 2 and 3 are additively 45.7% of yield 1 calculating cellular yield/brain which are harvested from intact cultures that would otherwise be discarded after the first yield. This suggests, for example, that a lab that normally requires 100 neonatal mice per month to generate microglia could decrease their number to only 48/month, a large decrease in animal usage.

3.2. Microglial expression of antigen presenting cell surface markers

In order to determine whether the successively isolated microglia were phenotypically similar to those acquired during our initial collection we first compared the morphology and specific cellular immunoreactivities of the first and third microglial collections to determine any dramatic differences existed between the culture isolations. Collections at 14 (yield 1) and 28 days *in vitro* (yield 3) were compared. Cells displayed similar morphologies but slightly altered intensity of immunoreactivities for several typical microglial marker proteins (Fig. 2). For instance, immunoreactivity intensity for CD68 and CD45 increased slightly at 28 days compared to 14 (Fig. 2B, D). On the other hand, immunoreactivity intensity for MHC class II antigen had an apparent decrease from 14 to 28 days (Fig. 2C). In order to avoid this qualitative assessment, we next quantitated the specific immunoreactivities. When normalized against cell number, there were no immunoreactivity changes from microglia harvested at 14 and 28 days (Fig. 3). These data demonstrate that microglia retain their morphologic phenotype across each yield suggesting that they have a similar basal activation state.

3.4. Microglia express lower levels of cytokine production with increased culture age

However, to insure that the cells truly maintain a comparable basal and stimutable phenotype across harvesting ages, we next assessed whether the successive microglial yields differed dramatically with respect to two standard activity assays, cytokine production and phagocytic ability. We first examined secretion of two cytokines, interleukin 6 (IL-6) and tumor necrosis factor α (TNF α), from 14 (yield 1) and 28 (yield 3) day harvested microglia following stimulation with lipopolysaccharide (LPS). Using equal number of cells per assay, secreted concentrations of both IL-6 and TNF α were significantly greater from yield 1 versus yield 3 stimulated microglia compared to their respective controls (Fig. 4). Interestingly, yield 3 microglia were able to secrete IL-6 but not TNF α following LPS stimulation indicating a rather specific decrease in secretory ability (Fig. 4). Importantly, the cell survival from yield 1 and 3 was not significantly different demonstrating that viability did not explain the decreased ability of the microglia to secrete cytokines with culture time (Fig. 4).

Lastly, we compared the phagocytic ability of yields 1 and 3 exposed to FITC-conjugated Bioparticle after 6 hours. Unlike the cytokine secretion analysis or immunohistochemistry, we found the equivalent numbers of cells from each culture age isolation had no significant differences in their ability to phagocytose bacterial derived bioparticles (Fig. 5). Again, this

demonstrates that the successively isolated amoeboid microglial population is useful for the study of typical microglial function assay employed *in vitro*.

4. Discussion

We have described a method that utilizes the intrinsic proliferative potential of the mixed murine glial cultures to repetitively harvest amoeboid microglia for use. We elected not to address changes in cells other than the amoeboid microglia as this was our cell type of interest although a ramified population of microglia as well as astrocytes are certainly in the cultures as well. Although the successive cell number is lower in subsequent yields compared to the initial harvest, we have determined that cells do retain a relatively similar phenotype, particularly with regard to morphology and phagocytic ability. For experimental protocols that do not require large numbers of cells we have found this derivation to be an excellent means to minimize the need for increased animal cost and use.

Of course we have only assessed the amoeboid phenotype cells in our experimental questions as this is the cellular population commonly employed in many protocols. We do not know the extent, if any, of phenotype changes that are occurring in the ramified microglia or astrocytes in the monolayer. We assume that the microglia are proliferating to provide us with our maintained ability to collect cells each week but it is possible that ramified cells are simply converting back to the amoeboid phenotype to allow for subsequent collection. We have elected not to address this issue in the current study since our primary concern was whether or not subsequent collections were possible and if so is the initial phenotype preserved.

