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Differential migration, LPS-induced cytokine, chemokine and NO expression in immortalized BV-2 and HAPI cell lines and primary microglial cultures

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Abstract

Microglial cells are hematopoietically derived monocytes of the CNS and serve important neuromodulatory, neurotrophic and neuroimmune roles. Following insult to the CNS, microglia develop a reactive phenotype, migrate to the site of injury, proliferate, and release a range of proinflammatory, anti-inflammatory and neurotrophic factors. Isolation of primary microglial cell cultures has been an integral step in elucidating the many roles of these cells. In addition to primary microglial cells, several immortalized cell lines have been created to model primary microglia *in vitro*, including murine derived BV-2 cells and rat derived HAPI cells. Here we compare rat primary microglial, BV-2 and HAPI cells in experiments assessing migration, expression of activation markers and production and release of NO (nitric oxide), cytokines and chemokines. BV-2 and HAPI cells responded similarly to primary microglia in experiments assessing migration, Iba1 expression, and NO release. However, BV-2 and HAPI cells did not model primary microglia in experiments assessing TNF α , IL-1 β , IL-6 and MCP-1 expression and release and pERK 44/42 (extracellular receptor kinase) expression following LPS treatment. These results indicate that BV-2 and HAPI cell cultures only partially model primary microglia and that their use should therefore be carefully considered.

Keywords

BV-2; HAPI; Microglia; Migration; Neuroimmune activation

INTRODUCTION

Although glial cells constitute roughly 70% of the total cell population in the Central Nervous System (CNS), they were once thought to be merely a physical and nutritional support system for neurons. In recent years, however, glia have been increasingly recognized as important neuromodulatory, neurotrophic and neuroimmune elements in the CNS. Microglia function as the immune surveillance cells in the brain and spinal cord and develop a reactive phenotype following insult (Hanisch & Kettenmann 2007, Streit *et al.* 1999). This reactivity is marked by proliferation, migration to the site of injury, altered

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morphology and surface marker expression, and release of proinflammatory cytokines, chemokines and NO (DeLeo & Yeziarski 2001, Nakajima & Kohsaka 2001).

Glial cell dysfunction has been implicated in a host of CNS diseases and syndromes. Glial modulating drugs, including minocycline and propentofylline have been shown to be neuroprotective in several CNS disease models and as a result their efficacy in the treatment of human disease is being investigated (Blum *et al.* 2004, Ringheim 2000). Minocycline has been investigated for the treatment of Alzheimer's disease, Parkinson's disease, Huntington's disease and Amyotrophic lateral sclerosis (2008, Bonelli *et al.* 2004, Gordon *et al.* 2007, Seabrook *et al.* 2006). Propentofylline has been studied for the treatment of Alzheimer's disease, stroke and vascular dementia (Bath & Bath-Hextall 2004, Frampton *et al.* 2003), Kittner *et al.* 2000). Conversely, LPS, a bacterial-derived endotoxin, is a potent immune system stimulant and has been considered the gold standard for inducing microglial activation (Hanisch & Kettenmann 2007, Kloss *et al.* 2001). For these reasons we used LPS as a stimulant and minocycline and propentofylline as modulators of the microglial reactivity in the current study. In order to identify future glial modulators, appropriate cell-based assays need to be characterized, standardized and optimized for ideal translation to *in vivo* disease model systems.

Primary microglial cells harvested from early postnatal rat cortical and hippocampal tissue have been widely used for *in vitro* pharmacologic and physiologic studies (Giulian & Baker 1986). When stimulated, cultured primary cells have been shown to retain a majority of the known physiologic activities of reactive microglia *in vivo*, including phagocytosis, migration (Nutile-McMenemy *et al.* 2007) and release of proinflammatory cytokines and chemokines (Boje & Arora 1992). However, harvesting primary cells for these studies is costly, time-consuming and yields only small quantities of purified microglia. As an alternative, several immortalized cell lines were created, including murine derived BV-2 cells (Blasi *et al.* 1990) and rat derived HAPI cells [Highly Aggressive Proliferating Immortalized, (Cheepsunthorn *et al.* 2001)]. These immortalized cells replicate readily and are easy to maintain in culture, allowing for convenient and cost-effective experimentation. However, apart from the genomic alterations that have rendered them immortal, other differences have been anecdotally observed in comparison to primary microglial cell cultures. These differences include increased variance of morphologies, increased adhesion properties and enhanced proliferation rates.

Despite these differences, many investigators continue to use these immortalized cell lines in experimental culture models under the assumption that they accurately represent primary microglial function. BV-2 cells have been extensively used as an *in vitro* culture system in published studies, including experiments examining the regulation of purinergic receptors (Brautigam *et al.* 2005, Raouf *et al.* 2007), NO (He *et al.* 2002), and the role of ERK 44/42 signaling on NO and IL-1 β production (Watters *et al.* 2002) following LPS stimulation. BV-2 cells have also been used in electrophysiological studies investigating the role of chloride influx on lamellipodium formation in migration (Zierler *et al.* 2008) and phagocytosis (Furtner *et al.* 2007). Several new discoveries have been identified using BV-2 cells, including the identification of a novel endocannabinoid-hydrolyzing enzyme (Muccioli *et al.* 2007) and the isolation and identification of secreted apolipoproteins (Xu *et al.* 2000).

A few reports of differences between primary microglia and BV-2 cells have been published, including a study that found that CXCL4 (chemokine C-X-C motif ligand 4) attenuated NO synthesis in BV-2, cells but not in primary microglia (de Jong *et al.* 2008). It has also been reported that although BV-2 cells were a suitable model system for studying proteosomal function in microglia, they are less responsive to LPS and interferon gamma

stimulation in comparison to primary mouse microglia (Stohwasser *et al.* 2000). Another study found that BV-2 cells display an impaired ability to release IL-12 and KC (keratinocyte-derived chemokine) and altered release of several other cytokines (Hausler *et al.* 2002). HAPI cells have not been used in published studies as frequently as BV-2 or primary cells. However, they have been the *in vitro* cell culture system used to assess the anti-inflammatory effect of *Curcuma comosa* (Jantaratnotai *et al.* 2006) and IL-10 (Kremlev & Palmer 2005) on NO and cytokine production after LPS stimulation. One study directly compared the expression of chemokines in BV-2 and HAPI cells following LPS treatment (Kremlev *et al.* 2004). This study found that LPS enhanced the release of MIP-1 α (macrophage inflammatory protein 1), RANTES (regulated upon activation, normal T cell expresses and secreted) and IP-10 (inducible protein 10) in BV-2 cells, however LPS only induced release of MIP-1 α and RANTES in HAPI cells. However, no study has directly compared the BV-2 and HAPI immortalized cell lines to primary rat microglia.

In the present study, we sought to characterize BV-2 and HAPI immortalized cell lines and directly compare them to primary cultures in assays for an array of reactive indices. Our aim was to determine if either immortalized cell line is an adequate model of cultured primary microglia. To this effect, we examined the three cell cultures in parallel assays for migration, increase of activation markers, release of NO, and production and release of cytokines and chemokines. In migration experiments, we tested the effects of two glial modulating drugs, minocycline and propentofylline, on the migration of each cell culture toward the chemoattractant ADP. To assess cell reactivity, we examined Iba1 (ionized calcium binding adaptor molecule 1) and pERK regulation in response to the proinflammatory agent LPS. Finally, we examined the production and release of proinflammatory factors in each cell culture in response to LPS. NO release was measured using Griess assays. TNF α , IL-1 β , and IL-6 production and release was measured via western blot and ELISA analyses and MCP-1 release was measured via ELISA.

