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Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia *in vitro*

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ABSTRACT

Microglia mediate multiple facets of neuroinflammation, including cytotoxicity, repair, regeneration, and immunosuppression due to their ability to acquire diverse activation states, or phenotypes. Modulation of microglial phenotype is an appealing neurotherapeutic strategy but a comprehensive study of classical and more novel microglial phenotypic markers *in vitro* is lacking. The aim of this study was to outline the temporal expression of a battery of phenotype markers from polarised microglia to generate an *in vitro* tool for screening the immunomodulatory potential of novel compounds. We characterised expression of thirty-one macrophage/microglial phenotype markers in primary microglia over time (4, 12, 36, and 72 h), using RT-qPCR or multiplex protein assay. Firstly, we selected Interleukin-4 (IL-4) and lipopolysaccharide (LPS) as the strongest M1–M2 polarising stimuli, from six stimuli tested. At each time point, markers useful to identify that microglia were M1 included iNOS, Cox-2 and IL-6 and a loss of M2a markers. Markers useful for quantifying M2b-immunomodulatory microglia included, increased IL-1RA and SOCS3 and for M2a-repair and regeneration, included increased arginase-1, and a loss of the M1 and M2b markers were discriminatory. Additional markers were regulated at fewer time points, but are still likely important to monitor when assessing the immunomodulatory potential of novel therapies. Further, to facilitate identification of how novel immunomodulatory treatments alter the functional affects of microglia, we characterised how the soluble products from polarised microglia affected the type and rate of neuronal death; M1/2b induced increasing and M2a-induced decreasing neuronal loss. We also assessed any effects of prior activation state, to provide a way to identify how a novel compound may alter phenotype depending on the stage of injury/insult progression. We identified generally that a prior M1/2b reduced the ability of microglia to switch to M2a. Altogether, we have characterised a profile of phenotype markers and a mechanism of assessing functional outcome that we can use as a reference guide for first-line screening of novel immunomodulatory therapies *in vitro* in the search for viable neuroprotectants.

1. Introduction

In response to insult or injury microglia and macrophages are capable of acquiring diverse and complex phenotypes, allowing them to participate in the cytotoxic response, immune regulation, and injury resolution. Nomenclature of these phenotypes varies across the literature (Kreider et al., 2007; Martinez et al., 2008) but can be characterised into four main states, classically activated M1 with cytotoxic properties; M2a with an alternate activation and involved in repair and regeneration; M2b with an immunoregulatory phenotype; or M2c with an acquired-deactivating phenotype.

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The concept of phenotype, including nomenclature, and its importance in our understanding of injury processes and drug design and has been extensively reviewed elsewhere (Mosser and Edwards, 2008; Martinez et al., 2009; Ransohoff and Perry, 2009; Perry et al., 2010; Weinstein et al., 2010).

Microglia are self-renewing and long-lived resident macrophage-like cells of the brain (Giulian and Baker, 1986; Glenn et al., 1992; Kettenmann et al., 2011). In addition to important roles in inflammation, microglia are also critical in developmental processes such as synaptogenesis, and are imperative for the maintenance of neural homeostasis (see, Tremblay et al., 2011; Ekdahl, 2012). Importantly, microglial phenotypes reflect expression of cell surface receptors and release of soluble factors with recognised functions. Over time following injury and over time *in vitro* in response to stimuli, expression of the markers used to characterise these states changes (Perego et al., 2011; Hu et al., 2012). Beneficial for our ability to study these populations, phenotypes have known prototypical inducers; Toll-like-receptor-4 agonist lipopolysaccharide (LPS), IFNγ and TNFα for M1; IL-4 and IL-13 for M2a; immune complexes and toll-like-receptor agonists, and IL-1R ligands for M2b; and IL-10, TGF-β and glucocorticoids for M2c. However, a great deal of the studies outlining these inducers were performed in peritoneal or blood derived macrophages, and microglia are known to differ in their responsiveness to stimuli (Schmid et al., 2009).

Inflammatory processes, including activation of microglia, are initiated in the adult and perinatal brain after most (if not all) types of insults and injury (see, Degos et al., 2010; Perry et al., 2010; Bilbo et al., 2012; Fleiss and Gressens, 2012; Hagberg et al., 2012). The importance of microglia in protecting the brain is illustrated by the observation that complete blockade of microglial activity exacerbates brain damage in adult and neonatal hypoxic ischemic injury models (Lalancette-Hebert et al., 2007; Faustino et al., 2011). Also, it is possible for neurotherapeutics to be effective in the neonatal brain without reducing microglial number, also suggesting that modulation rather than suppression may be important (Doverhag et al., 2010; Fleiss et al., 2012). In fact, cell therapy with microglia has been shown to reduce injury in a model of adult hypoxic/ischemic injury (Imai et al., 2007). All together these observations support the need for screening tools to identify neurotherapeutics that support a repair and regenerative microglial phenotype.

