Monocyte Chemotactic Protein-1 Regulates Voltage-Gated K^+ Channels and Macrophage Transmigration

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Abstract Progressive human immunodeficiency virus (HIV)-1 infection and virus-induced neuroinflammatory responses effectuate monocyte-macrophage transmigration across the blood–brain barrier (BBB). A key factor in mediating these events is monocyte chemotactic protein-1 (MCP-1). Upregulated glial-derived MCP-1 in HIV-1-infected brain tissues generates a gradient for monocyte recruitment into the nervous system. We posit that the interrelationships between MCP-1, voltage-gated ion channels, cell shape and volume, and cell mobility underlie monocyte transmigration across the BBB. In this regard, MCP-1 serves both as a chemoattractant and an inducer of monocyte-macrophage ion flux affecting cell shape and mobility. To address this hypothesis, MCP-1-treated bone marrow-derived macrophages (BMM) were analyzed for gene and protein expression, electrophysiology, and capacity to migrate across a laboratory constructed BBB. MCP-1 enhanced K^+ channel gene (KCNA3) and channel protein expression. Electrophysiological studies revealed that

MCP-1 increased outward K^+ currents in a dose-dependent manner. In vitro studies demonstrated that MCP-1 increased BMM migration across an artificial BBB, and the MCP-1-induced BMM migration was blocked by tetraethylammonium, a voltage-gated K^+ channel blocker. Together these data demonstrated that MCP-1 affects macrophage migratory movement through regulation of voltage-gated K^+ channels and, as such, provides a novel therapeutic strategy for neuroAIDS.

Keywords monocyte · monocyte chemotactic protein-1 · K^+ channels · blood–brain barrier

Introduction

Chemokines are structurally defined small proteins that serve as leukocyte chemoattractants for a variety of inflammatory diseases including those of the central nervous system (CNS; Rebenko-Moll et al. 2006; Callewaere et al. 2007; Mines et al. 2007). They affect a variety of divergent biological activities that include cell migration to sites of disease and inflammation, leukocyte activation, leukocyte secretions, and microbial activities (infection and cellular clearance; Callahan and Ransohoff 2004; Banisadr et al. 2005; Rebenko-Moll et al. 2006; Ubogu et al. 2006; Li and Ransohoff 2008). One of the most investigated and disease-associated chemokine is monocyte chemoattractant protein-1 (MCP-1), which plays a pivotal role in the recruitment of mononuclear phagocytes (MP; blood-borne monocytes, dendritic cells, and macrophages) to sites of inflammation and malignancies (de la Rosa et al. 2003; Garlet et al. 2003; Yao et al. 2006; Buhling et al. 2007; Tsai et al. 2008). The mechanism of such recruitment is well studied. Indeed, migration of monocytes across the vascular endothelium is linked to cells tethering to the

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endothelium, rolling along the vascular surface, firmly adhering to the endothelium, and finally diapedesis between tight morphologically structured endothelial cells, processes that are explicatively linked to both chemokine and adhesion molecule expression (Strell et al. 2007; Vestweber 2007; Crane 2008). After penetration of the endothelial basement membrane, monocytes migrate through the extracellular matrix of the tissues where they differentiate into tissue macrophages and migrate to sites of inflammation (Whatling et al. 2004; Fulcher et al. 2006; Reinhart et al. 2006; Cesar et al. 2008). Monocyte migration is mediated through selectins and their ligands or integrins interacting with endothelial vascular cell adhesion molecule 1 (VCAM-1). VCAM-1 is expressed at low levels on the endothelium and upregulated after immune activation (Lane et al. 1996; Nottet et al. 1996). On the apical endothelial surface, the chemokines MCP-1 and macrophage inflammatory proteins- α/β , (MIP-1 α/β) activate $\beta(2)$ integrins to facilitate firm adhesion (Biernacki et al. 2004; Maslin et al. 2005). Diapedesis by monocytes occurs through interaction between integrins on both the monocyte and the endothelial cells, followed by homophilic adhesion (Muller and Randolph 1999; Schenkel et al. 2002; Mamdouh et al. 2008). The intracellular mechanisms for these processes are linked to activation by tyrosine phosphorylation of cellular proteins and phosphorylation of the non-receptor tyrosine kinases Lyn, JAK2, the cytoskeletal binding protein paxillin, and downstream transcription factors STAT3 and STAT5 (Muller et al. 2005). In this manner, MCP-1 induces TNF- α and IL-1 production and promotes macrophage activation (Biswas and Sodhi 2002).

