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Age- and location-related changes in microglial function

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ABSTRACT

Inflammation in the central nervous system (CNS) is primarily regulated by microglia. No longer considered a homogenous population, microglia display a high degree of heterogeneity, immunological diversity and regional variability in function. Given their low rate of self-renewal, the microenvironment in which microglia reside may play an important role in microglial senescence. This study examines age-related changes in microglia in the brain and spinal cord. Using ex-vivo flow cytometry analyses, functional assays were performed to assess changes in microglial morphology, oxidative stress, cytokine production, and phagocytic activity with age in both the brain and spinal cord. The regional CNS environment had a significant effect on microglial activity with age. Blood-CNS barrier permeability was greater in the aging spinal cord compared with aging brain; this was associated with increased tissue cytokine levels. Aged microglia had deficits in phagocytosis at baseline and after stimulus-induced activation. The identification of age-specific, high scatter microglia together with the use of ex-vivo functional analyses provides the first functional characterization of senescent microglia. Age and regional-specificity of CNS disease should be taken into consideration when developing immune-modulatory treatments.

1. Introduction

Microglia, the resident macrophages of the central nervous system (CNS), actively survey the environment and rapidly respond to homeostatic disturbances. Although microglial responses have been widely studied in the context of injury, changes in microglial phenotype or activation status may also occur under normal conditions. These responses may also vary depending on cues from the local microenvironment (Zhang and Gensel, 2014). Microglia can have both proinflammatory (M1) and wound healing roles (M2) and their phenotype can vary in response to local cues (regional effects) and across the life span (aging effects) (Nayak et al., 2014; Ransohoff and Perry, 2009; Wong, 2013). The brain and spinal cord are distinct CNS environments that serve very specialized functions (Baskar Jesudasan et al., 2014). However, the degree to which heterogeneity and immunological diversity is present in the microglial population in each area remains an understudied area of glial biology (Hanisch, 2013).

Regional differences also occur in response to injury throughout the neuroaxis, with the spinal cord showing a more robust inflammatory response and a greater vulnerability to the detrimental effects of activated microglia (Batchelor et al., 2008; Schnell et al., 1999b). For instance, spinal cord lesions produce greater microglia activation and more extensive leukocyte recruitment and blood-CNS barrier breakdown than comparable lesions to the cortex (Bartanusz et al., 2011; Schnell et al., 1999a). Additionally, injury to white matter results in significantly more inflammation compared with an equivalent injury to gray matter, and caudal regions of the CNS respond with greater microglial activation than forebrain regions after focal cytokine injections (Phillips and Lampson, 1999; Phillips et al., 1999; Schnell et al., 1999b). These findings suggest that the brain and spinal cord, because of their unique structural and functional organization, harbor microglial populations with distinct activation thresholds that are primed to respond differently to stimuli. As many CNS diseases are age-related and manifest in specific CNS regions (e.g., amyloid deposition in Alzheimer's), furthering our knowledge of microglial diversity will increase our understanding of the role played by microglia in the pathogenesis of disease.

Given that microglia have a low rate of self-renewal, the environment in which they reside likely shapes their identity over time. In addition, aging itself has profound effects on cells of the myeloid

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lineage. Earlier studies have shown microglia senescence is manifested by morphological changes and alterations in immunophenotypic expression and inflammatory profile (Hart et al., 2012; Perry et al., 1993; Samorajski, 1976; Streit et al., 2004; Vaughan and Peters, 1974). A steady increase in the expression of markers that are upregulated on young, activated microglia after acute CNS injury is seen at baseline in aged brain. These observations lead to the hypothesis that age-induced microglial dystrophy results in chronic inflammation, a dysregulated or uncontrolled response to injury, and poorer recovery. To better understand the role played by microglia in age-related diseases, a clearer understanding of the effect of aging on baseline microglia function is required. Age-related transitions from ramified to amoeboid morphology have been described (von Bernhardi et al., 2010), but much less is known regarding the functional importance of this phenotypic shift.

The present study examined the effect of aging on mitochondrial activity, reactive oxygen species generation, and phagocytic potential of microglia within the 2 main CNS compartments, the brain and spinal cord. We hypothesized that microglial identity and function is specified by the environment, and that aging impairs niche function. Our findings indicate that microglia in the aging brain and spinal cord have distinct immunological profiles. A significant enhancement of inflammatory cytokines and markers of oxidative stress, coupled with a loss of phagocytic function was seen, which was more profound in the aged spinal cord. These changes may explain why microglial subsets can differentially contribute to the etiology and pathology of age-related neurodegenerative disease.

2. Materials and methods

2.1. Mice and/or animals

C57BL/6J male mice 8–12 weeks (young adult; 31.5 ± 0.8 g) and 20–22 months (aged; 37.8 ± 1.0 g) of age were pair-housed on sawdust bedding in a pathogen free facility (12-hour light-dark cycle) with free access to chow and water. Animal procedures were performed in accordance with National Institutes of Health guidelines for the Care and Use of Laboratory Animals and approved by the Animal Care Committee of the University of Connecticut Health Center. All studies were performed blinded to age.

