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High yield primary microglial cultures using granulocyte macrophage-colony stimulating factor from embryonic murine cerebral cortical tissue

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ABSTRACT

Background: Microglia play vital roles in neurotrophic support and modulating immune or inflammatory responses to pathogens or damage/stressors during disease. This study describes the ability to establish large numbers of microglia from embryonic tissues with the addition of granulocyte-macrophage stimulating factor (GM-CSF) and characterizes their similarities to *adult* microglia examined *ex vivo* as well as their responses to inflammatory mediators.

Method: Microglia were seeded from a primary embryonic mixed cortical suspension with the addition of GM-CSF. Microglial expression of CD45, CD11b, CD11c, MHC class I and II, CD40, CD80, and CD86 was analyzed by flow cytometry and compared to those isolated using different culture methods and to the BV-2 cell line. GM-CSF microglia immunoreactivity and cytokine production was examined in response to lipopolysaccharide (LPS) and interferon-γ (IFN-γ).

Results: Our results demonstrate GM-CSF addition during microglial culture yields higher cell numbers with greater purity than conventionally cultured primary microglia. We found that the expression of immune markers by GM-CSF microglia more closely resemble adult microglia than other methods or an immortalized BV-2 cell line. Primary differences amongst the different groups were reflected in their levels of CD39, CD86 and MHC class I expression. GM-CSF microglia produce CCL2, tumor necrosis factor-α, IL-6 and IL-10 following exposure to LPS and alter costimulatory marker expression in response to LPS or IFN-γ. Notably, GM-CSF microglia were often more responsive than the commonly used BV-2 cell line which produced negligible IL-10.

Conclusion: GM-CSF cultured microglia closely model the phenotype of adult microglia examined *ex vivo*. GM-CSF microglia are robust in their responses to inflammatory stimuli, altering immune markers including Iba-1 and expressing an array of cytokines characteristic of both pro-inflammatory and reparative processes. Consequently, the addition of GM-CSF for the culturing of primary microglia serves as a valuable method to increase the potential for studying microglial function *ex vivo*.

1. Introduction

Glial interactions within the neurovascular niche have become an area of increased research due to their importance in understanding disease pathogenesis. The central nervous system (CNS) consists of a heterogeneous population of cells, where microglial cells make up roughly 10% of the cell population (Lawson et al., 1992). Microglia are the main myeloid-derived immune cells found in the CNS, and are important in development and the neuromodulatory, neurotrophic, and neuroimmune responses to insult or disease (Dougherty et al., 2000; Elkabes et al., 1996; Garden and Möller, 2006; González et al., 2014). During embryogenesis, microglial progenitor cells infiltrate the brain from the yolk sac (Alliot et al., 1999). Microglia survey and scavenge the CNS for potential pathogens and play an important role in supporting neurons by synaptic pruning and the engulfing of synaptic material (Paolicelli et al., 2011).

Insults, such as spinal cord injury, can result in changes to the microenvironment that change the phenotype in microglia from a resting to an “activated” state, and have been thought to play a role in the neuropathogenesis of several neurodegenerative diseases (Popovich et al., 1997). This change in microglial phenotype has been linked to neurodegenerative disorders such as Parkinson's, Alzheimer's

Abbreviations: LPS, lipopolysaccharide; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha; IL, interleukin; CCL2, chemokine (C-C motif) ligand 2; CD, cluster of differentiation; GM-CSF, granulocyte macrophage-colony stimulating factor; Iba-1, ionized calcium-binding adaptor molecule 1; MHC, major histocompatibility complex; CNS, central nervous system; HAPI, highly aggressive proliferating immortalized; DIV, days *in vitro*; HBSS, Hank's balanced salt solution; DMEM, Dulbecco's modified eagle medium; ELISA, enzyme-linked immunosorbent assay