In addition, any mitogenic factor that is generated by the mixed cultures is likely made from the astrocytes in the cultures given the abundant literature already describing the ability of these cells to secrete mitogens including GM-CSF and M-CSF in culture. In light of the basal proliferative property of the cultures, it is possible that adding additional exogenous mitogens would further increase our microglial yield per harvest period but our goal in the current work was not to assess the proliferation stimulated by adding exogenous growth factors but rather the ability of the mixed glial culture alone to stimulate microglial proliferation. For this reason, we have also not elected in the current work to try to identify whether any specific factor was made by the mixed glial cultures to produce amoeboid microglial proliferation.

Although our protocol allows for prolonged utility of individual mixed glial cultures for the purpose of collecting microglia, it is important to point out that the system has limits. For example, it has been demonstrated previously that rat microglia have a limited proliferation period *in vitro* corresponding with progressive telomere shortening in the presence of GM-CSF (Flanary and Streit, 2004). Thus rat microglia undergo senescence *in vitro* after approximately 32 days of allowed proliferation.

In addition, although our data demonstrates a relative preservation of phenotype across yields it still remains possible that further collections beyond 28 days may result in phenotypes that differ dramatically from collection to collection. For example, although 28 day *in vitro* microglia secreted IL-6 in response to LPS stimulation, the amount of secretion was significantly less than that of 14 day *in vitro* microglia. Moreover, 28 day *in vitro* microglia were unable to secrete a significant increase in TNF α upon LPS stimulation. Also, prior work has demonstrated that mouse brain contains a precursor population that can be isolated *in vitro* to proliferate in the presence of M-CSF to generate cells that do or do not constitutively express high levels of CD45 and MHC II with the ability to present antigen to CD4 $^{+}$ T cells (Walker, 1999). Indeed others have reported that microglia isolated from adult mice undergo differentiation into a dendritic cell phenotype in the presence of exogenous GM-CSF while

assuming a proliferating microglial phenotype in the presence of M-CSF (Ponomarev et al, 2005).

With the understood caveats of using postnatal brain-derived cultured microglia, we propose the current protocol as a useful addition to standard microglia isolations from mixed glial culture preparations to provide a consistent population of cells useful for studying the biology of this cell with regard to a specific subset of assays including phagocytic ability and select cytokine secretion.

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References

- Aloisi F, De Simone R, Columba-Cabezas S, Penna G, Adorini L. Functional maturation of adult mouse resting microglia into an APC is promoted by granulocytemacrophage colony-stimulating factor and interaction with Th1 cells. *J Immunol* Feb 15;2000 164(4):1705–12. [PubMed: 10657614]
- Chung IY, Benveniste EN. Tumor necrosis factor- α production by astrocytes. Induction by lipopolysaccharide, IFN- γ , and IL-1 β . *J Immunol* Apr 15;1990 144(8):2999–3007. [PubMed: 2109008]
- Flanary BE, Streit WJ. Alpha-tocopherol (vitamin E) induces rapid, nonsustained proliferation in cultured rat microglia. *Glia* Apr 15;2006 53(6):669–74. [PubMed: 16419088]
- Flanary BE, Streit WJ. Progressive telomere shortening occurs in cultured rat microglia, but not astrocytes. *Glia* Jan 1;2004 45(1):75–88. [PubMed: 14648548]
- Fujita H, Tanaka J, Toku K, Tateishi N, Suzuki Y, Matsuda S, Sakanaka M, Maeda N. Effects of GM-CSF and ordinary supplements on the ramification of microglia in culture: a morphometrical study. *Glia* Dec;1996 18(4):269–81. [PubMed: 8972796]
- Gehrmann J. Colony-stimulating factors regulate programmed cell death of rat microglia/brain macrophages in vitro. *J Neuroimmunol* Dec;1995 63(1):55–61. [PubMed: 8557825]
- Giulian D, Baker TJ. Characterization of ameboid microglia isolated from developing mammalian brain. *J Neurosci* Aug;1986 6(8):2163–78. [PubMed: 3018187]
- Giulian D, Ingeman JE. Colony-stimulating factors as promoters of ameboid microglia. *J Neurosci* Dec; 1988 8(12):4707–17. [PubMed: 3058881]
- Giulian D, Johnson B, Krebs JF, George JK, Tapscott M. Microglial mitogens are produced in the developing and injured mammalian brain. *J Cell Biol* Jan;1991 112(2):323–33. [PubMed: 1988464]
- Giulian D, Li J, Bartel S, Broker J, Li X, Kirkpatrick JB. Cell surface morphology identifies microglia as a distinct class of mononuclear phagocyte. *J Neurosci* Nov;1995 15(11):7712–26. [PubMed: 7472522]
- Kalla R, Bohatschek M, Kloss CU, Krol J, Von Maltzan X, Raivich G. Loss of microglial ramification in microglia-astrocyte cocultures: involvement of adenylate cyclase, calcium, phosphatase, and Gi-protein systems. *Glia* Jan;2003 41(1):50–63. [PubMed: 12465045]
- Kawanokuchi J, Mizuno T, Kato H, Mitsuma N, Suzumura A. Effects of interferon- β on microglial functions as inflammatory and antigen presenting cells in the central nervous system. *Neuropharmacology* 2004;46:734–742. [PubMed: 14996551]
- Lee SC, Liu W, Brosnan CF, Dickson DW. GM-CSF promotes proliferation of human fetal and adult microglia in primary cultures. *Glia* Dec;1994 12(4):309–18. [PubMed: 7890333]
- Lee SC, Liu W, Roth P, Dickson DW, Berman JW, Brosnan CF. Macrophage colony-stimulating factor in human fetal astrocytes and microglia. Differential regulation by cytokines and lipopolysaccharide, and modulation of class II MHC on microglia. *J Immunol* Jan 15;1993 150(2):594–604. [PubMed: 8419491]

