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Validation of flow cytometry and magnetic bead-based methods to enrich CNS single cell suspensions for quiescent microglia

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Introduction

Microglia are resident CNS mononuclear phagocytes derived from myeloid progenitor stem cells in the yolk sac early in the course of embryogenesis (Ginhoux *et al.*, 2010). Microglia are required for many CNS functions, including immunosurveillance (Nimmerjahn *et al.*, 2005; Hanisch & Kettenmann 2007), cytokine production and cell signaling (Lee *et al.*, 1993; Hanisch 2002; Coull *et al.*, 2005; Liu *et al.*, 2011), clearing synaptic debris (Stevens *et al.*, 2007), and recovery from CNS insults (Chao *et al.*, 1993; Fitch & Silver 2008). Depending upon the specific environmental context and cellular activation state, microglia can further damage CNS ultrastructure through oxidative injury (ultimately leading to scarring, *e.g.* Dohi *et al.*, 2010; Block *et al.*, 2007) or help preserve and protect the CNS against these same types of injury (Polazzi *et al.*, 2001; Neumann *et al.*, 2006; Wyss-Coray 2006).

Significant progress has occurred toward understanding microglial function under both normal and pathological conditions. Microglial-neuronal interactions govern microglial activation state; once alerted, microglia coordinate chemotaxis (“find me”) and phagocytosis (“eat me”) signaling cascades to react in a rapid yet flexible manner to challenges of CNS integrity. Furthermore, tonic signals that inhibit microglial activation also inhibit microglial chemotaxis and phagocytosis, while signals triggering chemotaxis and phagocytosis

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Conflict of interest

The authors declare no conflicts of interest for this work.

Author contributions

All studies conceived, analyzed, reported by SJB. TAV, CDR, TAH performed and analyzed flow cytometry and *in vitro* microglial studies. TAH, JA collected and analyzed DIC and confocal microscopy images demonstrating microglial enrichment. TAV, TAH, JA assisted in manuscript preparation.

reinforce microglia to remain in an activated state. We depict the most significant of these mutual interactions in Figure 1.

Microglia and immunosurveillance

Despite the term “resting”, microglia in an unactivated state are highly dynamic, sending and withdrawing cytoplasmic processes that intermittently contact neuronal soma and synaptic networks (Nimmerjahn *et al.*, 2005; Wake *et al.*, 2009). Under normal surveillance conditions, neuronal-microglial interactions between CD200-CD200R1 (Hoek *et al.*, 2000; Wright *et al.*, 2003), CD47-SIRP α (Bamberger *et al.*, 2003; Gitik *et al.*, 2011) and CX3CL1-CX3CR1 (Harrison *et al.*, 1998; Cardona *et al.*, 2006) are particularly relevant. Microglia also express receptors for neurotransmitters; their sensing of ongoing synaptic activity may restrain microglial production of pro-inflammatory cytokines (Färber & Kettenmann 2005). Distinct signaling cascades mediate downstream effects of these different surveillance mechanisms, suggesting that the transition from normal to activated microglia is exquisitely regulated. For example, CD200 binding to CD200R1 (signaling a potential problem) autophosphorylates an NPxY phosphotyrosine binding domain recognition motif on CD200R1, triggering phosphorylation of the docking proteins *Dok1* and *Dok2* that attenuate signaling through protein tyrosine kinase (PTK) pathways (for review, Mashima *et al.*, 2009) and promote cytoskeletal reorganization (Master *et al.*, 2003). By contrast, under normal conditions there is tonic CD47 binding to SIRP α , leading to ITIM autophosphorylation (Kharitonov *et al.*, 1997) and recruitment of tyrosine protein phosphatases PTPN6 and PTPN11, ultimately biasing cell actions to prevent microglial phagocytosis (Yamao *et al.*, 2002). Similarly, tonic CX3CL1 binding to the CX3CR1 receptor prevents microglial activation while promoting neuronal survival (Cardona *et al.*, 2006). Under baseline immunosurveillance conditions, tonic SIRP α /CD47 and CX3CL1/CX3CR1 interactions not only maintain microglia in an inactive state, but also inhibit signaling cascades necessary for chemotaxis and phagocytosis. Several excellent reviews further detail steady-state microglial surveillance of the CNS (Wake *et al.*, 2013; Schafer *et al.*, 2013; Ousman & Kubes 2012).