MATERIALS AND METHODS

Cell culture

The procedures used in these studies were approved by the Dartmouth College Institutional Animal Care and Use Committee. Highly purified primary microglial cultures were prepared using P2-P3 Harlan Sprague Dawley pups (Indianapolis, IN) that were killed by decapitation (Nutile-McMenemy *et al.* 2007). The cerebral cortices were removed; meninges were dissected away; cortical tissue was minced with a sterile scalpel blade and digested with Trypsin/ EDTA 1X (Mediatech, Herndon, VA) for 15 min at 37°C. The tissue was allowed to settle and the supernatant was discarded. Five ml Dulbecco's modified Eagle's medium (DMEM, Mediatech) supplemented with 10% charcoal-stripped fetal bovine serum (FBS, Hyclone, Logan, UT), 1.1% GlutaMax (Gibco-Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin (P/S, 100 U/ml penicillin, 100 μ g/ml streptomycin, Mediatech) containing 2000 units DNase (Sigma, St. Louis, MO) were added to the tissue on ice. The tissue was triturated with a five ml pipette several times, the tissue clumps were allowed to settle and the supernatant was removed to a sterile 50 ml conical tube on ice between triturations. Triturations were repeated until no tissue clumps were observed. The final volume was diluted to 25 ml with media and centrifuged at 310 \times g for 15 min. The supernatant was discarded and the cells resuspended in media. A small aliquot of cells was stained for trypan blue exclusion and cells were plated at 1 \times 10⁶ cells per 75 cm² flask. Cultures were maintained at 37°C and 5% CO₂. Media was changed every 3-4 days. After eight days *in vitro* (DIV 8) the flasks were confluent with astrocytes and microglia. Flasks were lightly shaken by hand for one min and the media containing microglia was removed and centrifuged at 310 \times g for 15 min. Cultures were found to be 99% microglia by staining with

OX-42 antibody (generous gift from Dr. William Hickey) a marker for the microglial CR3/CD11b receptor.

The immortalized mouse microglial cell line BV-2 was developed in the laboratory of Dr. Blasi at the University of Perugia [Perugia, Italy; (Blasi et al. 1990)] and was a generous gift of Dr. Weihua Zhao (Methodist Hospital, Houston, TX). The cells were cultured in DMEM (Mediatech) supplemented with 10% charcoal-stripped FBS (Hyclone), 1.1% GlutaMax (Gibco-Invitrogen) and 1% P/S (100 U/ml penicillin, 100 µg/ml streptomycin, Mediatech) at a density not exceeding 5×10^5 cells/ml and maintained in 5% CO₂ at 37°C. To harvest BV-2 cells, cells were trypsinized (Trypsin/ EDTA, Sigma), then centrifuged ($310 \times g$ for 15 min) and resuspended in serum free DMEM. Cell concentration was determined by counting cells with a hemocytometer and viability was assessed by trypan blue staining (0.4% trypan blue in PBS, Sigma).

The immortalized rat microglial cell line HAPI was a generous gift of Dr. James R. Connor [M.S. Hershey Medical Center, Hershey PA, (Cheepsunthorn *et al.* 2001)]. The cells were cultured in DMEM (low glucose, Invitrogen), 5% FBS (Hyclone), 4 Mmol/l glutamine (Invitrogen), 100,000 U/l Penicillin G, 100 mg/l streptomycin (Mediatech) and maintained in 5% CO₂ at 37°C. To harvest HAPI microglia, cells were trypsinized, then centrifuged ($310 \times g$ for 15 min) and resuspended in SFM. Cell concentrations were determined by counting cells in a hemocytometer and viability was assessed by trypan blue staining (0.4% trypan blue in PBS, Sigma).

Migration

To assess what factors influence BV-2, HAPI, and primary cell migration we first examined the time course needed optimum cell migration using Costar Transwell® plates (6.5 mm diameter insert, 8.0 µm pore size, polycarbonate membrane, Corning Inc., Corning, NY). For this group of experiments, the bottom chamber of these plates contained either: 0, 0.1, 1, or 10 µM ADP. Confluent BV-2 or HAPI or DIV 8 microglia were trypsinized or shaken, cells were washed with PBS, counted using trypan blue (Sigma), then placed in serum free medium (SFM). Cells were resuspended at 100×10^3 (primary microglia) or 50×10^3 (BV-2 and HAPI) cells in 100 µl SFM and allowed to migrate for 30, 60, or 120 min at 37°C at 5% CO₂. It was determined that 10 µM ADP and 120 min yielded the best migration, so in subsequent migration experiments these conditions were used. To assess the effects of propentofylline or minocycline treatment on primary microglia, BV-2 or HAPI migration, cells were pretreated with minocycline (0, 1, 10, or 100 µM), or propentofylline (0, 1, 10, or 100 µM) for 1 h then allowed to migrate towards 10 µM ADP for 2 h at 37°C and 5% CO₂. Cells were counted using trypan blue to insure survival post treatment (>95% viability), and then added to the top chamber, 100×10^3 cells (primary cells) or 50×10^3 cells (BV-2 and HAPI cells) in 100 µl of a transwell plate with FN coated membranes with 10 µM ADP in 500 µl SFM in the bottom chamber. Following migration, the medium in the top chamber was aspirated and the membrane gently wiped with a cotton swab to remove the cells that did not migrate. The membranes were first rinsed with PBS, the cells were then fixed with 2% formaldehyde in PBS, permeabilized with 0.01% Triton X-100 (Sigma) in PBS and finally stained with crystal violet (Sigma). Cells that migrated across the membrane were counted. Nine random fields at 40x were counted for each condition using phase contrast microscopy. Each experiment was repeated at least three times. Results are expressed as mean cell migration normalized to media control \pm SEM.

Western blot analysis

Confluent DIV 8 primary microglia, BV-2 or HAPI cells were shaken or trypsinized, counted and plated overnight in complete medium at a density of 400×10^3 cells/ml for

BV-2 or primary cells and 250×10^3 cells/ml for HAPI cells. Complete media was removed and replaced with SFM containing 5 or 50 ng/ml LPS (0111:B4 serotype, Sigma) for 24 h or 0, 1 or 5 μ g/ml LPS for 24 or 48 h. Following treatment, the culture plates were briefly centrifuged; supernatants were removed and saved for NO, chemokine and cytokine expression measurements; and 100 μ l of 1x Laemmli buffer (Bio-Rad, Hercules, CA) containing 2-ME (Sigma) was added to each well. Protein expression was assessed using western blot analysis. Briefly, approximately 40-50 μ g of protein and standard protein markers were subjected to SDS polyacrylamide gel electrophoresis (10% or 18% gels, Bio-Rad) and transferred to polyvinylidene difluoride (PVDF, Bio-Rad) membranes. Nonspecific binding was blocked by incubation with 5% BSA in TBS-Tween 20 (0.05%, Sigma) at room temperature, then 18% membranes were incubated overnight at 4°C with rabbit anti-Iba1 primary antibody (1:3000, Wako, Richmond, VA) and 10% membranes were incubated overnight at 4°C with rabbit anti-phospho-ERK 44/42 (Phospho-MAP Kinase 1:500, Cell Signaling, Danvers, MA). The next day, blots were incubated for 1 h at room temperature with goat anti-rabbit HRP-conjugated secondary antibody (1:3000, Pierce, Rockford, IL), visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) for five minutes and imaged using the Syngene G-box (Synoptics, Frederick, MD). Finally, blots were incubated for 30 minutes at 37°C in stripping buffer and 18% blots re-probed with a monoclonal mouse anti- β -actin antibody (1:3000, Abcam) or 10% blots re-probed with mouse anti-ERK 44/42 (Total MAP Kinase 1:1000, Cell Signaling). Ten percent blots were subsequently stripped and re-probed with mouse anti- β -actin antibody (1:3000, Abcam) and rabbit anti-TNF α (1:1000, Peprotech, Rocky Hill, NJ). Eighteen percent blots were subsequently stripped and re-probed with rabbit anti-IL-1 β and with anti-IL-6 (1:1000, Peprotech). Band intensity was assessed using the analysis software package provided with the Syngene G-Box and data was quantified as relative intensity of band of interest divided by intensity of β -actin. All treatments were completed at least three times and data was expressed as relative intensity normalized to media control \pm SEM.

Griess assay - Nitric oxide production

Supernatants collected from the protein experiments above were collected and assessed for nitric oxide (NO) production using the Griess Assay (Promega, Madison, WI) following the manufacturer's protocol. All treatments were completed at least three times and data are expressed as mean μ M \pm SEM.