Monocyte trafficking is of special interest in studies of the neuropathogenesis of HIV infection, including establishment of viral reservoirs and the development of HIV-associated neurocognitive disorders (HAND; Venneti et al. 2004; Williams et al. 2005; Berman et al. 2006). However, the process of monocyte migration across the vascular endothelium and changes in migration that occur during HIV infection are incompletely understood. We theorized that since MCP-1 can elicit activation responses for macrophages, the actual influence of the chemokine on monocyte-macrophage physiology would be profound. In this manner, a linkage between MCP-1 and potassium (K⁺) channel expression would be apparent. Blockers of these channels altered the organization and structure of the cytoskeletal proteins actin, tubulin, and vimentin; adherence-mediated macrophage activation; and maintenance of cytoskeletal integrity and cell shape. Such cytoskeletal changes were associated with cell morphology changes. We now demonstrate that MCP-1 activation of monocytes leads to morphological alterations that are directly linked to K⁺ channels. Chemokine-mediated responses lead to a dosage escalation of channel responses and to alterations in size, shape, volume, and ingress across an artificial BBB. The

observations, taken together, provide unique insights into how MCP-1 can affect movement of monocytes across barriers and most notably into the CNS.

Materials and methods

BMM cultivation Bone marrow cells were obtained from femurs of 6-week-old C57/BL6 or BALB/c male mice (purchased from Jackson Laboratory, Bar Harbor, MN). Cells were cultured in Teflon flasks at 2×10^6 cells/ml in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin/streptomycin in the presence of mouse macrophage colony stimulating factor (M-CSF) 2 $\mu\text{g/ml}$ (a gift from Wyeth, Cambridge, MA). After 5–6 days in culture, BMM were determined to be >95% CD11b⁺ by flow cytometry and utilized thereafter.

Whole-cell patch clamp recording BMM were plated onto sterile glass coverslips at a density of 0.5×10^6 cells/slip. Whole-cell patch-clamp recordings were made using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) interfaced with a Digidata 1322A digitizer (Molecular Devices) and controlled with pClamp Version 8.1 software (Molecular Devices). Whole-cell currents were filtered at 1 kHz, digitized at 10 kHz, and stored on a computer hard disk. Patch pipettes were fabricated from borosilicate glass capillaries using a Sutter P97 microelectrode puller, with tip resistances of 4–8 M Ω when filled with pipette solution containing (in mM) 130 K⁺ gluconate, 10 EGTA, 1 CaCl₂, 1 MgCl₂, and 10 HEPES (pH 7.2, 280 mOsm). The bathing solution contained (in mM) 135 Na⁺ + gluconate, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose (pH 7.4, 290 mOsm). Values of access resistance ranging from 8 to 18 M Ω were compensated at 60–70% in most cells. Whole-cell voltage-dependent outward K⁺ currents were recorded in response to a series of voltage steps from –160 to +100 mV in 20 mV increments, starting from a holding potential of –60 mV. The steady-state current magnitudes were measured and normalized as a percentage of the magnitudes generated by a voltage step from –60 mV to +100 mV in control (perfused with normal bath solution). For statistical analyses, paired Student's *t* tests were used with statistical significance at $p < 0.05$.

Isolation and culture of primary human monocytes Human monocytes were obtained from HIV-1, HIV-2, and hepatitis B seronegative adults by leukaphoresis and purified by countercurrent centrifugal elutriation. Monocytes were cultured in DMEM (Sigma, St. Louis) supplemented with 10% heat-inactivated human serum, 2 mM L-glutamine, gentamicin (50 $\mu\text{g/ml}$), and 2 $\mu\text{g/ml}$ M-CSF. After 7 days

in culture, monocytes differentiated into macrophages and the monocyte-derived macrophages (MDM) were infected with HIV-1_{ADA} at a multiplicity of infection of 0.01 for 24 h before being injected into the brain of severe combined immunodeficient (SCID) mice.

SCID mouse model of HIVE SCID mice (male C.B-17, 4 weeks old) were purchased from the Charles River Laboratories (Wilmington, MA). Each animal was inoculated in the basal ganglia (caudate and putamen) with 5 μ l of either an HIV-1_{ADA}-infected or uninfected human MDM suspension containing 3×10^5 cells. All animal procedures were approved by the Institutional Animal Care and Use Committee of University of Nebraska Medical Center.