Microglial chemotaxis

Microglial chemotaxis is also subject to complex, independent, and interacting feedback control. Robust microglial chemotaxis occurs after activation of purinergic- and/or complement- based signaling pathways. Following neuronal or astrocyte damage, there is purine leakage into the extracellular space (Honda *et al.*, 2001). Microglia have multiple receptors for purine sensing, including ionotropic P2RX₄ receptors and metabotropic P2RY₆ and P2RY₁₂ receptors. Chemotaxis responses occur with activation of the G_i α /Go α -coupled P2RY₁₂ receptor (Sasaki *et al.*, 2003; Haynes *et al.*, 2006), which increases intracellular cAMP and Ca⁺⁺ efflux. This signal coordinates microglial development of ruffled membranes and focal adherence of microglial processes to sites of damage (Ohsawa *et al.*, 2007). Increased cAMP and Ca⁺⁺ further activate microglial IP₃, PLC, and PKA signaling pathways, leading to β -arrestin-2-dependent activation of ERK1/2 (Lee *et al.*, 2012), VAST phosphorylation (Lee & Chung 2009), paxillin phosphorylation, and extension of ruffled membranes necessary for chemotaxis. Complement sensing pathways independently support microglial chemotaxis. Microglia express C5AR1 receptors that bind the cleavage product

complement 5a, evoking increased intracellular Ca^{++} flux, Rac1 signaling facilitation, and ruffled membrane extension (Lacy *et al.*, 1995; Nolte *et al.*, 1996; Möller *et al.*, 1997; Miller & Stella 2009). Chemokine- and cannabinoid- based signaling systems contribute additional chemotactic regulatory control. For example, chemokines CCL2, CCL3, CCL4, CCL5, and CXCL1 (following binding to their cognate receptors) all facilitate microglial chemotaxis (Cross & Woodroffe 1999; Škuljec *et al.*, 2011) through G protein mediated cascades (Patel *et al.*, 2013 for review), including Gi_α (inhibition of adenylate cyclase, decreased cAMP, and increased intracellular Ca^{++} flux); and $\text{G}_{\beta\gamma}$ (activation of PIP_3 activation, increased intracellular IP_3 and Ca^{++} , downstream activation of PI3K, MAPK, and PIP_3 pleckstrin-homology domain pathways). By contrast, ligand binding at microglial CB_2 receptors attenuates both microglial activation and chemotaxis (Ehrhart *et al.*, 2005; Romero-Sandoval *et al.*, 2009) through $\text{Gi}_\alpha/\text{Go}_\alpha$ actions on adenylate cyclase and MAPK (Shoemaker *et al.*, 2005). Recent reviews offer additional in-depth treatment of microglial chemotaxis (Louboutin & Strayer 2013; Corriden & Insel 2012; Cabral *et al.*, 2008).

Microglial phagocytosis

After damage signals have appropriately activated microglia and recruited them to the site of injury, there are multiple mechanisms coordinating phagocytosis of cellular debris and trafficking this material for ultimate degradation. The interior surface of neuronal cell membranes is actively enriched for phosphatidylserine (Diaz & Schroit 1996), with little present on the membrane exterior. Extracellular MFG-E8 opsonizes free- and membrane-bound external phosphatidylserine (Hanayama *et al.*, 2002; Fricker *et al.*, 2012). In the presence of activated microglia, these complexes induce phagocytosis through the vitronectin receptor, initiating a signaling cascade involving Dock180 and Rac1 (Albert *et al.*, 2000). Microglia also express pattern recognition receptors (PRRs) specialized to trigger phagocytosis, including Trem2 (Neumann & Takahashi 2007), F_c receptors for Ig (Fcgr1, Fcgr3; Suemitsu *et al.*, 2010), and lectin receptors (Maneu *et al.*, 2011; Rotshenker *et al.*, 2008). All of these PRRs signal through Tyrobp, evoke ITAM phosphorylation, switch Syk into an autophosphorylated, constitutively active state, and thus activate multiple interacting second messenger pathways that ultimately converge upon NF κ B. Complement also participates in microglial phagocytosis. C1q, particularly in concert with calreticulin (Gardai *et al.*, 2005), binds to neurites that have lost sialic acid residues from the neuronal glycocalyx. This process marks neurites for phagocytic uptake through the CD11b receptor (Linnartz *et al.*, 2012) and generates C3 opsonins that further facilitate phagocytosis. In addition to triggering chemotaxis (as discussed above), extracellular purines also bind to metabotropic P2RY_6 receptors. As mentioned above, this event initiates Gi_α and $\text{G}_{\beta\gamma}$ signaling cascades that ultimately recruit multiple pathways responsible for cytoskeletal reorganization.