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disease, and amyotrophic lateral sclerosis (Barger and Harmon, 1997; Hall et al., 1998; Jackson-Lewis et al., 2002). Microglia exist in several states of activity, often reflected by changes in morphology. Examination in neurodegenerative and neuroinflammatory conditions and diseases have shown microglia become intensely ramified and positive for the expression of ionized-calcium binding adaptor molecule 1 (Iba-1), a classic marker of microglial activation (Ito et al., 1998). Amoeboid microglia are round in shape with small processes and are motile, for scavenging debris for phagocytosis (Ferrer et al., 1990). Quiescent ramified microglia have thin branching processes and are found throughout the CNS. However, unlike amoeboid microglia, the cell body is static while the processes are constantly extending and retracting to survey the CNS (Wake et al., 2013). Quiescent ramified microglia when activated will undergo a morphological change where their processes are retracted and thickened. Activation of microglia can result in antigen presentation and secretion of cytokines such as interleukin-1 beta (IL-1β), interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α), as well as secretion of several proteases and reactive oxygen species (Aloisi, 2001; Kreutzberg, 1996).

Cell culture models are amongst the most straightforward means to establish factors and conditions which can influence the phenotype and function of these cells. Commonly used *in vitro* models of microglia employ the murine BV-2 cell line or the rat-derived highly aggressive proliferating immortalized (HAPI) cells. The BV-2 cell line has been immortalized by infecting primary microglia with the J2 retrovirus carrying the oncogenes *v-raf* and *v-myc* (Blasi et al., 1990). The HAPI cell line was developed spontaneously through a mutation of a primary microglial enriched culture (Cheepsunthorn et al., 2001). However, the suitability of these cell lines as an appropriate model of microglia *in vivo* has been questioned. Studies have shown that neither the BV-2 nor HAPI cell lines respond similarly to freshly isolated microglia to relevant stimuli in terms of TNF-α, IL-1β, IL-6, and CCL2 production (De Jong et al., 2008; Häusler et al., 2002). Indeed, BV-2 and HAPI cell lines have differential expression of Iba-1, cytokines, chemokines, nitric oxide production, and altered migratory capacity compared to primary microglia (Horvath et al., 2008).

Perhaps the most accepted and widely used method for the isolation of primary microglia is the dissociation of newborn murine cerebral cortical tissue followed by plating for typically up to 3 weeks and collection of loosely adherent microglia from the established glial layer and further passaged to improve purity (Giulian and Baker, 1986). Mild trypsinization can be done to collect adherent microglia but increases contaminating astrocytes. Another method dissociates murine brains into their various cell types through discontinuous density gradients (Cardona et al., 2006). Magnetic separation with antibodies to the myeloid marker CD11b and sorting add cost and time. These adult microglia are appropriate for *in vitro* studies but given they are a small proportion of the cells within the CNS (Lawson et al., 1992) the yield remains low with potential for contamination from the vasculature.

Colony stimulating factors drive differentiation and proliferation of precursor cells into cells of the myeloid lineage (Fukunaga et al., 1993; Metcalf, 1986; Souza et al., 1986). Granulocyte macrophage colony stimulating factor (GM-CSF) promotes the proliferation of myeloid-derived cells such as microglia, frequently used alone (Heufler et al., 1996; Inaba et al., 1992) and in conjunction with IL-4 (Labeur et al., 1999; Lu et al., 1995) to generate primary bone marrow-derived dendritic cells from myeloid precursors. Although studies have shown that GM-CSF promotes the proliferation of microglia *in vitro* (Giulian and Ingeman, 1988; Lee et al., 1994), the phenotypic profile and likeness of these microglia to microglia *in vivo* and whether or not GM-CSF can be used as a means to establish microglia for studies *in vitro* has not been characterized.