ELISA assay

Supernatants collected from the protein experiments above were collected and assayed for chemokine and cytokine production. MCP-1 production was assessed using the BD OptEIA™ ELISA sets for mouse and rat MCP-1 (BD Biosciences, San Diego, CA) following the manufacturer's protocol. The cytokines IL-1 β , IL-6, and TNF α were assessed using R&D Systems DuoSet ELISA (R&D Systems, Minneapolis, MN) sets for mouse and rat IL-1 β , IL-6 and TNF α (R&D Systems) following the manufacturer's protocol. All treatments were completed at least three times and data are expressed as mean pg/ml \pm SEM.

Statistical analysis

All experiments were completed at least three times and data are expressed as mean \pm SEM. Statistical analyses were completed using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA). Unpaired t-tests were used to determine significance between groups except for western blot analyses where paired t-tests were used because of repeated values in media control. Significance was determined at a level of $p < 0.05$.

RESULTS

Migration

Microglial migration has been shown to play an integral role in the development of chronic pain in nerve ligation (Tsuda *et al.* 2003) and inflammatory pain models (Zhang *et al.* 2008). In previous studies, we have shown that primary microglia migrate towards various chemotactic agents including L-glutamate, MCP-1 and ADP. We have also shown that this migration can be inhibited by the glial modulating agent minocycline (Nutile-McMenemy *et al.* 2007). In this series of experiments, we assessed the effects of two glial modulating agents, minocycline and propentofylline on migration of primary microglia, BV-2 and HAPI cells. Cells were first incubated in media containing the glial modulating agents for one hour then allowed to migrate towards 10 μ M ADP for two hours.

In all three cell cultures, pretreatment with 1, 10, and 100 μ M minocycline inhibited microglial migration towards ADP in a dose dependent manner (Fig 1a, 1c, 1e). Pretreatment of cells with 100 μ M minocycline reduced migration from 100% in control to $40 \pm 2.12\%$ ($p < 0.001$) in primary microglia (Fig 1a), $21 \pm 1.56\%$ ($p < 0.001$) in BV-2 cells (Fig 1c) and $24 \pm 3.36\%$ ($p < 0.001$) in HAPI cells (Fig 1e).

Pretreatment with 1, 10, and 100 μ M propentofylline decreased migration in a dose dependent manner only in primary microglia (Fig 1b). Pretreatment of BV-2 cells with 1, 10, and 100 μ M propentofylline reduced migration, from $100 \pm 7.80\%$ in control to $44 \pm 3.79\%$ ($p < 0.001$), $67 \pm 2.78\%$ ($p < 0.01$) and $14 \pm 2.50\%$ ($p < 0.001$) respectively (Fig 1d), however not dose dependently. In HAPI cells, pretreatment with 1 and 10 μ M propentofylline decreased cell migration from $100 \pm 7.88\%$ to $49 \pm 4.11\%$ ($p < 0.001$) and $52 \pm 4.93\%$ ($p < 0.001$) respectively. However, treatment with 100 μ M propentofylline ($103 \pm 2.92\%$) failed to reduce microglial migration compared to control ($100 \pm 7.88\%$, $p = 0.78$, Fig 1f). Cell viability was found to be $>95\%$ for all treatments across the three cell cultures.

Iba1 Expression

Iba1 immunoreactivity is a common measure of microglial reactivity following insult to the CNS (Romero-Sandoval & Eisenach 2007). LPS, which binds to the TLR4/CD14 complex, is a proinflammatory agent used *in vivo* and *in vitro* as a model of CNS inflammation (Kloss *et al.* 2001). In these studies we treated the cell cultures with 0, 1 or 5 μ g/ml LPS for 24 or 48 h, then probed for Iba1 immunoreactivity via western blot analysis.

In primary microglial cells, the only significant increase in Iba1 expression compared to media control was a one fold increase following 48 h treatment with 5 μ g/ml LPS (2.08 ± 0.12 , $p < 0.05$, Fig 2a). Treatment of BV-2 cells with 1 μ g/ml LPS for 24 h increased Iba1 expression more than three fold (3.85 ± 0.45 , $p < 0.05$) compared to media control (1.00 ± 0.00 , Fig 2b). Finally, treatment of HAPI cells with 5 μ g/ml LPS for 48 h produced a slight decrease in Iba1 expression from 1.00 ± 0.00 to 0.69 ± 0.07 ($p < 0.05$) compared to media control (Fig 2c).

Nitric Oxide Release

NO is released from microglia following insult to the CNS or exposure to LPS (Boje & Arora 1992). Nitrite (NO_2^-) is the terminal product of NO oxidation and can be measured in cell culture media via Griess assay to assess NO release from cells. In this series of experiments, the three cell cultures were treated with 0, 1 or 5 μ g/ml LPS for 24 or 48 h and then the media was removed and assessed for NO secretion. Primary microglial cells increased NO release at both 24 and 48 h of LPS treatment. Peak release of $14.29 \pm 0.34 \mu\text{M}$ ($p < 0.001$) NO was observed following 48 h treatment with 5 μ g/ml LPS (Fig 3a). BV-2 cells

showed small but significant increases in NO secretion in response to LPS treatment (Fig 3b). Similar to primary microglia, HAPI cells robustly increased NO release into the culture media following 24 and 48 h LPS treatment. Peak release of $28.69 \pm 0.75 \mu\text{M}$ ($p < 0.001$) NO was observed following 48 h treatment with 1 $\mu\text{g/ml}$ LPS (Fig 3c).

TNF α

TNF α is a prototypical proinflammatory cytokine and is released in high concentration in culture media following LPS administration (Zuckerman *et al.* 1989). In this series of experiments, we assessed the intracellular production of TNF α (via western blot) and extracellular release (via ELISA) in the three cell cultures following 24 h exposure to 0, 5ng/ml, 50 ng/ml, 1 $\mu\text{g/ml}$ or 5 $\mu\text{g/ml}$ LPS or 48 h exposure to 0, 1 or 5 $\mu\text{g/ml}$ LPS.

Following LPS treatment, primary microglia increased expression of TNF α to a small but significant extent and very robustly increased TNF α release into the media supernatant. TNF α expression peaked at 24 h with an increase from 1.00 ± 0.00 in control to 2.94 ± 0.48 ($p < 0.01$) in the 1 $\mu\text{g/ml}$ LPS group (Fig 4a). TNF α released from primary microglia into the media supernatant was undetectable in media control, however after 24 h treatment 5 $\mu\text{g/ml}$ LPS, TNF α release increased to a maximum value of $3074.20 \pm 193.34 \text{ pg/ml}$ ($p < 0.001$, Fig 4b).

BV-2 cells responded similarly to primary microglia, with small changes in TNF α expression and more robust changes in TNF α release. Twenty-four hour treatment with 5 $\mu\text{g/ml}$ LPS increased expression of TNF α from 1.00 ± 0.00 to 1.46 ± 0.05 ($p < 0.05$). However, 48 h treatment with 1 and 5 $\mu\text{g/ml}$ LPS decreased TNF α expression from 1.00 ± 0.00 to 0.51 ± 0.07 ($p < 0.05$) and 0.62 ± 0.05 ($p < 0.05$) respectively (Fig 4c). Despite the small change in intracellular expression, TNF α release was increased following ng/ml and $\mu\text{g/ml}$ LPS treatment from $195.51 \pm 66.13 \text{ pg/ml}$ in media control to a maximum of $1974.85 \pm 176.13 \text{ pg/ml}$ ($p < 0.001$) following 48 h treatment with 5 $\mu\text{g/ml}$ LPS (Fig 4d).

Unlike primary microglia and BV-2 cells, HAPI cells expressed high amounts of the active form of TNF α protein intracellularly following $\mu\text{g/ml}$ LPS treatment. Peak TNF α expression was observed after 24 h treatment of HAPI cells with 1 $\mu\text{g/ml}$ LPS, increasing expression from 1.00 ± 0.00 in media control to 12.33 ± 1.43 ($p < 0.01$, Fig 4e). Released TNF α in the media supernatant of HAPI cells was undetectable following any LPS treatment (Fig 4f).

