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Aging and peripheral lipopolysaccharide can modulate epigenetic regulators and decrease IL-1β promoter DNA methylation in microglia

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ABSTRACT

In aged mice, peripheral stimulation of the innate immune system with lipopolysaccharide (LPS) causes exaggerated neuroinflammation and prolonged sickness behavior due in part to microglial dysfunction. Epigenetic changes to DNA may play a role in microglial dysfunction; therefore, we sought to determine whether aged microglia displayed DNA hypomethylation of the interleukin-1 beta (IL-1β) promoter and altered expression of epigenetic regulators. We further examined whether the demethylating agent 5-azacytidine induced IL-1β expression in BV2 and primary microglia similar to microglia from aged mice. Novel findings indicated that aged mice had decreased methylation of the IL-1β gene promoter in primary microglia basally or following systemic LPS that is associated with increased IL-1β mRNA, intracellular IL-1β production, as well as prolonged sickness behavior. Last, 5-azacytidine increased IL-1β gene expression and decreased DNA methylation of BV2 and primary microglial cells similar to microglia from aged mice. Taken together, these data indicate that DNA methylation promotes heightened microglial activation in the aged brain.

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1. Introduction

In the aged brain, it is common for microglia to have an inflammatory gene expression profile and a deramified morphology comparable to the morphology of activated adult microglia (Damani et al., 2011; Sierra et al., 2007; Tremblay et al., 2012). What's more, microglia in the brain of aged animals are hypersensitive to signals emerging from the peripheral immune system during infection, resulting in an aberrant neuroinflammatory response that is more intense and longer lasting (Dilger and Johnson, 2008; Henry et al., 2009; Sierra et al., 2007). This exaggerated proinflammatory response can be neurotoxic, lead to behavioral pathology (e.g., anorexia, acute cognitive disorders, and delirium) and exacerbate neurodegenerative diseases (e.g., Alzheimer's disease), greatly enhancing morbidity and mortality in older adults (Franceschi and Campisi, 2014). Why microglia become proinflammatory during aging and why they are hypersensitive to signals from the peripheral immune system is not known.

One way that microglial dysfunction may occur is through epigenetic modifications. Epigenetics refers to changes in gene expression that are independent of changes in the DNA sequence; it is thought of as a mediator between gene–environment interactions (Bird, 2007; Jaenisch and Bird, 2003). Epigenetics impact gene regulation though mechanisms such as DNA methylation and posttranslational modifications to histone tails, with DNA methylation being the most well-studied mechanism. DNA methylation is catalyzed by DNA methyltransferases (DNMTs) that includes DNMT1, DNMT3a, and DNMT3b (Moore et al., 2013). DNMT1 is a maintenance methyltransferase that maintains DNA methylation patterns during the DNA replication process, whereas DNMT3a and DNMT3b are de novo methyltransferases, capable of catalyzing new methylation patterns of previously unmethylated sequences. Another protein associated with DNA methylation is MeCP2, which binds to methylated cytosines and recruits histone deacetylases (HDACs) and other corepressors. This promotes higher-affinity interaction between DNA and histone core, condensing the chromatin, and typically suppresses transcription factor binding and gene expression (Bird, 2002; Strahl and Allis, 2000). In addition,

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Gadd45b protein plays an important role in active DNA demethylation (Ma et al., 2009).

DNA methylation depends on a precise balance of methylation and demethylation reactions that are nicely balanced in mature cells, but with age, there is a strong shift to favor DNA hypomethylation (Pogribny and Vanyushin, 2010). Age-related DNA hypomethylation may lead to redistribution of heterochromatin, impair normal gene responsiveness to environmental signals, and increase genomic instability that could compromise proper cell function. Aging could therefore be thought of as a time-dependent, epigenetically mediated loss of phenotypic plasticity (Gravina and Vijg, 2010), suggesting that aberrant DNA methylation patterns may be candidate mechanisms for explaining how microglia from aged mice seem to be "stuck" in a proinflammatory phenotype.

A handful of studies have demonstrated gene expression regulation by DNA methylation in microglia. One using BV2 cells, a murine microglial cell line, reported that DNA methylation influences the expression of several genes associated with the pathology of Alzheimer's disease (Byun et al., 2012), and another revealed that promoting increased maternal care alters the methylation pattern of the *IL-10* gene, leading to increased IL-10 expression in the nucleus accumbens and a reduction in morphine-induced addiction behavior (Schwarz and Bilbo, 2013). Very recently, it has also been determined that SIRT1 deficiency in aging microglia is related to increased IL-1β transcription and decreased methylation of CpG sites within the IL-1β proximal promoter (Cho et al., 2015).

