



Increased expression of M1 and M2 phenotypic markers in isolated microglia after four-day binge alcohol exposure in male rats



Hui Peng, Chelsea R. Geil Nickell, Kevin Y. Chen, Justin A. McClain¹, Kimberly Nixon^{*}

University of Kentucky, College of Pharmacy, Department of Pharmaceutical Sciences, Lexington, KY 40536, USA

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ABSTRACT

Microglia activation and neuroinflammation are common features of neurodegenerative conditions, including alcohol use disorders (AUDs). When activated, microglia span a continuum of diverse phenotypes ranging from classically activated, pro-inflammatory (M1) microglia/macrophages to alternatively activated, growth-promoting (M2) microglia/macrophages. Identifying microglia phenotypes is critical for understanding the role of microglia in the pathogenesis of AUDs. Therefore, male rats were gavaged with 25% (w/v) ethanol or isocaloric control diet every 8 h for 4 days and sacrificed at 0, 2, 4, and 7 days after alcohol exposure (e.g., T0, T2, etc.). Microglia were isolated from hippocampus and entorhinal cortices by Percoll density gradient centrifugation. Cells were labeled with microglia surface antigens and analyzed by flow cytometry. Consistent with prior studies, isolated cells yielded a highly enriched population of brain macrophages/microglia (>95% pure), evidenced by staining for the macrophage/microglia antigen CD11b. Polarization states of CD11b⁺CD45^{low} microglia were evaluated by expression of M1 surface markers, major histocompatibility complex (MHC) II, CD32, CD86, and M2 surface marker, CD206 (mannose receptor). Ethanol-treated animals begin to show increased expression of M1 and M2 markers at T0 ($p = \text{n.s.}$), with significant changes at the T2 time point. At T2, expression of M1 markers, MHC-II, CD86, and CD32 were increased ($p < 0.05$) in hippocampus and entorhinal cortices, while M2 marker, CD206, was increased significantly only in entorhinal cortices ($p < 0.05$). All effects resolved to control levels by T4. In summary, four-day binge alcohol exposure produces a transient increase in both M1 (MHC-II, CD32, and CD86) and M2 (CD206) populations of microglia isolated from the entorhinal cortex and hippocampus. Thus, these findings that both pro-inflammatory and potentially beneficial, recovery-promoting microglia phenotypes can be observed after a damaging exposure of alcohol are critically important to our understanding of the role of microglia in the pathogenesis of AUDs.

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Introduction

According to new Diagnostic and Statistical Manual V criteria, an even larger portion of the U.S. population, nearly 14%, meets the diagnostic criteria for an alcohol use disorder (AUD) in any given year (Grant et al., 2015). Excessive alcohol (ethanol) use, characteristic of an AUD, causes significant neuropathology throughout the brain, including the limbic system, cerebellum, and cerebral

cortex, according to both human studies and animal models (Crews & Nixon, 2009; de la Monte & Kril, 2014; Sullivan & Pfefferbaum, 2005). While human imaging studies have shown that both white and gray matter loss occurs in a variety of brain regions in alcoholics (Beresford et al., 2006; Mechtcheriakov et al., 2007; de la Monte & Kril, 2014; Pfefferbaum et al., 1992; Sullivan & Pfefferbaum, 2005), animal models of AUDs have been necessary to support the causal relationship between high blood alcohol concentrations characteristic of binge/bender pattern drinking and neurodegeneration (Collins, Corso, & Neafsey, 1996; Crews, Braun, Hoplight, Switzer, & Knapp, 2000; Crews & Nixon, 2009; Hunt, 1993; Kelso, Liput, Eaves, & Nixon, 2011). Binge drinking has been defined as imbibing 4 (women) or 5 (men) standard drinks within 2 h to produce blood ethanol concentrations (BECs) greater than 0.08 mg/dL (NIAAA, 2017), whereas a bender is repeated days of excessive intake including binge drinking. Over the last several

^{*} Corresponding author. University of Kentucky, Department of Pharmaceutical Sciences, 789 S. Limestone, TODD 473, Lexington, KY 40536, USA. Fax: +1 859 257 7585.