IL-1 β

We next assessed the production and release of IL-1 β following treatment with ng/ml and $\mu\text{g/ml}$ LPS for 24 or 48 h. We probed for both the pro-form (31kD) and the cleaved-form (17kD) of IL-1 β via western blot and released IL-1 β protein via ELISA. All three microglial cultures released IL-1 β , however, the amounts expressed intracellularly and released into the culture media varied widely.

Primary microglia increased expression of the pro-form of IL-1 β (31kD) following $\mu\text{g/ml}$ LPS treatment, however, no cleaved-form (17kD) was observed. Peak IL-1 β (31kD) expression of 22.51 ± 1.44 ($p < 0.001$) was observed after treatment with 1 $\mu\text{g/ml}$ LPS for 48 h (Fig 5a). There was no detectable immunoreactivity for the cleaved-form of IL-1 β (17kD) in primary microglial cells (Fig 5b). Primary microglia treated with 5 $\mu\text{g/ml}$ LPS for 24h robustly increased IL-1 β protein release from undetectable levels to a maximum of $2608.67 \pm 187.74 \text{ pg/ml}$ ($p < 0.001$, Fig 5c).

BV-2 cells expressed both forms of IL-1 β (31kD and 17kD) and released IL-1 β protein into the media supernatant in the unstimulated state. Treatment of BV-2 cells with LPS failed to change the expression of the pro-form of IL-1 β (31kD, Fig 5d). However, 24 h treatment

with 1 $\mu\text{g/ml}$ LPS significantly increased the cleaved-form of IL-1 β (17kD) from 1.00 ± 0.00 to 15.11 ± 2.28 ($p < 0.01$, Fig 5e). None of the LPS treatments altered BV-2 release of IL-1 β compared media control (Fig 5f).

Similar to BV-2 cells, HAPI cells expressed both pro- and cleaved-forms of IL-1 β (31kD and 17kD) and released low levels of IL-1 β protein. Treatment of HAPI cells with LPS did not significantly alter the expression of the pro-form of IL-1 β (31kD, Fig 5g), however, $\mu\text{g/ml}$ LPS treatment robustly increased the expression of the cleaved-form of IL-1 β (17kD) to a maximum of 44.36 ± 5.26 pg/ml ($p < 0.001$) following 48 h treatment with 5 $\mu\text{g/ml}$ LPS (Fig 5h). Treatment with $\mu\text{g/ml}$ LPS significantly increased IL-1 β release, however, not as robustly as observed in primary cells (Fig 5i).

IL-6

We next assessed the intracellular expression and extracellular release of IL-6 in response to ng/ml and $\mu\text{g/ml}$ LPS treatment in the three cell cultures. In primary cells, maximum IL-6 expression of 77.59 ± 6.79 ($p < 0.001$) was observed after treatment with 1 $\mu\text{g/ml}$ LPS for 48 h (Fig 6a). LPS treatment also enhanced IL-6 release from primary microglial cells from undetectable levels in control to a maximum of 1473.13 ± 27.39 pg/ml ($p < 0.001$) after treatment with 5 $\mu\text{g/ml}$ LPS for 24 h (Fig 6b).

BV-2 cells did not robustly alter IL-6 expression following LPS treatment (Fig 6c). However, these cells did increase release of IL-6 protein with maximum response of 1099.58 ± 51.53 pg/ml ($p < 0.001$) following treatment with 1 $\mu\text{g/ml}$ LPS for 48 h (Fig 6d). HAPI cells subjected to the same LPS treatments for 24 and 48 h did not alter expression of IL-6 (Fig 6e). Additionally, released IL-6 was undetectable in HAPI cell media following all LPS treatments (Fig 6f).

MCP-1

MCP-1 protein release from all three cell cultures was assessed following ng/ml and $\mu\text{g/ml}$ LPS treatment. In primary cells, all LPS treatments increased MCP-1 release with maximum of 3435.27 ± 61.73 pg/ml ($p < 0.001$) observed following 24 h treatment with 5 $\mu\text{g/ml}$ LPS (Fig 7a). BV-2 cells also increased MCP-1 release, however, not as robustly as observed in primary microglia. In BV-2 cells, peak MCP-1 release of 874.95 ± 47.95 pg/ml ($p < 0.01$) was observed following 48 h treatment with 1 $\mu\text{g/ml}$ LPS (Fig 7b). HAPI cells released lower concentrations of MCP-1 than primary and BV-2 cells following $\mu\text{g/ml}$ LPS treatment. Maximal MCP-1 release was only 18.68 ± 1.43 pg/ml ($p < 0.01$) following treatment for 24 h with 5 $\mu\text{g/ml}$ LPS (Fig 7c).

pERK

After assessing the effect of LPS treatment on cell migration and protein production and release, we next assessed activation of the extracellular signal-regulated kinase (ERK) pathway. Phosphorylation of ERK is a marker of cellular activation and is involved in many cellular processes including migration and cytokine production (Watters et al. 2002). In primary microglial cells, 24 h treatment with LPS did not alter ERK 44 or 42 phosphorylation (pERK). However, treatment for 48 h with 5 $\mu\text{g/ml}$ LPS increased pERK 44 and 42 expression to 40.40 ± 7.88 ($p < 0.001$, Fig 8a) and 39.53 ± 0.69 ($p < 0.001$, Fig 8b) respectively.

In BV-2 cells, there was no discernable increase in pERK 42 or 44 expression at either time point following LPS treatment (Fig 8c, 8d). In HAPI cells, only 48 h treatment with 1 $\mu\text{g/ml}$ LPS significantly increased pERK 44 expression from 1.00 ± 0.00 to 8.48 ± 2.71 ($p < 0.05$,

Fig 8e). Expression of pERK 42 was unchanged for all treatment groups in HAPI cells (Fig 8f).

DISCUSSION

The major finding of our study is that neither BV-2 nor HAPI immortalized cell lines consistently modeled the responses of primary microglial cells in the assays presented here. While all three cell cultures responded similarly in experiments assessing migration, Iba1 expression and NO secretion, BV-2 and HAPI cells responded differently than primary microglia in measures of TNF α , IL-1 β , IL-6 and MCP-1 release and pERK regulation. In experiments with BV-2 and HAPI cells, migration was inhibited by minocycline and propentofylline, but with slightly different dose-dependencies as compared to primary cells. In analyses of Iba1 expression, there was no consistent pattern of Iba1 expression following LPS treatment in the three cell cultures. Although primary microglial expression of Iba1 was increased by nearly two fold at 48 h and HAPI cell expression was increased by about four fold at 24 h, these results were only for single time-points and single doses. This result is not surprising given that, although LPS has been considered the gold standard for microglial activation, microglial activation is no longer considered an 'all-or-none event' (Hanisch & Kettenmann 2007). Whereas LPS is a potent inducer of NO and proinflammatory cytokine production, our results reveal that it does not substantially increase microglial Iba1 expression in our culture system. LPS treatment significantly increased NO release from all three cell cultures; however, the concentrations of NO differed widely among them. Primary microglia increased NO release by greater than 40 fold following LPS treatments, whereas HAPI cells increase NO release only by 15-20 fold and BV-2 cells by barely 2 fold.

Primary microglial cells responded to LPS stimulation with increased expression or release of TNF α , IL-1 β , IL-6, MCP-1 and pERK. However, BV-2 and HAPI cells did not respond similarly across the wide dosage range of ng/ml to μ g/ml LPS used in these experiments. These data suggest that BV-2 and HAPI cells are inadequate culture systems for assessing microglial cell responses to LPS stimulation.

The BV-2 cell line was derived from murine primary microglial cultures transformed with a J2 retrovirus carrying a *v-raftl v-myc* oncogene (Blasi *et al.* 1990). These immortalized cells exhibit the morphological, phenotypical and functional properties of activated microglial cells. Morphologically, they have short, thick processes similar to activated primary microglial cells. They express microglial markers including MAC 1 and MAC 2 antigens and do not express MAC 3, glial fibrillary acidic protein (GFAP, astrocyte marker) or galactocerebroside (GC, oligodendrocyte marker). Functionally, BV-2 cells are capable of phagocytic activity and respond to LPS by increasing lysozyme activity.

