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A novel cell line from spontaneously immortalized murine microglia

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HIGHLIGHTS

- A novel spontaneously immortalized microglial cell line, SIM-A9, was characterized.
- SIM-A9 cells express macrophage/microglia-specific proteins, CD68 and Iba1.
- LPS and β-amyloid elicit microglia-like immunological behavior in SIM-A9 cells.
- SIM-A9 cells can be induced to pro- or anti-inflammatory microglial phenotypes.

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ABSTRACT

Background: Purified microglia cultures are useful tools to study microglial behavior *in vitro*. Microglial cell lines serve as an attractive alternative to primary microglia culture, circumventing the costly and lengthy preparation of the latter. However, immortalization by genetic or pharmacologic manipulations may show altered physiology from primary microglia.
New method: A novel microglial cell line was isolated from a primary glial culture of postnatal murine cerebral cortices. The culture contained a population of spontaneously transformed microglia that continued to divide without genetic or pharmacological manipulations. After several clones were isolated, one particular clone, SIM-A9, was analyzed for its microglial characteristics.
Results: SIM-A9 cells expressed macrophage/microglia-specific proteins, CD68 and Iba1. SIM-A9 cells were responsive to exogenous inflammatory stimulation with lipopolysaccharide and β-amyloid, triggering tyrosine kinase-based and NFκB signaling cascades as well as TNFα secretion. SIM-A9 cells also exhibited phagocytic uptake of fluorescent labeled β-amyloid and bacterial bioparticles. Furthermore, lipopolysaccharide increased the levels of inducible nitric oxide synthase and cyclooxygenase-2, whereas IL-4 stimulation increased arginase-1 levels demonstrating that SIM-A9 cells are capable of switching their profiles to pro- or anti-inflammatory phenotypes, respectively.
Comparison with existing methods: The use of SIM-A9 cells avoids expensive and lengthy procedures required for the preparation of primary microglia. Spontaneously immortalized SIM-A9 cells are expected to behave more comparably to primary microglia than virally transformed or pharmacologically induced microglial cell lines.
Conclusions: SIM-A9 cells exhibit key characteristics of cultured primary microglia and may serve as a valuable model system for the investigation of microglial behavior *in vitro*.
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Abbreviations: SIM, spontaneously immortalized microglia; LPS, lipopolysaccharide; Aβ, beta-amyloid; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; IκB, inhibitor of κB; TNFα, tumor necrosis factor alpha; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; Arg-1, arginase-1; IL-4, interleukin-4; ELISA, enzyme-linked immunosorbent assay.

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1. Introduction

Microglia are the resident immune cells of the nervous system that continuously survey their local environment and become activated upon detection of abnormal and/or offensive signals to initiate inflammatory processes. Activated microglia have been described in a number of neurological conditions such as traumatic brain/spinal cord injury, ischemic strokes, infections, and neuroinflammatory diseases. Although microglial activation has been classically associated with inflammation and therefore implicated in pathogenesis of chronic neurodegenerative diseases such as Parkinson's and Alzheimer's diseases (McGeer and McGeer, 1995), newer evidence also supports a role of differentially activated microglia in regenerative processes with the expression of a distinct set of anti-inflammatory molecular markers (Chhor et al., 2013). Thus, understanding the mechanisms regulating specific phenotypes of microglia is of great interest to the field of neuroinflammatory diseases.

Purified microglia cultures are frequently used for the characterization of stimulus-triggered changes in their behavior. This *in vitro* model system provides a versatile tool, allowing direct application of exogenous stimulating or inhibiting agents to microglia, collection of secreted factors, and observation of microglial activities such as migration, proliferation and phagocytosis. In our laboratory, primary microglial cultures are routinely isolated from mixed cultures of neonatal mouse cerebral cortices and utilized to investigate responses to inflammatory stimuli as well as efficacy of anti-inflammatory agents (Dhawan et al., 2012; Floden et al., 2005; Nagamoto-Combs and Combs, 2010; Rojanathammanee et al., 2013; Sondag et al., 2009; Woster and Combs, 2007). However, isolation of primary microglia cultures requires a cumbersome procedure involving dissociation of brain tissue and 14 days of pre-culturing as a mixed cell population (Floden et al., 2005). Furthermore, the preparation method does not typically result in a large number of purified microglia thus requiring a substantial amount of brain tissue to obtain a sufficient number of microglia needed for an experiment.