Although increasing evidence suggests microglial phenotype is regulated by epigenetic mechanisms, little is known about DNA methylation of proinflammatory cytokines of healthy microglia from aged animals and the contribution of enzymes that significantly impact the epigenetic landscape. Because it is thought that microglia are the major producers of IL-1β in the brain and one of the most consistently upregulated cytokines with age (Burton et al., 2016; Chen et al., 2008), the objectives of this study were to determine whether there are promoter IL-1β DNA methylation modifications as well as altered gene expression of epigenetic regulators present between brain immune cells of young and old mice exposed to an immune challenge. Using the demethylating drug 5-azacytidine (5-aza), we also tested whether and how IL-1β gene expression is regulated in BV2 and primary microglial cells. We hypothesized that aged mice and those stimulated with an immune challenge would have IL-1β promoter hypomethylation associated with increased IL-1β gene expression, decreased expression of epigenetic regulators that promote DNA methylation, and that BV2 and adult primary microglial cells would have IL-1β promoter hypomethylation similar to an "aging" phenotype with the treatment of 5-aza.

2. Methods

2.1. Animals

Adult (4- to 6-month old) *C57BL/6* mice were either purchased from Charles River or reared in-house, and aged (24- to 26-month old) *C57BL/6* mice were purchased from the National Institute on Aging. They were individually housed in a temperature-controlled environment with a reversed-phase light/dark cycle (lights on 9 PM). Mice that were purchased were allowed to acclimate to their environment for at least 4 weeks before experimentation. All studies were carried out in accordance with United States National Institutes of Health guidelines and were approved by the University of Illinois Institutional Animal Care and Use Committee.

2.2. Immune challenge

Escherichia coli lipopolysaccharide (LPS; serotype 0127:B8, Sigma, St. Louis, MO, USA) was dissolved in sterile saline before experimentation. Mice from both age groups were given LPS (0.33 mg/kg body weight) or saline intraperitoneal. This dose of LPS was selected based on previous studies demonstrating that 0.33 mg/kg LPS produced prolonged sickness behavior in aged compared with young mice (Godbout et al., 2005). Treatments were administered at 9 AM for all cohorts.

2.3. Behavioral testing

Sickness behavior was assessed by changes in body weight and locomotor activity. Body weight and locomotor behavior were measured at baseline and 4 or 24 hours after treatment. Mice were placed in clear plexiglass cages identical to their home cage but devoid of bedding or nesting material. Clear plexiglass lids were placed on top of test cages to prevent escape while facilitating video recording. Locomotor activity was evaluated by virtual division of the cage into 4 equal quadrants and then tallying the number of line crossings and rearings each mouse displayed during the 5-minute test period. Videos were scored by a trained experimenter blinded to treatment.

2.4. Microglia isolation

Animals were euthanized via CO_2 asphyxiation 4 or 24 hours after treatment, perfused with sterile ice-cold saline, and brain tissue was collected and used immediately for microglia isolation using a procedure adapted from Nikademova & Watters (Nikodemova and Watters, 2012). To ensure a sufficient number of cells were recovered, tissue samples from 2 mice were pooled within a given experimental group. Brains were enzymatically digested using the Neural Tissue Dissociation Kit (Miltenyi Biotec, Germany) for 35 minutes at 37 °C. Further processing was performed at 4 °C. Tissue debris was removed by passing the cell suspension through a 40-μm cell strainer. After myelin removal using 30% Percoll Plus (GE Healthcare, Princeton, NJ, USA), cells in phosphate-buffered saline supplemented with 0.5% bovine serum albumin (BSA) and 2-mM EDTA were incubated for 15 minutes with anti-fluorescein isothiocyanate (FITC) magnetic beads and anti-Cd11b-FITC antibody (BD Biosciences) for flow cytometry or anti-Cd11b magnetic beads (Miltenyi Biotec, Germany) for gene expression and DNA methylation arrays. $CD11b^+$ cells were extensively washed and separated in a magnetic field using MS columns (Miltenyi Biotec, Germany). Cell yields for isolated microglia were around 1×10^6 cells per 2 brains and were not different between treatments.

2.5. Extracellular and intracellular flow cytometric analysis

Flow cytometric analysis of CD11b+ cell surface and intracellular markers was performed based on BD Cytofix/Cytoperm Plus fixation/permeabilization protocol (BD Biosciences, San Jose, CA, USA), as described previously, with a few modifications (Henry et al., 2009). Isolated cells were incubated in Dulbecco's Modified Eagle Medium (DMEM) (Bio-Whittaker, Cambrex, MD, USA) with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 100 units/mL penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), and brefeldin A (BD Biosciences) at 37 °C in a humidified incubator under 5% CO_2 for 4 hours. After incubation cells were washed, resuspended in phosphate-buffered saline/0.5% BSA/0.01% sodium azide solution (flow buffer), and Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience, San Diego, CA, USA). Cells were then

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