BMM tracking by CT/SPECT BMM were labeled with ¹¹¹indium (¹¹¹In) oxyquinoline (Indium oxine, GE Healthcare, Arlington Heights, IL) at a dose of 600 μ Ci per 30×10^6 cells in 1 ml RPMI 1640 supplemented with 10 mM HEPES for 45 min at 37°C. Cells were washed and resuspended in Hank's buffered salt solution. Labeling efficiency was determined by γ -scintillation spectrometry (Packard Instrument Co., Meriden, CT) and was >75% of total input isotope. Each recipient mouse received ¹¹¹In-labeled BMM by i.v. injection. Replicate groups of animals were simultaneously injected intraperitoneally (i.p.) with 4-aminopyridine daily (4-AP, 1 mg/kg) dissolved in phosphate-buffered saline (PBS). Mice were anesthetized with 0.5–1% isoflurane delivered in a 2:1 mixture of nitrous oxide and oxygen. Image acquisition was accomplished by a computed tomography/single photon emission computed tomography system (CT/SPECT; FLEX Triumph, Gamma Medica-Ideas, Northridge, CA). CT images were acquired by an X-ray detector, while SPECT images were acquired with a γ -scintillation camera detector fitted with 5 pinhole collimator. Co-registration of anatomical CT images and functional SPECT was performed by 3D image visualization and analysis software VIVID Gamma Medica-Ideas). The specific tissue and region of interest (ROI) of active viral encephalitis were defined and relative radioactivity for each area determined.

RNA extraction and real-time RT-PCR Total RNA was isolated from cells using TRIzol Reagent (Invitrogen, Carlsbad, CA) and was purified by RNeasy Mini Kit (Qiagen, Inc., Valencia, CA). Real-time reverse transcription polymerase chain reaction (RT-PCR) for mouse KCNA3, KCND1, and KCNN4 was performed using the one-step quantitative TaqMan real-time RT-PCR system (Applied Biosystems, Inc., Foster City, CA). Gene expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control.

Western blots Total proteins were prepared from BMM treated with MCP-1 or lipopolysaccharide (LPS) and equivalent amounts were electrophoretically separated on 4–15% sodium dodecyl sulfate polyacrylamide gel then transferred to Trans-Blot nitrocellulose membrane (BioRad, Hercules, CA). The membranes were probed with rabbit polyclonal antibodies to Kv1.3 and Kv1.5 (Almonade Lab, Israel) and images visualized with a chemoilluminescent horseradish peroxidase substrate kit (Pierce Biotechnology, Rockford, IL).

Immunohistochemistry BMM were fixed in 3.7% formaldehyde solution for 10 min at room temperature, permeabilized in 0.1% Triton X-100 for 3 to 5 min. Nonspecific binding on BMM was blocked with 1% bovine serum albumin in phosphate-buffered saline for 30 min and stained for actin expression with rhodamine phalloidin. All samples were viewed on a confocal microscope.

Chemotaxis assay BMM were labeled with 2.5 μ M 5-chloromethylfluorescein diacetate (CMFDA) (Invitrogen) in serum-free DMEM at 37°C for 15 min, washed, and adjusted to 3×10^6 cells/ml in complete DMEM. Tetraethylammonium chloride (TEA) was used to inhibit K⁺ channels. The CMFDA-labeled cells were loaded into chemotaxis μ -slides (ibidi, GmbH, Munich, Germany) in 10 μ l per chamber and incubated at 37°C for 35 min to allow cell attachment. Fresh or conditioned media (CM) was loaded into adjacent reservoirs of central channel, and the cells were imaged continuously for 2 h using a Nikon swept-field laser confocal microscope (Nikon Instruments, New York, NY). Images were analyzed using different plugins interfaced with ImageJ 1.38X software (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, MD, USA, <http://rsb.info.nih.gov/ij/>, 1997–2007). Migration coordination data for each observed cell was acquired with the Manual Tracking plugin (Fabrice Cordelières, Institut Curie, Orsay, France). Chemotaxis plots and migration velocities of each cell were determined with the Chemotaxis and Migration Tool (ibidi).

Transendothelial migration Transendothelial migration of BMM assay was measured using a quantitative fluorescence-based assay. First, mouse brain endothelial cells b. END3 (ATCC, Manassas, VA) were plated on rat-tail collagen type-I-coated FluoroBlok tinted tissue culture inserts (with 3- μ m pores; BD Biosciences, San Diego, CA) at a density of 1.5×10^4 cells/insert (Ramirez et al. 2008; Yamamoto et al. 2008). Thereafter, endothelial cells and BMM were treated with TEA for 30 min, and recombinant mouse MCP-1 (CCL2/MCP-1, 30 ng/ml; R&D Systems, Minneapolis, MN) was introduced into the lower chamber to create a chemokine gradient similar to that present in neuroinflammatory disorders. Labeled BMM

(8×10^4 cells/insert loaded with calcein-AM at $5 \mu\text{M}/1 \times 10^6$ cells for 45 min) were placed in the upper chamber, and migration was allowed to continue up to 4 h at 37°C . Migration was monitored by measuring the relative fluorescence of the labeled BMM found in the lower chamber by fluorescence spectrometry (Molecular Devices). The numbers of migrated BMM were derived from a standard curve of the relative fluorescence as a function of known numbers of labeled BMM.