This study outlines a comprehensive phenotypic characterization of embryonic microglia established with and without the addition of GM-CSF compared to adult microglia examined *ex vivo* and the BV-2 cell line. Surface marker expression of microglia cultured in the presence of GM-CSF with and without the addition of the anti-inflammatory cytokine IL-4 was compared to that of primary microglia cultured from murine embryos without those factors as well as that of healthy adult microglia to determine the suitability of GM-CSF cultured microglia to model their *in vivo* counterparts and to serve as an appropriate *in vitro* model. With the intent to focus on preparations that give the greatest purity and yield, microglial inflammatory marker and cytokine expression was characterized following exposure of GM-CSF microglia to the inflammatory stimuli lipopolysaccharide (LPS) and IFN-γ compared to similarly treated BV-2 cells.

2. Materials & methods

2.1. Microglia cell culture and treatment

Glial cultures were prepared from E18 C57BL/6 mouse cerebral cortices according to a previously established protocol (Aiga et al., 2011). Similar procedures to establish neuronal cultures for *in vitro* analyses typically isolate hippocampal or striatal regions to enrich for populations of interest from embryonic cortices. We chose to build upon these protocols and start with the removal of these neuronal-rich regions as the first step of establishing enriched cultures of glial cells.

All experiments were carried out in compliance with the 1996 Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research and protocols approved by the University of British Columbia Animal Care Committee per the Canadian Council on Animal Care Guidelines. Briefly, whole brains were isolated from E18 pups (Fig. 1). The cerebral cortices were removed and the hippocampus and striatum were dissected away before washing in Hank's balanced salt solution (HBSS) (Invitrogen, Carlsbad, CA) and trypsinized with 0.05% trypsin (Sigma, St. Louis, MO) before mechanical dissociation into a single cell suspension. Cells were seeded at 100,000 cells/cm^2 in plating media [Dulbecco's modified eagle medium (DMEM) (Invitrogen)], completed with 0.5% wt/vol glucose (Bio Basic Inc., Markham ON), 100 μg/mL penicillin and 100 U/mL streptomycin (Sigma), 10% horse serum (Invitrogen) on 25 or 75 cm^2 poly-L-lysine (Sigma)–coated flasks (Corning Inc., Corning, NY). The cells were maintained at 37 °C with 5% CO_2. Plating media was changed with the removal of non-adherent cells the next day and followed by the addition of fresh media or media containing 50 ng/mL murine GM-CSF (Peprotech, Rocky Hill, NJ) with or without 25 ng/mL of murine IL-4 (Peprotech). Developing microglia appear as lightly tethered amoeboid cells above a mixed glial cell monolayer and expand until day *in vitro* (DIV) 21 when the microglia approach peak density. GM-CSF microglia were harvested by gentle pipetting to dislodge non- and loosely adherent microglia then washed and analyzed by flow cytometry or used for further studies. For comparison to our GM-CSF cultured microglia, primary microglia were also prepared using a common method to establish microglia from embryonic tissues (Théry et al., 1991). Briefly, E18 mixed cerebral cortical cells were seeded into plates (Corning) at 100,000 cells/cm^2 with weekly media changes. After 3 weeks in culture, loosely adherent microglia were dislodged by gently pipetting up and down over the surface of the glial monolayer to then collect and pellet the detached microglia by centrifugation prior to flow cytometry. Adult microglia were examined *ex vivo* in whole murine brain single cell suspensions isolated by passing minced adult mouse brain (8 weeks of age) through a 70 μm filter and washing with HBSS. Cells were separated from myelin, other cells and cellular debris using a 30%/70% Percoll (GE Health Sciences, Mississauga, ON) gradient and after washing in PBS with 2% fetal bovine serum (FBS) were stained for flow cytometry. The BV-2 cell line was cultured according to previous studies (Blasi et al., 1990) in DMEM media supplemented with 10% heat-inactivated FBS, 100 μg/mL penicillin, 100 U/mL streptomycin, and L-glutamine (4 mM). Cells were passaged weekly and reseeded at a density of 10,000 cells/cm^2.