E-mail address: kim-nixon@uky.edu (K. Nixon).

¹ Current address: Division of Natural and Computational Sciences, School of Arts and Sciences, Gwynedd Mercy University, 1325 Sumneytown Pike, Gwynedd Valley, PA 19437, USA.

years, studies have revealed that excessive alcohol exposure results in immune activation in the central nervous system (CNS), a phenomenon that several groups suggest is driving the pathogenesis of AUDs (Chastain & Sarkar, 2014; Crews & Vetreno, 2014; Cui, Shurtleff, & Harris, 2014; Davis & Syapin, 2004; Marshall et al., 2013; Vallés, Blanco, Pascual, & Guerri, 2004).

Microglia are the myeloid-lineage, resident immune cells of the CNS that become activated in response to insult (Ransohoff & Perry, 2009). Similar to what has been described in macrophages, microglia are often characterized as two distinct activated phenotypes: the M1 pro-inflammatory/classically activated phenotype, and the M2 anti-inflammatory/alternative activated phenotype (Benarroch, 2013; Beynon & Walker, 2012; Carson et al., 2007; Colton & Wilcock, 2010; Graeber, 2010; Raivich et al., 1999). While the M2 phenotypes promote tissue repair and phagocytosis of protein aggregates and cell debris, the M1 phenotypes are more likely to be detrimental to the brain by inducing neuronal toxicity through secretion of pro-inflammatory cytokine and chemokine and production of reactive oxygen species (ROS). Although we and others have documented the effect of ethanol on various neuro-immune activation markers and microglia in animal models of alcohol use and abuse (Alfonso-Loeches, Pascual-Lucas, Blanco, Sanchez-Vera, & Guerri, 2010; Blednov et al., 2005; Crews, Zou, & Qin, 2011; Fernandez-Lizarbe, Pascual, & Guerri, 2009; Marshall et al., 2013; McClain et al., 2011; Nixon, Kim, Potts, He, & Crews, 2008; Qin et al., 2008; Suk, 2007; Vallés et al., 2004), specific phenotypic M1 versus M2 markers have not been examined in the context of determining microglia phenotype. Therefore, the specific role of microglia in alcoholic neuropathology remains unclear. Identifying microglia activation states (phenotypes) is critical for understanding the role of microglia in the pathogenesis of AUDs.

Microglia are routinely enumerated and classified by morphology and cell-surface markers using immunohistochemistry, and indirectly through assessment of cytokine expression (though multiple cell types could be the source, such as astroglia; Bedi, Smith, Hetz, Xue, & Cox, 2013; Beynon & Walker, 2012; Colton & Wilcock, 2010). For example, upregulation of ionized calcium binding adaptor molecule 1 (Iba-1) and monocyte chemoattractant protein 1 (MCP1) immunoreactivity (He & Crews, 2008) and observation of proliferating microglia (Dennis et al., 2013; Sutherland et al., 2013) in human alcoholic brain provide the most compelling evidence for at least some level of activation, but neither of these phenomena are associated specifically with an M1 state (Raivich et al., 1999). Our recent work using these two methodologies has shown that four-day binge alcohol exposure, a model of an AUD, shows that microglia are activated but potentially not to a classically activated or M1 phenotype (Marshall et al., 2013; McClain et al., 2011). However, the specific and well-accepted phenotypic markers have not been examined. Therefore, the current study was designed to use Percoll separation/enrichment followed by three-color flow cytometric analysis of fluorescently labeled surface markers on microglia to enumerate microglia phenotypes from different brain regions, 48 h after four-day binge alcohol exposure. The fresh isolation of enriched cell suspensions enabled us to accurately quantify microglia activation states in entire populations of cells from regions of interest (hippocampus and entorhinal cortex) without reliance on manual morphometric counting of serial immunohistochemistry slides.