Results

MCP-1 increased outward K^+ currents in BMM

Voltage-gated K^+ channels (Kv) play a key role in cell volume regulation and as such, cell movement. Under whole-cell voltage clamp recording, outward currents were evoked on BMM by voltage steps (Fig. 1A). These voltage sensitive outward currents were blocked by TEA, a voltage-gated K^+ channel blocker (Fig. 1B), suggesting the expression of Kv channels in BMM. Because MCP-1 is expressed in inflamed tissues serving as a pivotal chemo-attractant for cell migration and Kv channels are involved in controlling macrophage migration and proliferation (Gallin 1991), we investigated its influence on BMM Kv channels. As shown in Fig. 1A, bath perfusion of MCP-1 increased the outward K^+ currents induced by a voltage step from -60 to $+100$ mV with the holding potential of -60 mV in a dose-dependent manner. The average magnitude of steady-state outward K^+ currents before

application of MCP-1 was $100.0 \pm 2.6\%$ of control ($M \pm \text{SD}$, $n=9$). In contrast, bath application of MCP-1 at concentrations of 2, 20, and 200 ng/ml increased the steady-state K^+ currents to $164.1 \pm 1.75\%$, $191.7 \pm 4.2\%$, and $344.0 \pm 24.0\%$ of control, respectively ($n=9$, Fig. 1B). After washout of MCP-1, the outward K^+ currents returned to control levels (before application of MCP-1), with an average magnitude of $106.7 \pm 6.4\%$ of control ($n=9$). Based on these observations, we next examined whether the Kv channel blocker, TEA, could block K^+ currents induced by MCP-1. In another set of BMM, bath application of MCP-1 (20 ng/ml) alone increased the steady-state outward currents to an average of $167.3 \pm 18.8\%$ of control ($n=5$, Fig. 2). However, when MCP-1 was co-applied with TEA (20 mM), the average steady-state outward K^+ currents decreased ($p < 0.05$) by $83.5 \pm 15.6\%$ of control ($n=5$, Fig. 2), suggesting that MCP-1-mediated enhancement of outward K^+ current is sensitive to Kv channel blockers. After washout of MCP-1 and TEA, the outward K^+ currents returned to control levels, with an average of $96.6 \pm 9.7\%$ of control (Fig. 2, $n=4$).

MCP-1 regulates K^+ channel gene expression

To determine the responsiveness of K^+ channel to MCP-1, we investigated the messenger RNA (mRNA) abundance and protein expression level of genes related to K^+ channel. As shown in Fig. 3, we measured KCND1 ($\text{K}_{\text{v}}4.1$), KCNA3 ($\text{K}_{\text{v}}1.3$), and KCNN4 ($\text{K}_{\text{Ca}}3.1$) mRNA levels of BMM after treatment with MCP-1 and LPS. The results showed that KCNA3 gene expression is significantly increased 30 and 60 min after treatment with MCP-1

Fig. 1 MCP-1 enhanced outward K^+ currents in C57BL/6 mouse bone-marrow-derived macrophages (BMM). **A** Representative recordings from BMM before (control), during, and after (wash) bath perfusion with MCP-1 were assessed at different concentrations of 2, 20, and 200 ng/ml. Note that MCP-1 enhanced outward K^+ currents in a concentration-dependent manner. **B** Concentration-dependent effects of MCP-1 were assessed on the steady-state outward K^+ currents induced by a voltage step from -60 mV (holding potential) to $+100$ mV. Bath application of MCP-1 significantly increased K^+ currents, as compared with control ($n=9$). **C** The voltage jump protocol employed in this study is shown. * $p < 0.05$, ** $p < 0.01$