2.2. Tissue harvesting

Mice were euthanized, transcardially perfused with 60 mL cold, sterile phosphate-buffered saline (PBS), and the brains and spinal cords were harvested. The brainstem, cerebellum, and optic nerve were removed. The brain was then divided along the interhemispheric fissure into 2 hemispheres. Spinal cords were removed by flushing the spinal column with sterile PBS by hydrostatic pressure and subsequent rinsing with PBS to remove contaminant cells. Samples were then processed in a blinded fashion.

2.3. Flow cytometry

Brains and spinal were placed in complete Roswell Park Memorial Institute medium 1640 (Lonza) medium and mechanically and enzymatically digested in Collagenase/Dispase (1 mg/mL) and DNase (10 mg/mL; both Roche Diagnostics) for 1 hour at 37 °C. The cell suspension was filtered through a 70 μm filter. Leukocytes were harvested from the interphase of a 70% to 30% Percoll gradient. Cells were washed and blocked with mouse Fc Block (eBioscience) before staining with primary antibody-conjugated flourophores: CD45-eF450, CD11b-APC-eF780, Ly6C-PerCP-Cy5.5, and SIRPα-PE. All primary-conjugated antibodies were purchased from eBioscience. For live or dead discrimination, a fixable viability dye, carboxylic acid succinimidyl ester (CASE-AF350, Invitrogen), was diluted at 1:300 from a working stock of 0.3 mg/mL. Cells were briefly fixed in 2% paraformaldehyde. Data were acquired on an LSR II using FACSDiva 6.0 (BD Biosciences) and analyzed using FlowJo (Tree Star Inc). No less than 100,000 events were recorded for each sample. Fluorescence minus one (FMO) controls were used to determine the positivity of each antibody. Microglia were identified as $CD45^{int}CD11\ b^{+}$. Subsequently, to ensure that the microglia population was pure, we excluded all $Ly6C^{+}$ bone marrow-derived myeloid populations with overlapping CD45 expression.

To study the phagocytic activity of microglia, fluorescent latex beads (Fluoresbrite Yellow Green (YG) Carboxylate Microspheres; 1 μm diameter; Polysciences) were added to freshly isolated microglia in a final dilution of 1:100 as described (Voss et al., 2012). After 1 hour at 37 °C in an air incubator, the cells were washed 3 times with flow cytometry (FACS) staining buffer, re-suspended in FACS buffer, stained for surface markers, and fixed in paraformaldehyde (N = 7 per group). For beta-amyloid peptide and cell stimulation experiments, cells were incubated for 1 hour in 37 °C water bath. Lyophilized fluorescein-labeled (synthetic human) beta-amyloid (1–42) was dissolved in dimethyl sulfoxide (DMSO) to a final stock concentration of 500 μg/mL (rPeptide, Bogart, GA, USA). This was further diluted 1:1 in PBS to obtain a working stock from which 1 μL was added to every 100 μL of sample and vortexed to mix. Cell Stimulation Cocktail containing phorbol 12-myristate 13-acetate (PMA) and ionomycin was purchased from eBioscience. Cells were treated with stimulation cocktail (500X) after the addition of phagocytic substrate and subsequently incubated (N = 6 per group).

To determine mitochondrial activity, MitoTracker Red CM-H2XRos (Life Technologies, Invitrogen; Ex/Em: 579/599), a reduced, nonfluorescent dye that fluoresces on oxidation was used to stain mitochondria in live cells. Its accumulation is dependent upon membrane potential. MitoTracker was diluted in DMSO to a working concentration of 500 nM, at a final volume of 300 μL. Cells were incubated for 20 minutes at 37 °C and subsequently washed 3 times with FACS buffer (without NaAz) to remove residual dye (N = 6 per group).

For detection of reactive oxygen species, microglial cells were incubated with redox-sensitive CM-H2DCFDA (5μM; Ex/Em: 495/520) fluorogenic cell-permeant dye (Life Technologies, Invitrogen). Cells were incubated for 20 minutes at 37 °C, washed 3 times with FACS buffer (without NaAz), and then stained for surface markers including carboxylic acid succinimidyl ester (N = 5–7 per group).

For intracellular cytokine staining, an in vivo brefeldin A (BFA) protocol was followed. Briefly, 10 mL/kg of BFA (Sigma, 0.5 mg/mL in DMSO) was injected via tail vein. Ten hours later, animals were sacrificed and tissue was harvested as noted previously. Before staining, 1 μL of GolgiPlug containing BFA (BD Biosciences) was added to 800 μL complete Roswell Park Memorial Institute medium and cells were incubated for 2 hours at 37 °C (5% CO_2). Afterward, cells were resuspended in Fc Block, stained for surface antigens, and washed in 100 μL of Fixation/Permeabilization Solution (BD Biosciences) for 20 minutes. Microglia were then washed twice in 300 μL permeabilization/wash buffer (BD Biosciences) and resuspended in an intracellular antibody cocktail containing TNF-PE-Cy7 (eBioscience), IL-1β-fluorescein isothiocyanate (eBioscience), and MMP-9-PE (StressMarq Biosciences, Inc) and subsequently fixed (N = 5 per group).

ImageStream data were acquired using the Amnis ImageStream Analyzer (Amnis Corporation, WA, USA) equipped with the Amnis INSPIRE software.