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Neurons and astroglia govern microglial endotoxin tolerance through macrophage colony-stimulating factor receptor-mediated ERK1/2 signals

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ABSTRACT

Endotoxin tolerance (ET) is a reduced responsiveness of innate immune cells like macrophages/monocytes to an endotoxin challenge following a previous encounter with the endotoxin. Although ET in peripheral systems has been well studied, little is known about ET in the brain. The present study showed that brain immune cells, microglia, being different from peripheral macrophages, displayed non-cell autonomous mechanisms in ET formation. Specifically, neurons and astroglia were indispensable for microglial ET. Macrophage colony-stimulating factor (M-CSF) secreted from these non-immune cells was essential for governing microglial ET. Neutralization of M-CSF deprived the neuron-glia conditioned medium of its ability to enable microglia to form ET when microglia encountered two lipopolysaccharide (LPS) treatments. Recombinant M-CSF protein rendered enriched microglia refractory to the second LPS challenge leading to microglial ET. Activation of microglial M-CSF receptor (M-CSFR; also known as CSF1R) and the downstream ERK1/2 signals was responsible for M-CSF-mediated microglial ET. Endotoxin-tolerant microglia in neuron-glia cultures displayed M2-like polarized phenotypes, as shown by upregulation of M2 marker Arg-1, elevated production of anti-inflammatory cytokine interleukin 10, and decreased secretion of pro-inflammatory mediators (tumor necrosis factor α, nitric oxide, prostaglandin E2 and interleukin 1β). Endotoxin-tolerant microglia protected neurons against LPS-elicited inflammatory insults, as shown by reduced neuronal damages in LPS pre-treatment group compared with the group without LPS pre-treatment. Moreover, while neurons and astroglia became injured during chronic neuroinflammation, microglia failed to form ET. Thus, this study identified a distinct non-cell autonomous mechanism of microglial ET. Interactions of M-CSF secreted by neurons and astroglia with microglial M-CSFR programed microglial ET. Loss of microglial ET could be an important pathogenetic mechanism of inflammation-associated neuronal damages.

1. Introduction

In response to a potent immune challenge, activated innate immune cells (e.g. monocytes and macrophages) can produce various inflammatory mediators such as tumor necrosis factor α

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(TNF-α), interleukin 1β (IL-1β), IL-6, eicosanoids, nitric oxide (NO), and reactive free radicals. When in excess, these inflammatory mediators can cause serious systemic disorders with a high mortality rate (Gordon and Taylor, 2005). Pre-exposure to endotoxin can induce transient unresponsive or reduced sensitivity to a subsequent endotoxin challenge, as shown by decreases in production of inflammatory mediators, febrile reaction, and lethality rate (Mendez et al., 1999). This phenomenon is termed endotoxin tolerance (ET). Although the incidence of ET in peripheral immune systems has been observed both *in vitro* and *in vivo* (Lopez-Collazo and del Fresno, 2013), whether the central immune system exhibits similar immune tolerance has not been well investigated. Furthermore, the regulatory mechanism of ET in peripheral immune cells such as macrophages has been well established at multiple levels from negative signal transduction, transcriptional network to post-translational modifications (Biswas and Lopez-Collazo, 2009; Hoppstadter et al., 2015; Lopez-Collazo and del Fresno, 2013). However, molecular mechanisms by which microglia program ET are still unclear. In addition, the physiological and pathological role of microglial ET in brain health and diseases warrants further investigation.

ET can be modeled by two consecutive lipopolysaccharide (LPS) challenges with an interval of hours to days. Historically, a large body of knowledge on the development of ET in peripheral innate immune cells, especially monocytes and macrophages, has been obtained through the study of responses of enriched cell culture systems or experimental animals to repeated LPS stimulation. Such experimental designs did not allow for a readily examination of the temporal and possibly causative relationship between non-immune cells and ET-development immune cells. As a result, the contribution of non-immune cells to ET formation still remains an open question.

Ample evidence has demonstrated important functions of neurons and astroglia in keeping microglia in a quiescent state and reducing their activation upon immune challenge. Indeed, the low turnover rate and limited replenishment mechanism of microglia (the sole type of immune cells in the brain) demand tight spatial and temporal regulation for maintaining immune homeostasis as well as structural and functional integrity of the central nervous system (CNS). Up to now, how neurons and astroglia affect the response of microglia to repeat endotoxin exposure has not been investigated.