In order to overcome the disadvantages of microglial culture, several research groups have created a few cell lines by transforming primary microglia with viral vectors (Blasi et al., 1990; Briers et al., 1994; Peudenier et al., 1991; Righi et al., 1989) or other genetic (Ohsawa et al., 1997) or pharmacological (Kanzawa et al., 2000) inductions. However, some issues with the long-term retention of primary microglia properties in these transformed cell lines have been reported (Ohsawa et al., 1997). Non-induced cell lines have also been isolated from spontaneously immortalized primary microglia from a mouse cerebellar organ culture (Alliot et al., 1996) and rat cerebral tissue culture (Cheepsunthorn et al., 2001). The novel microglial cell line described in this report is a cell line in this category, and has been isolated from a mixed glial culture of postnatal murine cerebral cortices that continued to proliferate for a number of passages without any genetic or pharmacologic manipulations. To our knowledge, this is the first spontaneously immortalized microglial cell line cloned from mouse cerebral tissue. In order to test whether our microglial cell line is a suitable alternative to the use of primary microglia culture, we have determined its phenotypic and functional properties that are characteristics of cultured primary microglia at rest as well as in response to exogenous proinflammatory stimuli.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12) was purchased from Life Technologies (Carlsbad, CA, USA). Mouse TNFα ELISA kit was obtained from R&D Systems (Minneapolis, MN, USA). Lactate dehydrogenase assay (LDH) and Griess assay were purchased from Promega (Madison, WI, USA).

Primary antibodies against inducible nitric oxide synthase (iNOS; NOS2 [C-11])), cyclooxygenase-2 (COX-2, [N-20]), arginase I (Arg-I), α-tubulin, and horseradish peroxidase conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-amyloid antibody was obtained from Covance (Emeryville, CA, USA). Anti-TNFα antibody was from Abcam (Cambridge, MA, USA) and anti-CD68 antibody was purchased from Serotec (Raleigh, NC, USA). The antibodies for phospho-IκB, IκB, and glial fibrillary acidic protein (GFAP) were acquired from Cell Signaling Technology (Danvers, MA, USA). Anti-phospho-tyrosine (4G10) antibody was from EMD Millipore (Billerica, MA, USA), and anti-Iba1 antibody was from Wako Chemicals USA, Inc (Richmond, VA, USA). All the biotinylated secondary antibodies, Elite Vectastain ABC Kit, VIP Peroxidase Substrate Kit were obtained from Vector Laboratories, Inc. (Burlingame, CA, USA). Anti-microtubule associated protein 2 (MAP2), lipopolysaccharide (LPS) and other chemicals were obtained from Sigma (St. Louis, MO, USA).

2.2. Animals

The use of mice was approved by the University of North Dakota (UND) Institutional Animal Care and Use Committee (IACUC). The C57BL/6 strain of mice were housed in a room with 12-hr light/dark cycle and food and water were provided *ad libitum* in accordance with the National Research Council of the National Academies Guide for the Care and Use of Laboratory Animals (8th edition). Mice were bred in the UND animal facility, and newborn pups were housed in the same cage with their mother until sacrificed for tissue culture preparation.

2.3. Tissue culture

Mixed glial cultures were prepared as previously described (Floden et al., 2005). Briefly, cortical tissues were collected from mouse pups at postnatal day 1 (P1). The tissues were pooled and trypsinized after the removal of meninges, then the dissociated cells were plated in DMEM/F12 supplemented with L-glutamine (EMD Millipore; Billerica, MA, USA), 10% heat-inactivated fetal bovine serum and 5% heat-inactivated horse serum (Serum Source International, Charlotte, NC, USA) in a 75-mm culture flask. The cells were fed every 3 days for 14 days and microglia were harvested by vigorously shaking the flask at 120 rpm on a rotary shaker for 30 min. The detached microglia were resuspended in DMEM/F12 serum containing media, plated in a 6-well culture dish. Typically, purified microglia are used (*i.e.*, treated, harvested or fixed) within 48 h of plating. At one particular occasion, however, the cells were maintained for an additional 2 weeks. At this time, we unexpectedly observed extensive proliferation of the plated microglia. The cells were gently detached from the dish with phosphate buffered saline (PBS) containing 1 mM EDTA, 1 mM EGTA and 1 mg/mL glucose, and replated to test whether they had the ability to further propagate. The cells were passaged a total of 7 times over the course of an additional 4 weeks, each time aggressively proliferating to confluency. The cells were determined to have become spontaneously immortalized, and clonal populations of these cells were established. The cell line has been disclosed to the University of North Dakota Intellectual Property Commercialization and Economic Development Office, which can be contacted for material transfer agreements.

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