Fig. 2 Blockade of MCP-1-induced enhancement of outward K⁺ current by TEA. **A** Whole-cell outward K⁺ currents were recorded from a bone-marrow-derived macrophage before (control), during bath application of MCP-1 (MCP-1, 20 ng/ml), TEA+MCP-1 (20 ng/ml of each) (TEA+MCP-1), and washout period (washout). Note that the blockade of MCP-1-induced increase of outward K⁺ currents was significantly blocked by TEA (20 mM). **B** *I*-*V* curves were constructed from the results shown in **A**. **C** The steady-state K⁺ current induced by

a voltage step from -60 to +100 mV was significantly increased by MCP-1 ($p < 0.05$, $n = 5$). The MCP-1-induced increase of outward K⁺ currents was significantly blocked by bath application of TEA ($p < 0.01$, $n = 5$). Voltage jump protocol employed was the same as shown in Fig. 1C. Asterisks Compared with control group; number sign compared with MCP-1 alone group

(Fig. 3C, $p < 0.01$). Considering that Kv1.3 and Kv1.5 account for most of the voltage-gated K⁺ currents expressed by BMM, we tested the protein expression of Kv1.3 and Kv1.5. After treatment with MCP-1 for 30 and 60 min, Kv1.3 and Kv1.5 protein expression was readily increased by BMM (Fig. 3D). The results suggested that some K⁺ channel genes are regulated by MCP-1.

MCP-1 affects BMM F-actin reorganization

F-actin reorganization is essential for cell morphogenesis and migration. Based on morphology and F-actin distribution within BMM, macrophages can be categorized into four groups showing: (1) ramified macrophages with more than two protrusions, (2) elongated cells with two protrusions, (3) round cells with no protrusions, and (4) migratory cells with one or more rich lamellae and a tail (polarized F-actin). Migratory morphology was present in $25 \pm 2.9\%$ of unstimulated control group; whereas, after stimulation with 20 ng/ml MCP-1 for 1 h, $48.56 \pm 1.7\%$ of the cells exhibited a migratory morphology ($p < 0.05$, Fig. 4A,B).

MCP-1 facilitated BMM migration and is blocked by K⁺ channel antagonists

To investigate how MCP-1 influences BMM migration, we monitored the mobility of CMFDA-labeled BMM in chemotaxis chambers by laser confocal microscopy live imaging (Fig. 5A–C). Minimal BMM migration was observed in fresh media (Fig. 5A). However, levels of BMM migration significantly increased when MCP-1 was introduced to the migratory system (Fig. 5B). This level of BMM migration was attenuated by TEA, a non-specific voltage-gated K⁺ channel blocker (Fig. 5C). These results were consistent with the measured mean migration speed. In control media, BMM migrated at 138.5 ± 29.9 nm/min (mean $\pm$ SEM), which increased to 368.6 ± 37.5 nm/min in MCP-1-conditioned media (Fig. 5D). However, after co-culture with TEA and MCP-1, BMM migration speed was reduced to 143.0 ± 18.6 nm/min, levels comparable to those obtained in control media. Together, these observations demonstrated that MCP-1 significantly affects BMM mobility by activating K⁺ channels.

Fig. 3 MCP-1 altered K⁺ channel gene and protein expression in mouse BMM cells. BMM were stimulated with LPS or MCP-1 and mRNA was extracted at 0, 30, and 60 min after stimulation, respectively. Abundance of mRNAs for individual samples was quantified using quantitative RT-PCR and normalized to GAPDH. Data were presented as fold-increase of specific mRNA species

encoding for **A** KCND1, **B** KCNA3, and **C** KCNN4. **D** BMM cells were stimulated with LPS or MCP-1. Protein was extracted at 0, 30, and 60 min after stimulation, respectively, for Western blot analysis. Western blot analysis results show that MCP-1-induced increases in Kv1.5 and Kv1.3 protein expression

K⁺ channels regulate BMM BBB transmigration

Since MCP-1 increased BMM mobility and TEA attenuated mobility, we determined the biological importance of these

observations by assessing whether MCP-1-induced BMM migration across an *in vitro* BBB was affected by a K⁺ channel antagonist. We used a mouse brain endothelial cell to construct the *in vitro* barrier system and evaluated BMM

Fig. 4 MCP-1 increased the migratory morphology of BMM. **A** BMM cells were stimulated with LPS or MCP-1 for 60 min, fixed with formaldehyde, and stained with rhodamine phalloidin. Examples of round, migratory, ramified, and elongated BMM cells are indicated by blue, yellow, green, and white arrows, respectively. **B** BMM numbers were counted from slides based on different morphologies: round, migratory, ramified, and elongated for BMM cells treated with MCP-1 (gray bar), LPS (black bar), or media alone (control) (white bar). BMM numbers were normalized to percent of total number of BMM. **P*<0.01 compared with control