Microglia and neurodegenerative diseases

Microglia are important contributors to the pathogenesis of many neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and multiple sclerosis. Alzheimer's disease is characterized by amyloid β plaques surrounded by activated microglia and astrocytes. Microglia bind to the A β fibrils and initiate phagocytosis, triggering tyrosine kinase-mediated cascades, and ultimately, production of IL-1 (Huseman

et al., 2002; Fu *et al.*, 2014). In Parkinson's disease, oxidative stress and an accumulation of α -synuclein lead to dopaminergic neuronal loss in the substantia nigra, resulting in characteristic motor impairments. Further release of α -synuclein, neuromelanin, and MMP3 from dopaminergic neurons activates microglia and generates neuroinflammatory molecules such as nitric oxide and superoxide. Because the products of dopaminergic neuronal degeneration are difficult to clear, a chronic state of inflammation is induced (Huseman *et al.*, 2002; Hwang 2013). Multiple sclerosis arises from demyelination and formation of sclerotic lesions in the CNS. In mouse models, several immune cells, including microglia, are aggregated around the sclerotic lesions. Changes in myelin appear to induce microglial phagocytosis. Microglia remove degenerated myelin, which helps restore the environment necessary for myelin regeneration. However, this process is not vigorous enough to lead to full recovery of myelin loss (Huseman *et al.*, 2002; Hendrickx *et al.*, 2014).

Methodological approaches to obtain microglia for study

Microglia were first purified from mixed glial cultures through differential adhesion (Giulian & Baker 1986). Alternatively, microglial F_c receptors were cross-linked into rosettes and then separated from the remaining cell suspension through a centrifugation gradient (Hayes *et al.*, 1988). Immunopanning with antibodies against targets expressed by specific cells within a CNS suspension was initially described to purify retinal ganglion cells (Barres *et al.*, 1988), and was soon modified to isolate microglia (Fulton *et al.*, 1992). Further studies demonstrated that microglia could reliably be differentiated from CNS-associated macrophages on the basis of CD45 expression (Ford *et al.*, 1995). These findings suggested that the cell surface expression phenotype of perivascular microglia was CD11b(+)/CD45(+), compared to the CD11b(+)/CD45(low) phenotype of parenchymal microglia and the CD11b(-)/CD45(+) phenotype of macrophages.

Immune-based separation methods have been refined to shorten protocol time and increase yields of desired target populations (Marek *et al.*, 2008). These methods use small specific-antibody-coated iron beads that bind large quantities of antigen while in suspension, yet can be rapidly removed from suspension with a weak magnetic field to elute captured cells (Reynolds *et al.*, 1985). We present further validation justifying magnetic bead separation approaches to obtain enriched populations of quiescent perivascular and parenchymal microglia. We replicate several studies demonstrating the flow cytometry phenotype of microglia, and provide novel data showing that the microglia are in a quiescent state following purification (as assessed by a sensitive activation marker), yet undergo changes in gene expression concordant with a microglial proinflammatory phenotype following stimulation with lipopolysaccharide (LPS).

Methods

Mouse husbandry

Mice (strain C57BL/6, BALB, male) were obtained from the NIA aged rodent colony; animals with obvious medical problems (cataracts, skin lesions, tooth malocclusion, *etc.*) were excluded. Mouse ages included 2–3 mo old, 12–13 mo old, and 21–24 mo old. Mice were sacrificed by decapitation followed by rapid brain dissection and placement of tissue

into phosphate-buffered saline (PBS) for preparation of single cell suspensions. Both institutional and federal regulations (2011) regarding animal care and welfare were followed.