HAPI cells were derived from spontaneously immortalized primary rat microglial cell cultures (Cheepsunthorn *et al.* 2001). These cells were found to express isolectin B4, OX-42 (CR3/ CD11b), and Glut 5 (microglial markers), but did not label for GFAP (astrocyte marker) or A2B5 (oligodendrocyte lineage marker). Morphologically, HAPI cells were shown to have several forms, including cells with thick, short processes and cells with no processes. The exact mutation that has immortalized these cells is unknown. Therefore there may be changes in intracellular pathways compared to primary microglia which could account for the differences observed in our experiments.

Cheepsunthorn *et al.* (2001) reported that HAPI cells produce and release NO and TNF α following stimulation with 1 μ g/ml LPS. Our results presented herein support their finding in regards to NO release, however, contradict their data for TNF α expression and release. Cheepsunthorn *et al.* (2001) report that LPS stimulation increased TNF α transcript levels in

a time-dependent manner up to 24 h. However, they reported no TNF α prior to LPS stimulation and a peak secretion of TNF α at 6 h. By 24 h, they reported that the secretion of TNF α fell by nearly 40%. These findings indicate a large discordance between transcript expression and TNF α release in HAPI cells. In our experiments, HAPI cells increased intracellular TNF α expression at 24 and 48 h following LPS treatments, but there was no detectable release in the media at either time point. Possible reasons for this discrepancy include: genetic drift of the HAPI cells, the confluency of the cultures use, or that Cheepsunthorn et al. (2001) did not completely serum starve the cells during LPS treatment. Future studies may reveal that HAPI cells require higher levels of the LPS binding protein present in serum to effectively respond to LPS stimulation. However, it is also possible that had we measured LPS-induced TNF α release at earlier time-points we may have detected some TNF α in the media. A more recent study showed that mRNA for TNF α was enhanced in HAPI cells following LPS treatment, however, TNF α release was only reported in primary microglia (Kremlev & Palmer 2005). Regardless, the results taken together indicate that HAPI cells have significantly altered temporal expression profile of TNF α compared to primary microglia.

One of the strengths of our study was that the experiments were specifically designed to examine the potential differences between primary microglia, BV-2 and HAPI cells, unlike many of the studies which use these cell lines as surrogates for primary cells and only report limited deviations (de Jong et al. 2008, (Hausler et al. 2002, Stohwasser et al. 2000). We were also able to probe each experimental treatment both for multiple intracellularly expressed proteins, via several stripping/ reprobing of western blots, and for numerous extracellularly released proteins, via multiple ELISAs of the same stock supernatant. This approach allows for direct comparison of expression and release without the normal variability of many samples, thereby increasing our confidence in the results presented here. The results of our study reveal some interesting trends. Only primary microglia were found to release the three pro-inflammatory cytokines TNF α , IL-1 β and IL-6 in response to LPS stimulation. BV-2 cells increased release of TNF α and IL-6, following LPS treatment, whereas there was no observed release in HAPI cells. HAPI cells did, however, release IL-1 β following LPS treatment. TNF α and IL-6 are vesicularly released cytokines, indicating the possibility that HAPI cells have some form of vesicular dysfunction. Additionally, both BV-2 and HAPI cells also displayed very little pERK activation after LPS stimulation. LPS binds to toll-like receptor 4 and increases pro-inflammatory cytokine production via ERK phosphorylation. The reduced pERK levels in BV-2 and HAPI cells might, therefore, account for the reduced release of cytokines, chemokines and NO observed.

Although both BV-2 and HAPI cells have been used as models of primary microglia, our results are the first to compare the reactive and inflammatory profiles of these cell cultures across a broad range of assays including cell migration and cytokine/chemokine production and release. Despite some similarities in individual experiments, a comparison of the entire profiles of the three cell cultures forces us to reject the assumption that the immortalized BV-2 and HAPI cell lines model primary microglial cells. It is important that any cell line must first be shown to respond similarly to the primary cells they are to model before they are used in experiments. Based on our findings, we suggest that primary microglia should be used in lieu of these immortalized cell lines in experiments designed to model microglial morphology, phenotype and function *in vivo* and *in vitro*.

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Abbreviations

BSA	Bovine Serum Albumin
FBS	Fetal Bovine Serum
FN	Fibronectin
NGS	Normal Goat Serum
PBS	Phosphate Buffered Saline
LPS	Lipopolysaccharide
Iba1	Ionized calcium Binding Adaptor molecule 1
NO	Nitric Oxide
TNFα	Tumor Necrosis Factor Alpha
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
MCP-1	Monocyte Chemotactic Protein 1
ERK	Extracellular Receptor Kinase

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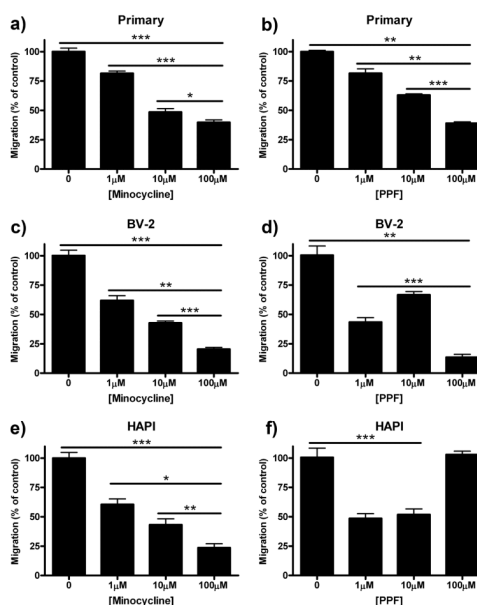


Figure 1.

Minocycline and propentofylline inhibited migration in all three cell types. Primary microglial, BV-2 or HAPI cells were pretreated for 1 h with 0, 1, 10 or 100 μM minocycline or 0, 1, 10, or 100 μM propentofylline, then allowed to migrate towards 10 μM ADP for 2 h. (a) Minocycline (1, 10, 100 μM) dose dependently decreased migration of primary microglia towards ADP. (b) Primary microglial migration towards ADP was dose dependently inhibited by propentofylline (1, 10, 100 μM). (c) BV-2 cell migration towards ADP was dose dependently inhibited by minocycline (1, 10, 100 μM). (d) Propentofylline (1, 10, 100 μM) reduced BV-2 migration towards ADP, however, not in a dose dependent manner. (e) HAPI cell migration was dose dependently inhibited by minocycline (1, 10, 100 μM). (f) HAPI cell migration was reduced by 1 and 10 μM propentofylline, however, 100 μM had no effect. Results are shown as mean cell counts per 40x field as percentage of control \pm SEM for all treatment groups. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

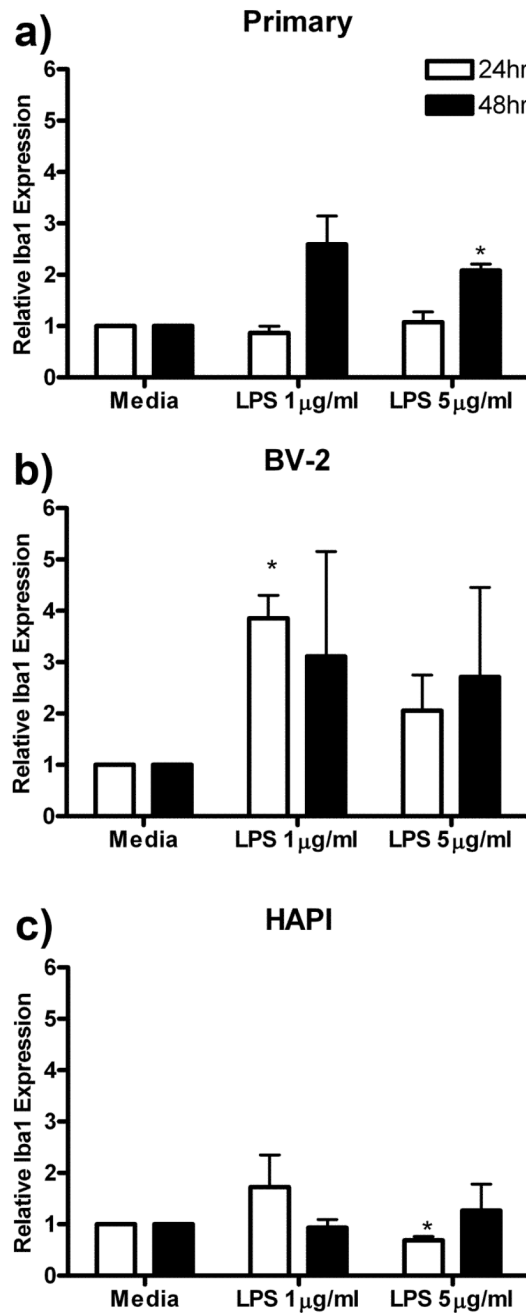


Figure 2.