- Liu W, Brosnan CF, Dickson DW, Lee SC. Macrophage colony-stimulating factor mediates astrocyte-induced microglial ramification in human fetal central nervous system culture. *Am J Pathol* Jul;1994 145(1):48–53. [PubMed: 8030755]
- McCarthy KD, de Vellis J. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J Cell Biol* Jun;1980 85(3):890–902. [PubMed: 6248568]
- Murabe Y, Sano Y. Morphological studies on neuroglia. VII. Distribution of "brain macrophages" in brains of neonatal and adult rats, as determined by means of immunohistochemistry. *Cell Tissue Res* 1983;229(1):85–95. [PubMed: 6339067]
- Nakamura Y, Si QS, Kataoka K. Lipopolysaccharide-induced microglial activation in culture: temporal profiles of morphological change and release of cytokines and nitric oxide. *Neuroscience Research* 1999;35:95–100. [PubMed: 10616913]
- Ponomarev ED, Novikova M, Maresz K, Shriver LP, Dittel BN. Development of a culture system that supports adult microglial cell proliferation and maintenance in the resting state. *J Immunol Methods* May;2005 300(1–2):32–46. [PubMed: 15893321]Epub 2005 Apr 26
- Schilling T, Nitsch R, Heinemann U, Haas D, Eder C. Astrocyte-released cytokines induce ramification and outward K⁺ channel expression in microglia via distinct signalling pathways. *Eur J Neurosci* Aug;2001 14(3):463–73. [PubMed: 11553296]
- Sondag CM, Combs CK. Amyloid precursor protein cross-linking stimulates beta amyloid production and pro-inflammatory cytokine release in monocytic lineage cells. *J Neurochem* 2006;97:449–61. [PubMed: 16539666]
- Tomozawa Y, Inoue T, Takahashi M, Adachi M, Satoh M. Apoptosis of cultured microglia by the deprivation of macrophage colony-stimulating factor. *Neurosci Res* May;1996 25(1):7–15. [PubMed: 8808795]
- Tanaka J, Maeda N. Microglial ramification requires nondiffusible factors derived from astrocytes. *Exp Neurol* Feb;1996 137(2):367–75. [PubMed: 8635553]
- Tanaka J, Toku K, Sakanaka M, Maeda N. Morphological differentiation of microglial cells in culture: involvement of insoluble factors derived from astrocytes. *Neurosci Res* Sep;1999 34(4):207–15. [PubMed: 10576543]
- Tseng CY, Ling EA, Wong WC. Scanning electron microscopy of amoeboid microglial cells in the transient cavum septum pellucidum in pre- and postnatal rats. *J Anat* Mar;1983 136(Pt 2):251–63. [PubMed: 6853343]
- Walker WS. Separate precursor cells for macrophages and microglia in mouse brain: immunophenotypic and immunoregulatory properties of the progeny. *J Neuroimmunol* Feb 1;1999 94(1–2):127–33. [PubMed: 10376945]
- Xie Z, Morgan TE, Rozovsky I, Finch CE. Aging and glial responses to lipopolysaccharide in vitro: greater induction of IL-1 and IL-6, but smaller induction of neurotoxicity. *Experimental Neurology* 2003;182:135–141. [PubMed: 12821383]