Fig. 5 BMM mobility increased after treated with MCP-1 and blocked by TEA. Chemotaxis Assay 2-Reservoir μ -slides (ibidi, LLC) was utilized to generate MCP-1 gradient and monitor BMM mobility by confocal microscopy live imaging. BMM migration over a defined area was determined for **A** both reservoirs filled with fresh media to generate a control environment; **B** MCP-1 loaded one reservoir with media and BMM in the other, and **C** MCP-1 applied to one reservoir,

BMM introduced into the other, and TEA (20 mM) applied to both reservoirs. BMM migration was assessed by live imaging over 2 h and analyzed by Image J software with chemotaxis tools plugins. **D** BMM migration in the presence of media alone, MCP-1, and MCP-1 in the presence of TEA was evaluated as a function of speed. $*p < 0.05$ compared to MCP-1-treated BMM

trans-endothelial migration in the absence or presence of MCP-1 and/or TEA. BMM cultured with MCP-1 increased BMM migration across the endothelium compared to migration in the absence of MCP-1 (Fig. 6A). In contrast, the ability of the BMM to migrate across the endothelium in the presence of TEA was significantly decreased. Similar results were obtained when both cell types were exposed to TEA (Fig. 6B). Taken together, these results suggested that K^+ channels play a central role in MCP-1-induced BMM migration.

CT/SPECT analysis of ^{111}In -labeled BMM transmigration in peripheral tissues

To assess real-time migration of macrophages, 1×10^7 ^{111}In -labeled BMM were adoptively transferred to normal recipients or recipients treated i.p. with 4-AP, and migration was assessed by CT/SPECT at 2, 24, 48, and 72 h following adoptive transfer. At 2 h post-transfer time point, radio-labeled BMM were detected in lung tissue with reduced signal in spleen and liver (Fig. 7A); however, by 24 h post-transfer, the predominant signal was localized to spleen and

liver. To quantify labeled BMM within each tissue, regions of interest were electronically mapped and total radioactivity counts determined. Relative number of counts/tissue volume confirmed that BMM levels in lungs were higher by 2 h then decreased quickly thereafter (Fig. 7B). Labeled BMM levels in liver and spleen were lower at the first 2 h; however, by 24 h, higher levels were observed in liver and spleen and diminished thereafter, indicating that most BMM migrated to liver and spleen (Fig. 7C,D). Comparable levels of labeled BMM migration in animals were observed regardless of treatment with or without AP-4 K^+ channel ($p > 0.05$). These results suggest that K^+ channel blocker had no effect on BMM migration into peripheral tissues under non-inflammatory conditions.

CT/SPECT analysis of ^{111}In -labeled BMM transmigration into brains of HIVE mice

We next assessed the effects of K^+ channel blockade on BMM migration to the brain and across the BBB under inflammatory conditions exhibited in a murine model of

Fig. 6 Effects of MCP-1 on BMM migration across a murine BBB. BMM migration across mouse brain endothelial cells toward MCP-1 (20 ng/ml) was evaluated after 2 h. **a** BMM were pretreated with TEA for 30 min and then removed before migration. **b** Both BMM and mouse brain endothelial cells were pretreated with TEA for 30 min. Data are represented as mean $\pm$ SEM for numbers of migrated BMM. * $P < 0.05$ for comparisons of groups attached by indicated lines

HIVE. Here, HIV-1_{ADA}-infected MDMs were injected intracranially by stereotactic measures within the caudate and putamen. After 3 days, 1×10^7 ¹¹¹In-labeled BMM were adoptively transferred i.v. to control, HIVE, and HIVE mice treated with AP-4 K⁺ blocker. BMM transmigration into the brain and the brain subregion with active encephalitis were tracked by CT/SPECT at 2, 24, and 48 h post-transfer. Whole-brain and injection site were electronically bitmapped in reconstructed 3D images, and relative radioactive levels were determined for each recipient (Fig. 8A). Counts within the whole brain (Fig. 8B) and ROI area (Fig. 8C) are shown. Quantitative analyses of those bitmapped tissues demonstrated that radioactive counts of BMM from HIVE mice treated with AP-4 K⁺ blocker were significantly diminished within whole brains by 24 h post-transfer compared to control or HIVE mice (Fig. 8B). Upon closer examination of the area containing the injection site and thus the focal HIVE, relative counts from K⁺ blocker-treated animals significantly diminished by 2 h and times thereafter when compared to the HIVE animals (Fig. 8C). These results suggest that K⁺ channels clearly affect the migration of BMM to the brain within the context of HIVE inflammatory conditions.