LPS enhanced Iba1 expression in primary microglia and BV-2 cells. Primary microglia, BV-2 or HAPI cells were treated with 0, 1 or 5 μg/ml LPS for 24 or 48 h. (a) Five μg/ml LPS enhanced primary microglial expression of Iba1 after 48 h treatment. (b) One μg/ml LPS enhanced BV-2 expression of Iba1 after 24h treatment. (c) Five μg/ml LPS decreased Iba1 expression in HAPI cells after 24 h treatment. Results are shown as mean Iba1 band immunoreactivity relative to β-actin and normalized to media controls ± SEM. * p<0.05 compared to media control.

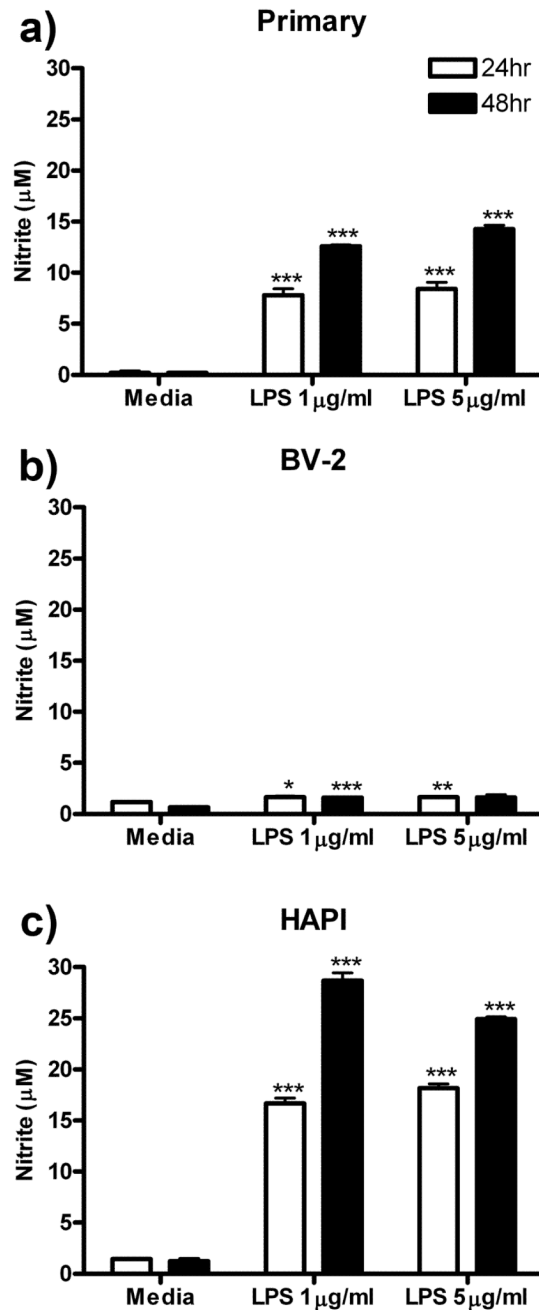


Figure 3.

LPS treatment induced NO release from all three cell cultures. Primary microglia, BV-2 or HAPI cells were treated for 24 or 48 h with 0, 1 or 5 $\mu\text{g/ml}$ LPS. (a) Primary microglia robustly increased NO release following treatment with LPS. (b) BV-2 cells slightly but significantly increased NO release following LPS treatment. (c) LPS treatment induced robust increases in NO release in HAPI cells. Results are expressed as mean nitrite (μM) \pm SEM. * p<0.05, ** p<0.01 and *** p<0.001 compared to media control.

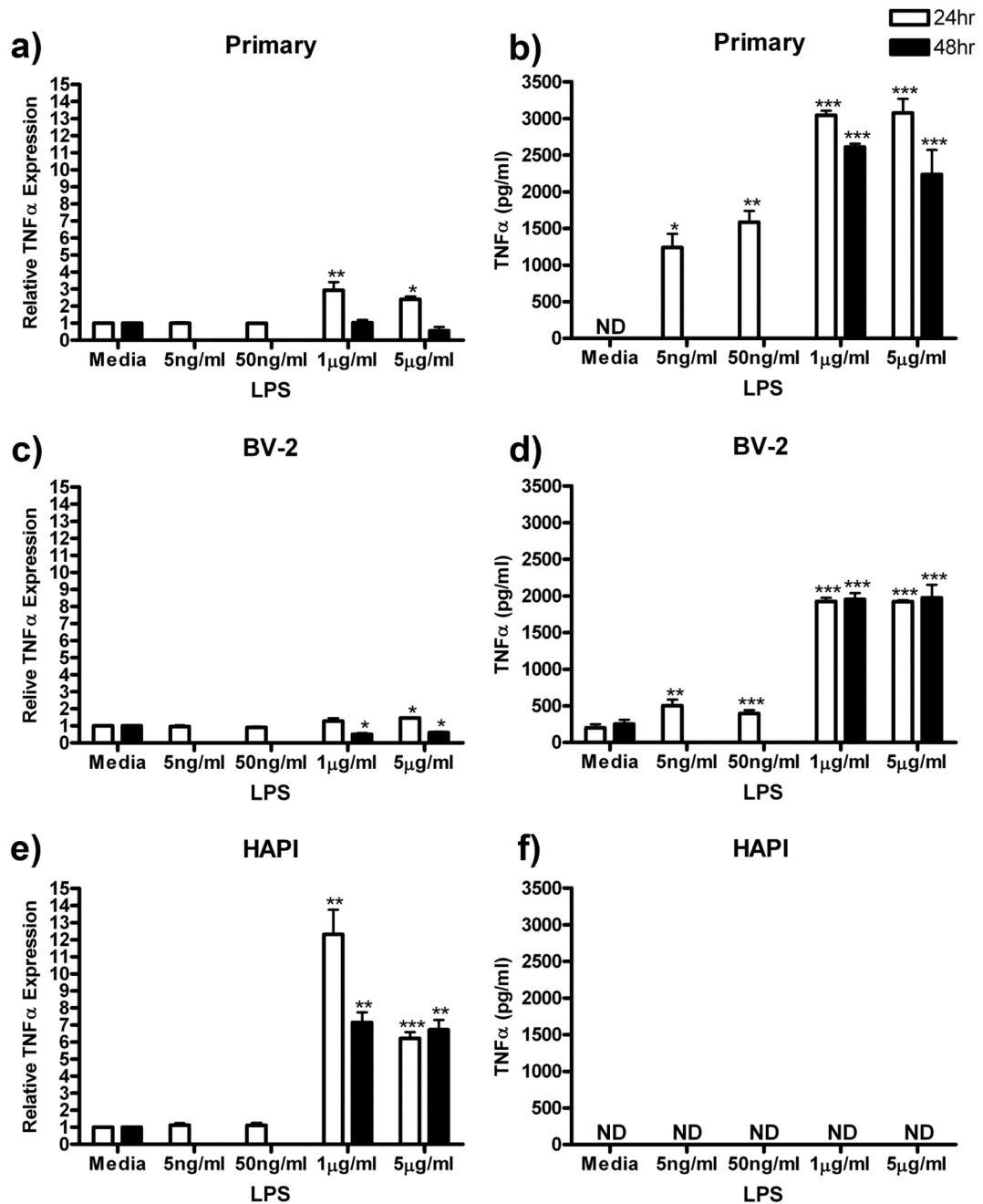


Figure 4.