Fig 1. Microglia can be repetitively harvested from murine mixed glial cultures

At 14 days *in vitro*, microglia were removed from mixed glial cultures and the total number of microglia isolated per flask was calculated from yield 1. Fresh media was added to the flasks for 1 week before microglia were collected again (yield 2). This was repeated a third time for yield 3. Results are the average of 3 independent experiments expressed as mean values $\pm$ S.D. (*= $p < 0.001$ from yield 1).

Fig 2. Microglia undergo little change in specific immunoreactivities during repetitive harvesting from mixed glial cultures

Microglia were collected from yields 1 (14 days *in vitro*) and 3 (28 days *in vitro*) and plated onto glass chamberslides at 40,000 cells/500mL DMEM/F12 for 24hours. The cells were fixed in 4% paraformaldehyde and immunostained using (A) anti-CD11b, (B) -CD68, (C) -MHCII, or (D) -CD45 antibodies. Antibody binding was visualized using Texas Red (A,B) and FITC (C,D) -conjugated secondary antibodies. Images are representative of 3 independent experiments. Scale bar represents 50 μ m.

Fig 3. Microglia express quantitatively similar immunoreactivities during repetitive harvesting from mixed glial cultures

Microglia were collected from yields 1 (14 days *in vitro*) and 3 (28 days *in vitro*) and plated onto black 96 well clear bottomed plates with 20,000 cells/75 μ L DMEM/F12 for 24 hours. The cells were fixed in 4% paraformaldehyde and immunostained using anti-CD11b, -CD68, -CD45 or -MHCII, antibodies. Antibody binding was visualized using VIP as the chromagen. Cells were counterstained with DAPI. Immunostaining absorbance values were normalized against their respective nuclear DAPI relative fluorescence units and averaged $\pm$ S.D. Data shown are representative of three independent experiments.

Fig 4. Microglia have decreased ability for stimulated secretion of specific cytokines during repetitive harvesting from mixed glial cultures

Microglia were collected from yields 1 (14 days *in vitro*) and 3 (28 days *in vitro*) and plated onto 96 well plates with 20,000 cells/75μL DMEM/F12 with or without 25ng/mL LPS for 24 hours. The media was collected and used to quantitate (A) IL-6, (B) TNFα, and (C) LDH release. Results are representative of 3 independent experiments and are expressed as mean values + S.D. (*=p<0.001 from the respective control, **=p<0.001 yield 1+LPS vs yield 3 +LPS).

Fig 5. Microglia have no decrease in phagocytic ability during repetitive harvesting from mixed glial cultures

Microglia were collected from yields 1 (14 days *in vitro*) and 3 (28 days *in vitro*) plated onto 96 well plates with 20,000 cells/75 μ L DMEM/F12 media with or without 0.25mg/mL of FITC-*E. coli* Bioparticles. After 6 hours all media was removed and replaced with 0.25mg/mL of Trypan Blue to quench the extracellular FITC signal from unphagocytosed peptide. Intracellular fluorescence was read at 480/520 via plate reader. Results are expressed as relative fluorescence units (RFU) and are representative of 3 independent experiments expressed as mean values $\pm$ S.D. (*= $p < 0.001$ from respective control).