Materials and methods

Rat model of an AUD

All procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee and adhered to the

Guide for the Care and Use of Laboratory Animals (NRC, 1996). Thirty-three adult, male Sprague Dawley rats (275–300 g, Charles River Laboratories, Raleigh, NC) were pair-housed in a University of Kentucky AALAC accredited vivarium with a 12-h light:dark cycle. Rats were allowed to acclimate to the vivarium for 2 days followed by 3 days of handling before any experimentation. Except during the binge periods when chow was removed, animals had *ad libitum* access to food and water. Following acclimation, rats were gavaged with ethanol (25% ethanol w/v in Vanilla Ensure Plus[®], Abbott Laboratories, Abbott Park, IL; $n = 17$) or isocaloric control diet (added dextrose; $n = 16$) every 8 h for 4 days following a procedure modified from Majchrowicz (1975), as reported previously (Morris, Kelso, Liput, Marshall, & Nixon, 2010). Following an initial 5-g/kg ethanol dose, subsequent doses were titrated according to a 6-point intoxication behavior scale. BECs were determined in serum from tail blood collected 90 min following the seventh dose by an AM1 Alcohol Analyser (Analox, London, UK). Starting 10 h after the last dose of ethanol, withdrawal was observed for 30 min every 4 h for 16 h. Behaviors were scored based on a scale modified from Majchrowicz (1975) but identical to that reported previously (Morris et al., 2010).

Isolation of microglia

Microglia were isolated from brain tissue by Percoll gradient centrifugation as described previously (Frank, Wieseler-Frank, Watkins, & Maier, 2006), with slight modification. Based on the time course of microglia activation in this model of an AUD, rats were humanely killed at 0, 2, 4, and 7 days following the last dose of ethanol (i.e., T0, T2, T4, and T7): rats were deeply anesthetized and transcardially perfused with 0.9% NaCl containing heparin. Brains were harvested and the hippocampus and entorhinal cortex were dissected on ice. Tissue was finely minced with a razor blade and gently homogenized in Dulbecco's phosphate-buffered saline (DPBS), pH 7.4, then passed through a 70- μ m nylon cell strainer (VWR, Batavia, IL). Resulting homogenates were centrifuged at $400 \times g$ for 6 min and cell pellets were resuspended in 2 mL 50% isotonic Percoll (GE Healthcare, Piscataway, NJ). Two milliliters of 50% isotonic Percoll was gently layered on top of 1 mL 70% layer and then 1 mL $1 \times$ PBS was layered on top of the 50% Percoll layer. The density gradient was centrifuged at $1200 \times g$ for 45 min (minimum acceleration and brake) at 20 °C. Microglia were collected from the interphase between the 70% and 50% isotonic Percoll phases (Frank et al., 2006). Cells were washed in $1 \times$ PBS and then resuspended in sterile DPBS.

Microglia staining and flow cytometry

Microglia were surface-stained with conjugated monoclonal antibodies to assess microglia purity (CD11b-FITC, BD Pharmingen, San Jose, CA; CD45-APC, eBioscience, San Diego, CA) and M1 activation markers (CD86-PE, Fc γ RIII[CD32]-PE, or MHC-II-PE, BD Pharmingen) as previous described (Bedi et al., 2013). For the M2 marker, microglia were stained with rabbit anti-rat CD206 (Abcam, Cambridge, MA), followed by a secondary incubation with donkey anti-rabbit-PE (BD Pharmingen). In brief, microglia were suspended in 50- μ L incubation buffer ($1 \times$ PBS + 0.1% bovine serum albumin) for 30 min on ice, and Fc receptors were blocked with anti-CD32 antibody (except for CD32 staining, eBioscience). Cells were incubated with antibodies for 30 min on ice in the dark. Cells were washed with $1 \times$ PBS and fixed with a formaldehyde-based fixation buffer (eBioscience #00-8222) on ice.

Data were acquired with an Attune Acoustic Focusing Cytometer (ABI, Carlsbad, CA) and analyzed with Attune Acoustic software (ABI). Before each run, the cytometer was calibrated with

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