TNF α protein expression and release was increased in microglial cells following LPS treatment. Primary microglia, BV-2 or HAPI cells were treated with 0, 5 ng/ml, 50 ng/ml, 1 μ g/ml or 5 μ g/ml LPS for 24 h or 0, 1 or 5 μ g/ml LPS for 48 h. (a) TNF α protein expression was enhanced in primary microglial cells following μ g/ml LPS treatment. (b) Primary microglia robustly increased TNF α release following ng/ml and μ g/ml LPS treatment. (c) TNF α expression was increased in BV-2 cells following μ g/ml LPS for 24 h treatment and decreased after 48 h treatment. (d) LPS increased TNF α release in BV-2 cells after ng/ml and μ g/ml treatment. (e) HAPI cells expression of TNF α protein was significantly increased after μ g/ml LPS treatment, however, no released TNF α was observed these cells (f). Results

from western blot experiments (a, c, e) are expressed as mean density of TNF α immunoreactivity relative to β -actin and normalized to media control \pm SEM. Results from ELISA experiments (b, d, f) are shown as mean TNF α (pg/ml) \pm SEM. ND, non-detectable. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to media control.

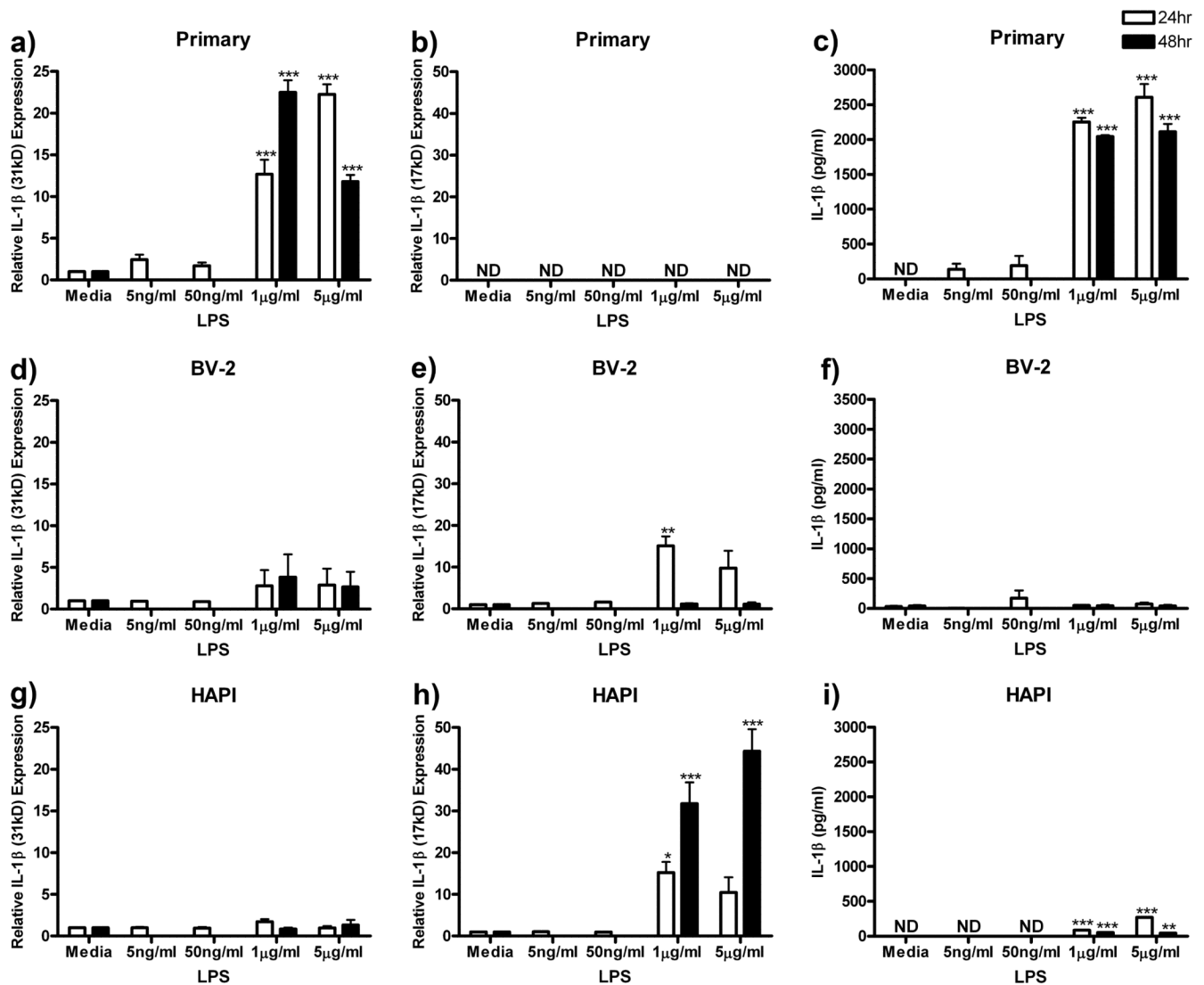


Figure 5.

LPS enhanced IL-1 β protein expression and release in microglial cells. Primary microglia, BV-2 or HAPI cells were treated with 0, 5 ng/ml, 50 ng/ml, 1 μ g/ml or 5 μ g/ml LPS for 24 h or 0, 1 or 5 μ g/ml LPS for 48 h. (a) Twenty-four and 48 h μ g/ml LPS treatment enhanced pro-IL-1 β (31kD) expression in primary cells. (b) No cleaved IL-1 β (17kD) was observed in primary cells. (c) Primary microglia robustly released IL-1 β following μ g/ml LPS treatment. (d) LPS treatment did not alter IL-1 β (31kD) expression in BV-2 cells. (e) BV-2 cells increased expression of IL-1 β (17kD) after 24 h treatment with 1 μ g/ml LPS. (f) LPS treatment did not alter IL-1 β release from BV-2 cells. (g) HAPI cells did not alter expression of IL-1 β (31kD) following LPS treatment. (h) HAPI cells increased expression of IL-1 β (17kD) after 24 and 48 h μ g/ml LPS treatment. (i) LPS treatment enhanced IL-1 β release from HAPI cells following μ g/ml LPS treatments. Results for western blot analysis (a, b, d, e, g, h) are shown as mean IL-1 β immunoreactivity relative to β -actin and normalized to media control \pm SEM. Results for ELISA experiments (c, f, i) are shown as IL-1 β (pg/ml) \pm SEM. ND, non-detectable. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to media control.

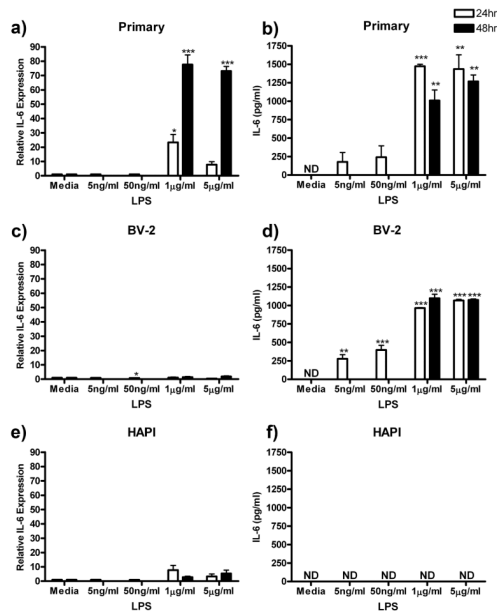


Figure 6.

LPS enhanced IL-6 expression and release in primary microglia and BV-2 cells. Primary microglia, BV-2 or HAPI cells were treated with 0, 5 ng/ml, 50 ng/ml, 1 µg/ml or 5 µg/ml LPS for 24 h or 0, 1 or 5 µg/ml LPS for 48 h. (a) LPS enhanced IL-6 expression in primary microglia after µg/ml treatment. (b) Primary microglial release of IL-6 was robustly increased by 24 and 48 h µg/ml LPS treatment. (c) No substantial changes in IL-6 protein were observed in BV-2 cells following LPS treatment. (d) BV-2 cells increased release of IL-6 following all LPS treatments. (e) No change in IL-6 protein was observed in HAPI cells following LPS treatment. (f) No IL-6 was observed in HAPI cell media following any treatment. Results for western blot (a, c, e) are expressed as mean IL-6 immunoreactivity relative to β-actin and normalized to media control ± SEM. Results for ELISA experiments (b, d, f) are expressed as mean IL-6 (pg/ml) ± SEM. ND, non-detectable. * p<0.05, ** p<0.01 and *** p<0.001 compared to media control.