Preparation of CNS single cell suspensions

Single cell suspensions (SCSs) of the hypothalamus and cerebellum were prepared using a commercially available papain-based enzymatic digestion kit (Worthington Biochemicals, Lakewood, NJ) per Huettner and Baughman (1986). Following sacrifice, CNS tissue was placed in 2 mL PBS, minced with a spatula, and allowed to settle for 5 min. PBS was removed and replaced with 1300 μ L of protease solution (20 U papain per mL Earle's Balanced Salt Solution (EBSS) in 1 mM L-cysteine with 0.5 mM EDTA). Tissue was placed in a 5% CO₂ incubator for 40 min at 37° C, and gently rocked every 10 min. After incubation, tissue was thoroughly triturated with a 1 mL pipette into a suspension, filtered with a 40 μ m cell strainer (Falcon), and centrifuged for 3 min at 300 $\times$ g at room temperature (RT). We then removed the supernatant from the tube, taking care not to break the pellet, and added EBSS/albumin/ovomucoid inhibitor/DNAase (23.44 μ g albumin, 23.44 μ g ovomucoid inhibitor, 7.5 U DNAase in 787.5 μ L EBSS). We then slowly added 1250 mL EBSS/albumin/ovomucoid inhibitor (390.6 μ g albumin, 390.6 μ g ovomucoid inhibitor in 1250 mL EBSS) to the bottom of the tube. Samples were then centrifuged for 10 min at 300 $\times$ g at RT (discontinuous density gradient separation). Following centrifugation, cells were rinsed in 2.25 mL PBS buffer (aliquoted from 1 L PBS, 0.5% bovine serum albumin, 2 mM EDTA, pH 7.2), centrifuged at 300 $\times$ g for 3 min at RT, and then resuspended in 250 μ L PBS buffer. SCSs at this stage consisted of dissociated neurons, astrocytes, microglia, oligodendrocytes, a small number of leukocytes, CNS endothelial, and ependymal cells, as well as cellular debris (Figure 2, top row, second column from left).

Microglial enrichment

We enriched the above SCS for microglia using a magnetic bead-based approach (Miltenyi Biotec, Auburn, CA). First, SCSs were mixed with anti-myelin magnetic beads (50 μ L young cohort, 75 μ L middle-aged cohort, 100 μ L aged cohort, 130-094-544, Miltenyi) and kept at 4° C for 15 min. Cells were washed with 2250 μ L PBS buffer and centrifuged at 300 $\times$ g for 10 min. Supernatant was removed, cells resuspended in 500 μ L PBS buffer, and filtered through an automatic magnetic cell sorter (autoMACS, DEPL05 setting, Miltenyi), keeping the flow-through and discarding the bead-bound elements. This procedure removed myelin debris from the SCS (Figure 2, third column from left). The flow-through was centrifuged at 300 $\times$ g for 10 min at RT. The supernatant was discarded, and cells resuspended in 90 μ L PBS buffer mixed with anti-CD11b magnetic beads (10 μ L, 130-093-634, Miltenyi). The suspension was placed at 4° C for 15 min, rinsed in 1 mL PBS buffer, and recentrifuged at 300 $\times$ g for 10 min at RT. We then discarded the supernatant, resuspended the cells in 500 μ L PBS buffer, and filtered this solution twice through the autoMACS (POSSEL setting), now discarding the flow-through for each run and keeping the bead-bound elements (Figure 2, fourth column from left). CD11b is a well-established surface antigen present on both quiescent and activated microglial cells (Sedgwick *et al.*, 1991; Frank *et al.*, 2006).

Flow cytometry identification of CD11b positive cells

Putative microglial suspensions were validated for successful enrichment by flow cytometry. A small aliquot of the post-MACS single cell suspension was reserved as a cells-only control. Compbeads (BD Biosciences, anti-rat Ig κ , 51-90-9001189) stained with FITC and APC were used for positive controls. SCSs (starting suspension, post-myelin removal, and post-CD11b enrichment) were stained with rat anti-mouse CD11b Ab (BD Pharmingen, 553310) directly conjugated to FITC. Primary antibodies were incubated for 1 hour at 4° C. Labeled cells were rinsed in 1 mL PBS buffer, centrifuged 300×g for 10 min at RT, and resuspended in 300–500 μ L PBS buffer. FITC-labeled cell populations were identified by FACS-Calibur (BD Biosciences).