Cultured primary microglia are a useful first line research tool with which to screen the immunomodulatory potential of novel therapeutics. Primary microglial cultures derived from early postnatal rodents have cell surface receptor expression and functional characteristics similar to that seen in microglia *in vivo* (Giulian and Baker, 1986; Henn et al., 2009). However, despite the enormous wealth of information regarding macrophage/microglial phenotype, to the best of our knowledge there is no comprehensive time and stimulus dependent data on the phenotype of primary microglia. In addition, a great deal of our understanding of phenotype is derived from studies of macrophages. However, the responses of resident microglia differ from that of the circulating macrophages that infiltrate the brain during insult/injury (Lalancette-Hebert et al., 2007; Schmid et al., 2009), suggested to relate to differing origin of these cell types (Saijo and Glass, 2011). As such, it is critical to understand the specific functions and responses of microglia. Thus, the aim of this study was to characterise in primary microglia the expression of a battery of classic (IL-6, iNOS etc.) and more novel phenotype markers (SphK1/2, FIZZ1 etc.) in a time and stimulus dependent manner and to create a functional output with which to evaluate the neuronotoxic effects of phenotype. We propose that this data set represents an *in vitro* model, useful as a first-line screening tool, to assess the immunomodulatory potential of novel neurotherapeutics in microglia of an M1 or M2 phenotype.

2. Methods

2.1. Animals

Animals were handled according to institutional guidelines of Institut National de la Santé et de la Recherche Scientifique (Inserm) France, or the Gothenburg University Sweden Animal Ethics Committee and met the guidelines for the Care and use of laboratory animals (NIH, Bethesda, Maryland, USA). Experiments were performed using OF1 strain mice from Charles River (L'Arbresle, France).

2.2. Drugs

Lipopolysaccharide (LPS; Sigma, Lyon, France, L2880, lot 050M4014) was diluted in PBS to a stock concentration of 0.1 mg/mL. Cytokines were from R&D systems (Lille Cedex, France), and diluted in PBS and 0.1% bovine serum albumin to create stock solutions; 5 μg/mL for IL-1β, 1 μg/mL for tumour necrosis factor-α (TNFα) and 2 μg/mL for IL-4; IL-10; and Interferon-γ (IFNγ).

2.3. Primary microglial culture

Primary mixed glial cell cultures were prepared from the cortices of postnatal day (P) 0–1 OF1 mice, as previously described (Thery et al., 1991). Pups of both sexes were included and on average an equal number of males and females were included in each culture. After dissection of the cortices in 0.1 M PBS with 6% glucose and 2% penicillin–streptomycin (PS; Gibco, Cergy Pontoise, France) and removal of the meninges, the cortices were chopped into small pieces and subsequently mechanically dissociated. The suspension was diluted in pre-cooled low glucose Dulbecco's modified Eagle's minimum essential medium (DMEM, 31885, Gibco) supplemented with 10% foetal bovine serum (FBS, Gibco) and 0.01% PS. Microglia were isolated from primary mixed glial cultures on day *in vitro* 14 (DIV14) using a reciprocating shaker (20 min at room temperature) and repeated rinsing with their medium using a 10 mL pipette. Media was subsequently removed, microglia pelleted via centrifugation (300*g* × 10 min) and following resuspension maintained in DMEM supplemented with 10% FBS at a concentration of 4×10^5 cells/mL in 6-well culture plates. Culture purity was verified by immunostaining ($n = 5$) using cell-type specific antibodies against tomato lectin (microglia), glial fibrillary acidic protein (GFAP; astrocytes) and neuronal nuclear antigen (NeuN; neurons) and revealed a >99% purity of microglia.

Based on previous reports, the day after plating, microglia were treated with PBS (vehicle; 10 μl/ml of culture media), LPS at 1 μg/mL (Takeuchi et al., 2006; Kaindl et al., 2012), IL-1β at 50 ng/mL (Street et al., 2003; Hjorth et al., 2010), IL-4 at 20 ng/mL (Butovsky et al., 2006; Kigerl et al., 2009), IL-10 at 20 ng/mL (Strle et al., 2002), TNFα at 10 ng/mL (Bernardino et al., 2005), or IFNγ at 20 ng/mL (Butovsky et al., 2006; Kigerl et al., 2009). After various exposure times (4, 12, 36, and 72 h) supernatant (conditioned media) was collected and stored at −80 °C until analysis of cytokine/chemokine levels or for use in neuronal viability studies, and cells were harvested and RNA extracted for gene expression analysis. A schematic representation of the experimental timelines is shown in Fig. 1.

2.4. Primary neuronal cultures

Cultured neurons were derived from the cerebral cortex of embryonic (E) 14.5 mice as previously described (Fontaine et al., 2008). After dissection of the cortices and removal of the meninges the cortices were minced into small pieces, chemically dissociated