In this study, using various re-constituted primary cultures, we investigated roles of brain non-immune cells in microglial ET formation and further studied mechanism of microglial ET. We show for the first time that neurons and astroglia regulate microglial ET development through the release of macrophage colony-stimulating factor (M-CSF) and consequent activation of its receptor (M-CSFR; also known as CSF1R) and downstream ERK1/2 signals. This study identified a novel molecular mechanism for ET development in central immune system.

2. Materials and methods

2.1. Animals

All the animals were treated humanely and with regard for alleviation of suffering following the *National Institutes of Health Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources 1996). All procedures were approved by the NIEHS Animal Care and Use Committee.

2.2. Recombinant proteins, protein kinase inhibitors, and other reagents

LPS (*E. coli* O111:B4, Cat# 437627, protein contaminants ⩽2.0%, nucleic acid contaminants ⩽2.5%), SP600125, and Bay 11-7821 were obtained from EMD Millipore Corporation (Darmstadt, Germany), Abcam Inc. (Cambridge, MA), and TOCRIS bioscience (Bristol, UK), respectively. U0126 and SB203580 were purchased from Cell Signaling Technology, Inc. (Danvers, MA). The following reagents were purchased from R&D Systems (Minneapolis, MN): anti-M-CSF antibody, recombinant mouse TNF-α, M-CSF, interleukin 34 (IL-34), and IL-1β. Cycloheximide, cytosine arabinoside (Ara-C), and L-leucine methyl ester (LME) were from Sigma-Aldrich (Saint Louis, MO).

2.3. Preparation of primary neuron-glia, mixed-glia, neuron-microglia, neuron-enriched, microglia-enriched, and astrocyte-enriched cultures

To investigate roles of different brain cells in the regulation of microglial ET, we prepared enriched cultures of single cell type and co-cultures/reconstituted cultures of multiple cell types. As seen below, although the percentage of microglia in different cultures seems quite different, the number of microglial cells in different cultures is similar. This makes TNF-α measurement and evaluation of microglial ET formation in the different experiment settings comparable. In addition, to reduce any possible influence of serum components on cell response to various treatments, we lowered serum concentration in the treatment media of various cultures to one fifth of their respective culture media, except where indicated otherwise.

Mesencephalic neuron–glia cultures were prepared from the mesencephalon of embryos at gestation day 14 ± 0.5 C57BL/6J mice as previously reported (Gao et al., 2002). Briefly, mesencephalic tissues were dissected and dissociated with a mild mechanical trituration. Cells were seeded to 24-well (5×10^5 cells/well) culture plates pre-coated with poly-D-lysine (20 μg/ml) and maintained in 0.5 ml/well of MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10% heat-inactivated horse serum (HS), 1 g/L glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air and were replenished with 0.5 ml/well fresh medium 3 days later. Seven-day after seeding, cultures were treated with vehicle or desired reagents in MEM treatment medium containing 2% FBS, 2% HS, 2 mM L-glutamine, and 1 mM sodium pyruvate. Immunocytochemical analysis indicated that, at the time of treatment, the neuron–glia cultures were made up of about 11% microglia (~5.5×10^4 microglia/well), 50% astrocytes, and 39% neurons that were immunoreactive (IR) to the antibody against ionized calcium binding adaptor molecule 1 (Iba1), glial fibrillary acidic protein (GFAP), and neuron-specific nuclear protein (NeuN), respectively.

Neuron-enriched cultures were prepared by using cytosine arabinoside (Ara-C; 5 μM) to suppress the proliferation of glial cells 2 days after seeding of disassociated mesencephalic cells as described above (Gao et al., 2011). At 4 days after the initial plating the cultures consisted of 1% GFAP-IR astrocytes, <0.1% OX-42-IR microglia, and ~99% NeuN-IR neurons.

Primary mixed-glia cultures were prepared from whole brains of postnatal day 1 pups from C57BL/6J mice (Chu et al., 2015). Disassociated brain cells were seeded onto 24-well (2.5×10^5 cells/well) and 6-well (1×10^6 cells/well) culture plates and maintained in 1 ml/well DMEM/F-12 supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. The medium was changed every 3 days. After reaching confluence at 11–12 days after plating, the cultures were treated with vehicle or desired reagents in DMEM/F-12 treatment medium containing 2% FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate and 0.1 mM nonessential amino acids. At the time of treatment, the cultures contained about 80% GFAP-IR astrocytes and 20% Iba1-IR microglia (~6×10^4 and ~2.5×10^5 microglia per well of 24-well plate and 6-well plate, respectively).

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