Immunofluorescent identification of CD11b and CD45 positive cells

Single cell suspensions were prepared from cerebellum as described above. To perform cytopins, 100 μ L of cells were collected after each of the following steps: tissue dissociation, post myelin removal, and post CD11b enrichment. Tubes were placed in a cytopsin chamber (Cytopro 7620, Wescor, UT) and spun for 5 min at 1000 rpm, adhering cells to the slides (Fisherbrand Superfrost™ Plus, #12-550-15). A circular hydrophobic barrier was created around cells (ImmEdge™, Vector, CA), after which cells were treated with 50 μ L 4% PFA/4%-sucrose warmed to 37°C, and incubated for 10 min at RT. Following incubation, cells were washed twice for 5 min in PBS. Cells were then covered with 50 μ L 0.1% triton-X in PBS, and incubated 10 min at RT. Following this incubation, cells were washed once for 5 min in PBS, and blocked in 5% BSA in PBS for 1 hr at RT. We added 50 μ L of primary antibody mix (in 1% BSA/PBS) containing anti-CD45 at 1:200 dilution (NBP2-15811, rabbit polyclonal, Novus Biologicals, CO) and anti-CD11b at 1:200 dilution (ab8878, rat monoclonal M1/70 IgG2b, Abcam, MA), and incubated overnight at 4 °C. Following incubation, cells were washed three times for 10 minutes each in PBS. We then added 50 μ L of secondary antibody mix (in 1% BSA/PBS) containing Alexa Fluor 488 goat anti-rabbit IgG (H+L) at 1:250 dilution (A11034, Invitrogen, NY) and Alexa Fluor 555 goat anti-rat IgG (H+L) at 1:250 dilution (A21434, Invitrogen, NY). We rinsed slides in PBS three times for 10 min each, and stained nuclei with 4',6-diamidino-2-phenylindole (DAPI) in PBS (1:4000 dilution) for 30 sec. DAPI was washed off slides with a 5 min PBS rinse. Excess fluid was removed, and slides were treated with antifade gold, coverslipped, dried in the dark, and sealed at the edges using nail polish. We stored slides at –80 °C until imaging with a confocal microscope (Zeiss LSM700).

Microglial functional assay

Aliquots of myelin-depleted, CD11b-enriched cells were divided into immune-activation and sham-activation groups. For both groups, cells were placed into modified Eagle medium and incubated for 4 hours under 5% CO₂ at 37° C. For immune activation, LPS (from *E. coli* 0111:B4, InvivoGen, San Diego) was added to the medium at 10 ng/ml. Given the incomplete understanding of which DAMP combinations most closely resemble the physiological situation that triggers microglial activation, we chose a lower-range LPS dosage for activation. Our major concern was to avoid potentially idiosyncratic microglial responses that might be observed at higher-than-physiological LPS dosages (Abd-El-Basset

& Federoff, 1995). RNA was harvested from SCS, and underwent RT-qPCR assessment for IL-1 β , Aif1 (Iba-1), CD45, CD11b, Annexin1, Coronin-1a, and GFAP. Presumptive microglia were expected to change expression of all these molecules except GFAP, which was included as a negative control, in response to immune activation (Cahoy *et al.*, 2008; Ahmed *et al.*, 2007; McArthur *et al.*, 2010).

RNA isolation from SCS

We employed magnetic bead separation to obtain mRNA from SCSs (μ MACS, 130-075-201, Miltenyi). Lysis/binding buffer (high salt, 1% SDS) was warmed to RT, and elution buffer was heated to 70° C. SCSs were centrifuged at 300 $\times$ g for 10 min at RT. Supernatant was removed, 1 mL of lysis/binding buffer added to cells, and tubes vortexed for 4 min to achieve thorough cell lysis. Lysed suspensions were then equilibrated at RT for 5 min. Kit-supplied lysate clear columns were placed in a 2 mL centrifuge tube. Lysate clear columns containing an integrated filter were loaded with lysate and centrifuged at 13000}g for 3 min at RT to remove cell debris. 50 μ L oligo-dT magnetic beads were added to the flow-through from the lysate clear column. We prepared the kit-supplied MACS micro column by mounting the column in a magnetic separator, and wetting the column with 10 μ L lysis binding buffer. The solution containing oligo-dT magnetic beads was then loaded onto the MACS micro column and allowed to flow through. The column was rinsed with 200 μ L lysis binding buffer x2 to remove protein and DNA contaminants, followed by 100 μ L wash buffer x4 to remove rRNA and DNA contaminants. MACS micro columns were cleared by adding 27 μ L elution buffer (discarding flow-through). mRNA was then eluted into a clean RNAase free Eppendorf tube by adding 75 μ L elution buffer to the MACS micro column. The beads captured messages possessing a 3' tail; smaller RNAs and immature or long noncoding RNAs (lncRNAs) without a tail are discarded in the flow-through.