Discussion

Several different subtypes of Kv channels are expressed in human and mouse monocytes and macrophages including, but not limited to Kv1.3, Kv1.5 (Gallin 1991; Mackenzie et al. 2003; Vicente et al. 2003). These channels play an important role in controlling macrophage functions such as proliferation, activation, migration, and cytokine production (Gallin 1991; DeCoursey et al. 1996; Qiu et al. 2002). Studies have shown that Kv channels in macrophages can be regulated by many factors including proinflammatory cytokines. In the present study, we have demonstrated that MCP-1, a glial-derived cytokine, enhanced outward K⁺ currents and macrophage transmigration via regulation of Kv channel expression and channel activity. Our results herein fully support works by others demonstrating the expression of Kv channels in monocytes and macrophages (Mackenzie et al. 2003; Vicente et al. 2005, 2006; Irvine et al. 2007; Villalonga et al. 2007). Voltage-gated K⁺ channels including Kv1.3 channels are operative in blood-borne macrophages, tissue macrophages, and microglia, and are prominently induced concomitant with M-CSF-dependent proliferation.

It is well known that differential channel regulation affects intracellular signals and is linked to macrophage activation and differentiation as well as to immune, morphological, and migratory responses of the cells (DeCoursey et al. 1996; Mackenzie et al. 2003; Fordyce et al. 2005; Rus et al. 2005; Vicente et al. 2005; Irvine et al. 2007). LPS activation, for example, differentially regulates channel expression, as demonstrated by the induction of Kv1.3 channels and down-regulation of Kir2.1 channels. This modulation is dependent on the mode of macrophage immune responses (Gerth et al. 2005; Lei et al. 2005; Hoa et al. 2007; Villalonga et al. 2007). In this report, we have shown by pharmacological, mRNA, and protein analyses that macrophage ion currents are operative through Kv1.3 and Kir2.1 channels. This provides further support for the physiological importance of potassium channels in macrophage immunity. For example, it is well known that Kv1.3 channels are those principally found in macrophages and in other blood leukocytes (Mackenzie et al. 2003; Vicente et al. 2003; Fordyce et al. 2005; Vicente et al. 2005). For T cells, these channels are important since they serve as a primary regulator and effector of cell activation, as well as in maintaining resting membrane potential and ion homeostasis (Estes et al. 2008; Pouloupoulou et al. 2008). Importantly, less is known about the functional roles of channels in disease, although Kv1.3 blockade has been shown to reduce proinflammatory cytokine secretion and cell proliferation (Irvine et al. 2007), as well as to slow cell movement. Such findings serve as a foundation for investigations into whether channel modulation can be utilized as a therapeutic target for

Fig. 7 Peripheral BMM migration in non-HIVE mice is not affected by K⁺ channel blockade. BMM were isolated from the femurs BALB/C mice and grown in MCSF-containing media. After 1 week of cultivation, 1×10^7 BMM cells (purity by CD11b staining of 95%) were recovered, labeled with ^{111}In oxine (indium), and transferred by i.v. injections to two groups of naive mice (four animals/group). Mice were then treated with either PBS (control) or 1 mg/kg of the K⁺ channel blocker, AP-4 (blocker), administered by daily i.p. injections. **a** A representative animal was imaged 2 h (left panel) and 24 h (right

panel) after injection of indium-labeled BMM and CT images were co-registered with SPECT images (Gamma Medica-Ideas Inc.). Intensities of radioactive signals from indium-labeled BMM are shown as *pseudo-colored blue-green* signals. Quantitative assessment of labeled BMM distributions in PBS (control) and AP-4 (blocker) treated mice were calculated for **b** lung, **c** liver, and **d** spleen by digital image software (VIVID, Gamma Medica-Ideas, Inc.). Relative radioactive counts as a function of pixel intensities were determined and presented as the means $\pm$ SEM for four mice/group

autoimmune disease such as multiple sclerosis as suggested from studies showing that selective Kv1.3 channel blockers diminish disease progression in experimental allergic encephalomyelitis (Gonsette 2004; Vianna-Jorge and Suarez-Kurtz 2004; Beeton and Chandy 2005; Panyi 2005; Rus et al. 2005; Beraud et al. 2006).