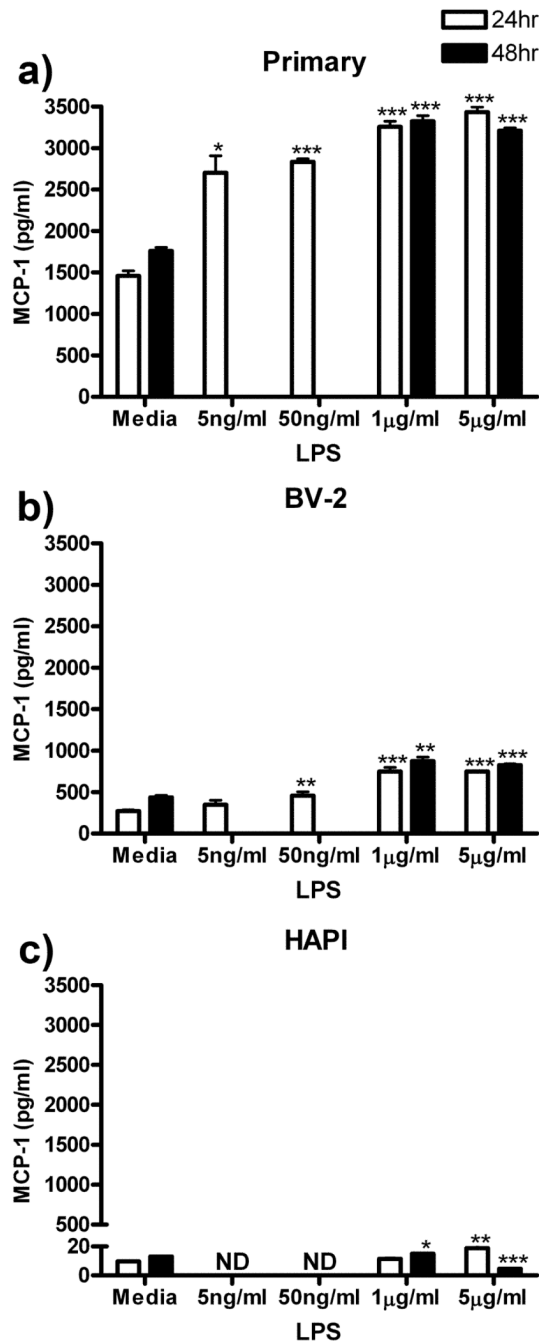


Figure 7.

LPS induced MCP-1 release from primary, BV-2 and HAPI, but by very different amounts. Primary microglia, BV-2 and HAPI cells were treated for 24 h with 0, 5 ng/ml, 50 ng/ml, 1 µg/ml or 5 µg/ml LPS or for 48 h with 0, 1 or 5 µg/ml LPS. (a) Primary microglia enhanced release of MCP-1 following all LPS treatments. (b) LPS treatment enhanced MCP-1 release in BV-2 cells. (c) In HAPI cells, LPS treatment increased or decreased MCP-1 release to a small but significant extent. Results are expressed as mean MCP-1 (pg/ml) \pm SEM. ND, non-detectable. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to media control.

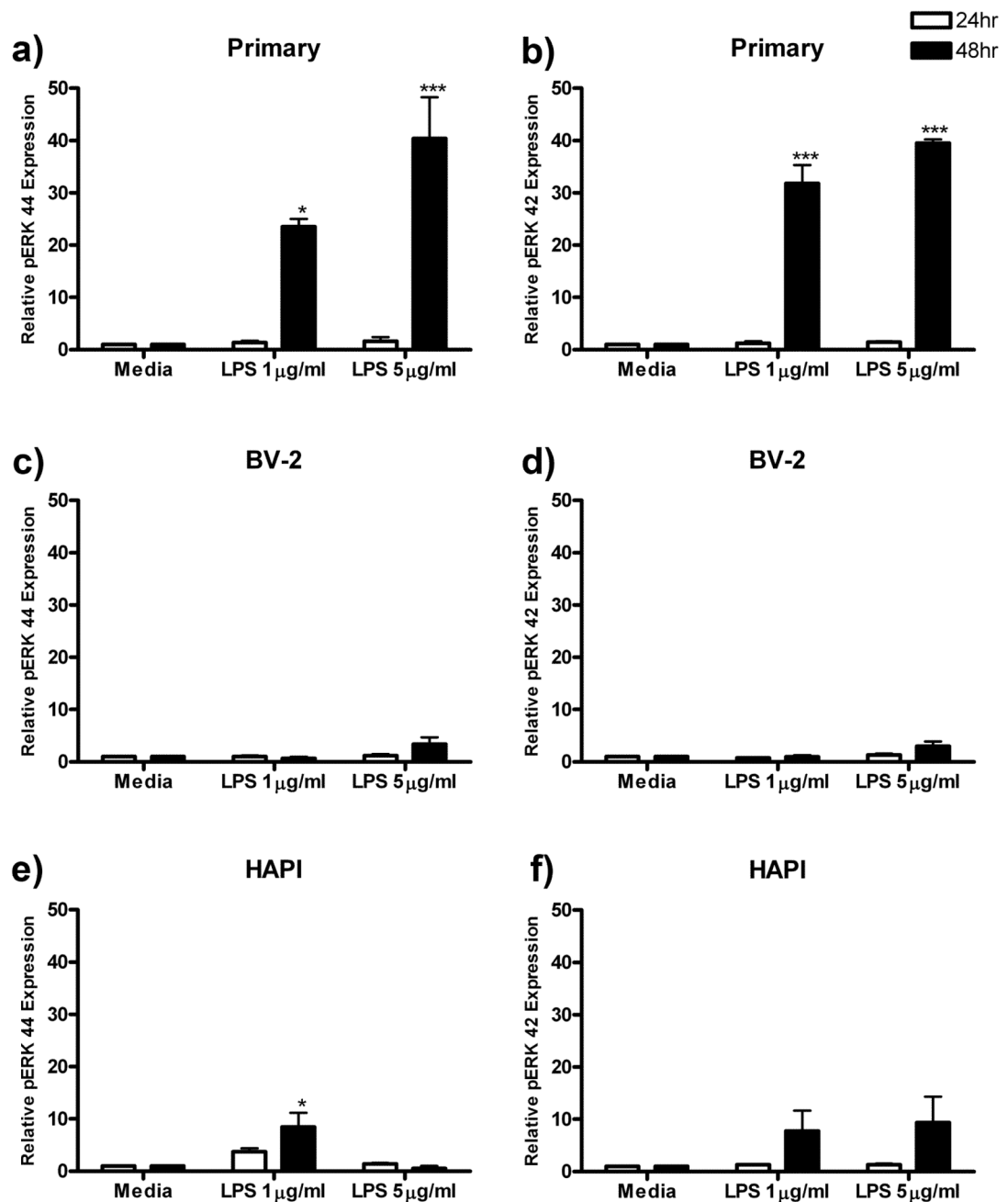


Figure 8.

pERK 42 and pERK 44 expression is enhanced in primary microglia following LPS treatment. Primary microglia, BV-2 and HAPI cells were treated for 24 or 48 h with 0, 1 or 5 μg/ml LPS. (a) Treatment for 48 h with LPS enhanced pERK 44 expression in primary microglial cells. (b) pERK 42 expression was also enhanced in primary microglia following 48 h LPS treatment. (c) In BV-2 cells, LPS treatment did not alter pERK 44 (c) or pERK 42 (d) expression in BV-2 cells. In HAPI cells, 48 h treatment with 1 μg/ml LPS enhanced pERK 44 expression (e), however, there was no alteration in pERK 42 expression (f) for any treatment. Results are expressed as mean pERK immunoreactivity relative to total ERK and

β -actin, then normalized to media controls \pm SEM. * $p < 0.05$ and *** $p < 0.001$ compared to media control.