RT-qPCR measures of gene expression

RT-qPCR (*Taq* polymerase, SYBR green) was performed per standard protocol in 96 well plates. Master mix was prepared per manufacturer's instructions and primers (QuantiTect[®], Qiagen) added at 0.5 μ L/reaction. Samples were run in triplicate (Mastercycler ep *realplex*, Eppendorf). Controls included (1) positive control, (2) no RT with RNA and master mix, and (3) no RNA with RT and master mix. We examined the melting curves for each reaction to ensure amplification of a single PCR product. RT-qPCR cycle was converted to relative gene expression by the comparative C_T method (Livak & Schmittgen 2001).

Statistics

Each sample post-LPS-activation value for C_T was normalized to that sample's pre-LPS-activation C_T value. Mean $\pm$ standard error of the mean were calculated for each gene/region. Significances were determined by z-score (testing for means greater than 1) followed by Bonferroni correction.

Results

Flow cytometry validation of microglia from hypothalamic and cerebellar single cell suspensions

Figure 2 depicts representative data obtained from different steps of the magnetic bead-based cell separation protocol for cerebellar-derived single cell suspensions (analogous data for hypothalamic-derived single cell suspensions not shown). The first and second row of panels demonstrate that in starting single cell suspensions, microglia (constituents with high CD11b fluorescence and low side scatter) constitute a small population (15%) of elements within the target gate. Following depletion of myelin debris, there is a significant decrease in suspension debris. Parenchymal microglia show significant enrichment, while perivascular microglial elements remain sparse. Following two rounds of enrichment for CD11b(+) cells, microglia remain the majority constituent providing a flow cytometric signal in the target gate (64.2% for depicted experiment). Furthermore, we note excellent enrichment of both parenchymal (50.2%) and perivascular (32.7%) microglia within the target gate. In our experience, we typically obtain microglial enrichments of 65–85% from our neuronal single cell suspensions, regardless of CNS region studied. These results suggest that magnetic bead-based purification of single cell CNS suspensions can be significantly enriched for putative microglia.

Functional validation of microglia from hypothalamic and cerebellar single cell suspensions

The flow cytometric studies show that cells expressing either CD11b (presumptive parenchymal microglia) or CD11b/CD45 (presumptive perivascular microglia) after magnetic bead-based separation are the dominant elements of the single cell suspension (Ford *et al.*, 1995; Zhang *et al.*, 2002). Further phenotyping of this population demonstrates a low expression level of Aif1 in cells from the enriched suspension (Figure 3A). However, following LPS treatment, expression levels of a number of microglial-associated molecules, including Aif1, Cd68, IL-1 β , Itgam, and Anxa1, demonstrate a marked change in expression (Figure 3B), while the astrocyte-specific marker Gfap remain at undetectable levels. Microglial responses to LPS also depend on their region of origin. Coron1 and Itgam expression increases and decreases (respectively) in hypothalamic-derived microglia, while Coron1 expression does not change, and Itgam expression significantly increases in cerebellar-derived microglia.

Discussion

We demonstrate that magnetic bead-based cell sorting of CNS single cell suspensions that depletes myelin debris and enriches for CD11b(+) elements is an effective way to obtain populations of quiescent parenchymal and perivascular microglia. Suspensions that have undergone this process have a high percentage of CD11b(+) cells that do not express genes associated with microglial activation after purification, but demonstrate a microglial phenotype following LPS stimulation.

This study provides further validation for using magnetic bead-based methods to isolate CNS microglia in a minimally disruptive manner. We provide the first data demonstrating a quiescent microglial phenotype (as determined by Aif1 expression) in suspensions following microglial enrichment. Similar findings have been noted when these methods were used to enrich for monocytes without evoking macrophage activation (Lyons *et al.*, 2007). We also provide the first data showing that following stimulation with lipopolysaccharide, quiescent enriched microglial suspensions appropriately alter expression of genes associated with microglial, but not astrocyte, identity. Finally, we confirm prior findings showing that flow cytometry gating characteristics of presumptive parenchymal microglia include cells with low side scatter, low CD45 surface expression, and high CD11b surface expression, while presumptive perivascular microglia include cells with low side scatter, high CD45 surface expression, and high CD11b surface expression (Bedi *et al.*, 2013; Harms *et al.*, 2012; Zhang *et al.*, 2002).