In hippocampal microglia, Kv channel toxins affect pathobiological responses and cell migration across tissue barriers (Bordey and Spencer 2003; Fordyce et al. 2005; Thomas et al. 2007). Cytokines, toll receptor activation, and phorbol esters upregulate macrophage expression of Kv channels. Kv channel expression has been shown to be strongly upregulated in macrophage populations and only recently has the functional role of these channels been shown. First, extracellular K⁺ can activate T cell $\beta 1$ integrin and mediate adhesion and cell migration (Levite et al. 2000; Artym and Petty 2002; Davis et al. 2002). The gating of leukocyte voltage-gated K⁺ channels (Kv1.3) affects phys-

iological responses, including elevated extracellular K⁺ levels, integrin functions, and cell volume changes since they are linked to cell migration (Colden-Stanfield and Scanlon 2000; Colden-Stanfield 2002). Ion channels are known to affect macrophage function. For human macrophages, the major ionic currents are carried by an outwardly rectifying K⁺ channel activated by adhesion onto a substrate stretch applied to membrane patches. While K⁺ channels govern properties of cell movement, chloride and K⁺ channels contribute to the regulation of biological responses including nitric oxide. Indeed, nitric oxide synthase is inhibited by channel blockers. Thus, a range of ion channels expressed by macrophages and microglia is linked to cell biology (Gerth et al. 2005; Lei et al. 2005; Hoa et al. 2007; Villalonga et al. 2007).

The linkages shown in this report between MCP-1, K⁺ channels, and macrophage migration across the BBB are significant in regards to the pathogenesis of HAND for a

Fig. 8 Brain BMM migration for HIV mice is affected by K⁺ channel blockade. HIV was induced by the stereotactic injection of virus-infected human monocyte-derived macrophages (MDM). Mouse BMM were labeled with ¹¹¹In oxine, washed and 1×10^7 labeled BMM were adoptive transferred to four animals/groups. One group of HIV mice received PBS and the other group received 1 mg/kg of the K⁺ channel blocker, 4-AP administered by daily i.p. injections. BMM ingress into the brain was tracked by CT/SPECT imaging (Gamma Medica-Ideas Inc.) for each animal 2, 24, and 72 h post-transfer. Radioactivity in whole brain and in the area of MDM injection was assessed by digital image analysis software (VIVID, Gamma Medica-Ideas, Inc.). **A** CT/SPECT image from control mouse showing electronically mapped whole brain (pink) and the region of interest (ROI) (broken white line) that encompasses the injection site within the basal ganglia. Total counts as a function of pixel intensity were calculated by digital image analysis (VIVID software, Gamma Medica-Ideas, Inc.) from whole brain (**B**) and the ROI comprising the injection site and area of inflammation (**C**) for HIV mice treated with PBS (control) or 4-AP (blocker). * $p < 0.05$, compared to the HIV group

variety of reasons. HIV-1 transactivator protein Tat or progeny virus released from infected macrophages significantly increases astrocyte production and release of MCP-1 (Eugenin et al. 2005; El-Hage et al. 2006). MCP-1 is also

expressed at high levels in the brains of patients with HIV-1-associated dementia (HAD), and MCP-1 levels within the cerebrospinal fluid correlate with the severity of neurological dysfunction (Sevigny et al. 2004; Maslin et al. 2005; Sevigny et al. 2007). Such observations are mirrored in simian immunodeficiency virus infected macaques with pathological features of HIVE (Zink and Clements 2002; Mankowski et al. 2004; Harrington et al. 2007). Homozygosity for the MCP-1-2578G allele was linked to a 50% reduction in the risk of HIV-1 acquisition. However, following HIV-1 infection, this MCP-1 genotype was associated with accelerated disease and a 4.5-fold increased risk of acquiring HAD. The mutant MCP-1-2578G allele yields increased transcriptional activity, protein production, and serum MCP-1 levels concomitant with accelerated MP tissue migration (Gonzalez et al. 2002).

To evaluate the biological significance of potassium channel influence on BMM migration into the brain, we investigated BMM migration in a SCID mouse model with HIVE using CT/SPECT. HIVE was induced by i.c. injection of HIV-1_{ADA}-infected MDM into the caudate and putamen of SCID mice and was characterized by robust inflammation and the generation of an MCP-1 gradient for BMM attraction into sites of active disease. In this work, comparisons of BMM brain migration between uninfected and HIVE mice that were either untreated or treated with 4-aminopyridine (4-AP), a K⁺ channel blocker, showed that Kv channel blocker had no effects on BMM distribution in the peripheral organs but significantly reduced BMM migration into the mouse brain. These results further reinforce the biological importance of potassium channels for macrophage migration and its links to MCP-1 and inflammatory responses.

The data, taken together, build on previous works demonstrating a link between MCP-1 induced biological responses and specific ion channels. MCP-1 certainly has a multifaceted role in HAND through its abilities to affect proinflammatory properties and contribute to disease progression and increased risk of dementia by affecting MP ingress into the brain. The importance of the current study rests in that these physiological responses to MCP-1 play a central role in HAND and as such opens up new therapeutic targets for disease.

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