We note four limitations in this study. First, it is highly likely that future studies will implicate more genes as specific markers of microglial identity or activation. Thus, future gene panels best suited to identify microglia may differ from the panel we chose. Second, for applications focused on obtaining either perivascular or parenchymal microglia, our approach should be modified to include magnetic bead separation (depletion or enrichment) by CD45 surface expression. Future investigators may also want to differentiate CD45(+) elements by CD16/CD68, F4/80, or CD11c/MHC-II expression to further identify macrophages or dendritic cells. Third, we limited our functional validation to gene expression changes in response to LPS stimulation. Additional assays of microglial function, including phagocytosis (Chan *et al.*, 2001; Pul *et al.*, 2013), cytokine production (Woodroffe *et al.*, 1991; Lee *et al.*, 2002), and cell motility (Davalos *et al.*, 2005; Haynes *et al.*, 2006) assays would provide further evidence that cells obtained by this method demonstrate a microglial phenotype. Finally, we did not perform confirmatory microglial imaging following enrichment for either quiescent or activated conditions.

In conclusion, we suggest that techniques for microglial enrichment of CNS cell suspensions have become streamlined such that laboratories with access to flow cytometry and magnetic bead separation can obtain high quality samples in a rapid, inexpensive, and reliable fashion. Given the significant role that microglia contribute to CNS homeostasis, function, injury, and repair, reliable purification of microglia will remain an important tool to advance our understanding of CNS function and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

(CCL1, <i>aka</i> SCYA1, I-309, TCA3, P500, SISE)	chemokine C-C motif ligand 1
(CCL2, <i>aka</i> MCP-1, MCP1, GDCF-2, HC11, HSMCR30, MCAF, SCYA2, SMC-CF)	chemokine C-C motif ligand 2
(CCL3, <i>aka</i> G0S19-1, LD78ALPHA, MIP-1 α , MIP1A, SCYA3)	chemokine C-C motif ligand 3
(CCL4, <i>aka</i> ACT2, AT744.1, G-26, HC21, LAG-1, LAG1, MIP-1 β , MIP1B, MIP1B1, SCYA2, SCYA4)	chemokine C-C motif ligand 4
(CCL5, <i>aka</i> D17S136E, RANTES, SCYA5, SIS-delta, SISd, TCP228, eoCP)	chemokine C-C motif ligand 5
(C5AR1, <i>aka</i> CD88)	complement 5a receptor
(cyclic AMP)	cAMP
(CD47, <i>aka</i> IAP, MER6, OA3)	cluster of differentiation 47
(CD200, <i>aka</i> MOX1, MOX2, MRC, OX-2)	cluster of differentiation 200
(CD200R1, <i>aka</i> CD200R, HCRTR2, MOX2R, OX2R)	cluster of differentiation 200 receptor
(chemokine C-X3-C motif ligand, <i>aka</i> fractalkine, neurotactin, ABCD-3, C3Xkine, CXC3, CXC3C, NTN, NTT, SCYD1)	CX3CL1
(CX3C chemokine receptor 1; <i>aka</i> fractalkine receptor, CCRL1, CMKBRL1, CMKDR1, GPR13, GPRV28, V28)	CX3CR1
(Dock180, <i>aka</i> Dock1)	dedicator of cytokinesis 180
(Dok1 <i>aka</i> P62DOK)	docking protein 1
(Dok2, <i>aka</i> p56DOK, p56dok-2)	docking protein 2
(ERK1/2)	extracellular signal-regulated protein kinases 1 and 2
(Fcgr1)	high affinity immunoglobulin gamma F _c receptor I

(Fcgr3)	low affinity immunoglobulin gamma F _c region receptor III
(ITAM)	immunoreceptor tyrosine-based activation motif
(ITIM)	immunoreceptor tyrosine-based inhibitory motif
(IP₃)	inositol triphosphate
(IL-1)	interleukin 1
(MMP3)	matrix metalloproteinase-3
(MFG-e8)	milk fat globule-EGF factor 8 protein
(PLC)	phospholipase C
(NFκB)	nuclear factor kappa-light-chain-enhancer of activated B cells
(PTPN6, aka HCP, HCPH, HPTP1C, PTP-1C, SH-PTP1, SHP-1, SHP-1L, SHP1)	protein tyrosine phosphatase non-receptor type 6
(PTPN11, aka BPTP3, CFC, NS1, PTP-1D, PTP2C, SH-PTP2, SH-PTP3, SHP2)	protein tyrosine phosphatase non-receptor type 11
(PKA)	protein kinase A
(PTK)	protein tyrosine kinase
(P2RX₄, aka P2X4, P2X4R)	purinergic receptor P2X ligand-gated ion channel, 4
(P2RY₆, aka P2Y6)	pyrimidinergic receptor P2Y G-protein coupled, 6
(P2RY₁₂, aka ADPG-R, BDPLT8, HORK3, P2T(AC), P2Y(12)R, P2Y(AC), P2Y(ADP), P2Y(cyc), P2Y12, SP1999)	purinergic receptor P2Y G-protein coupled, 12
(Rac1)	ras-related C3 botulinum toxin substrate 1
(SIRPα, aka SIRP, SHPS1, CD172A, PTPNS1, BIT, MFR, MYD-1, P84)	signal regulatory protein α
(Syk)	spleen tyrosine kinase
(Tyrobp)	TYRO protein tyrosine kinase-binding protein
(Trem2)	triggering receptor expressed on myeloid cells 2

(VASP)

vasodilator-stimulated phosphoprotein

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Figure 1. Overview of molecular systems regulating microglial activation state, chemotaxis, and phagocytosis

Processes regulating activation are in the light blue box, processes regulating chemotaxis are in the light purple box, and processes regulating phagocytosis are in the light green box. Pathways ending with an arrowhead indicate that the pathway activates its target; pathways ending with a T indicate that the pathway inhibits its target. Pathways colored green are tonically active in microglia that are not activated; pathways colored red are activated once their appropriate signal (ligand-receptor binding, *etc.*) occurs. For clarity, not all intermediate steps are shown in each pathway. SIRPα/CD47 and CX3CL1/CX3CR1 interactions are ongoing aspects of neuronal-microglial information exchange, reinforcing microglial inactive state.

Figure 2. Cerebellar single cell suspensions are enriched for perivascular and parenchymal microglia as assessed by flow cytometry

Top row: Cytospin images demonstrating progressive sample enrichment for CD11b(+) elements. Cells are stained for CD11b (Alexa Fluor 555; red), CD45 (Alexa Fluor 488; green) and cell nuclei (DAPI; blue). The leftmost panel suggests that most cells in the starting CNS single cell suspension do not stain for microglial markers. After myelin depletion, the suspension has been enriched for cells, but most of these cells do not express microglial markers (center panel). However, after bead enrichment for CD11b-expressing cells (right panel), there is a significant increase in cells staining for either CD11b (red only) or CD11b/CD45 (red and green). Inset is a 4X enlargement of cluster of 10 cells on right hand side of panel. Scale bar is 10 μ m. Second row: Forward scatter vs. CD11b-FITC fluorescence demonstrating progressive enhancement of CD11b(+) elements. Unstained panel estimates FITC autofluorescence, cells only panel estimates the percentage of microglia in whole cerebellar tissue (similar to values obtained by microscopic methods, Ashwell 1990). Suspensions following myelin removal and CD11b enrichment show significant increase in percentage of CD11b(+) elements. Third row: Contour maps for the corresponding within-gate scatterplots. Note that with each protocol step there is further suspension enrichment for CD11b(+)/CD45(+) cells (perivascular microglia). FITC and APC compbead controls for CD11b and CD45 staining (respectively) as labeled.

Figure 3. Microglia from CNS single cell suspensions are in a quiescent state, but readily activate following exposure to lipopolysaccharide

A. Baseline expression of genes consistent with microglial phenotype following magnetic bead-based enrichment. Ordinate depicts number of thermal cycles above minimum detection threshold (41 cycles). Error bars are ± 1 standard error of the mean. **B.** Following LPS exposure, suspensions with cells enriched for surface CD11b expression demonstrate changes in gene expression consistent with a microglial phenotype. Bars in blue derived from hypothalamic samples ($n=3$ for each condition); bars in red derived from cerebellar samples ($n=3$ for each condition). Dotted lines at 1 and -1 depict cutoffs for significant fold changes. Error bars are ± 1 standard error of the mean.