Inflammatory status of transmigrating primary rat monocytes in a novel perfusion model simulating blood flow

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ABSTRACT

It remains unclear whether monocyte infiltration plays a protective or detrimental role in neurodegenerative disease. The present study characterizes the inflammatory status of primary monocytes in a novel in vitro perfusion model. Monocytes under perfusion do not undergo elevated cell death. However, perfusion does lead to altered morphology, which can be counteracted by anti-inflammatory drugs. Functional studies indicate that cytokine levels are significantly reduced in perfusion compared to stationary conditions and enhanced with brain slices or capillary endothelial cells. Understanding monocyte properties could lead to refined treatment and new ways to interfere with inflammation in diseased brains.

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1. Introduction

Monocytes are bone marrow-derived hematopoietic cells that circulate throughout the bloodstream and eventually give rise to tissue macrophages and dendritic cells (Ziegler-Heitbrock, 2007). Peripheral monocytes consist of a wide variety of phenotypically and functionally distinct subpopulations varying in maturation, differentiation, and activation states. These heterogeneous subpopulations are characterized by their differential expression of cell surface markers (Auffray et al., 2009; Buckner et al., 2011). Inflammation and the recruitment of monocytes and monocyte-derived cells into the central nervous system (CNS) have been implicated in a number of neurological disorders including: Alzheimer’s disease (AD), Parkinson’s disease (PD), amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS) (Akiyama et al., 2000; Glass et al., 2010; Khandelwal et al., 2011). Upon pathological stimulation (including neurodegeneration or inflammation) or during normal immune patrol, monocytes adhere and transmigrate across the blood–brain barrier (BBB) into the brain where they execute effector functions and differentiate into cells with a microglia-like phenotype. This migration is mainly regulated by cell adhesion molecules (CAMs) and chemokines (Auffray et al., 2007; D’Mello et al., 2009; Prinz and Priller, 2010).

Extensive evidence indicates that inflammation can contribute to further exacerbation of neurodegenerative diseases. In the healthy brain and CNS tissue, inflammatory mediators (cytokines and their receptors) are expressed constitutively at low or undetectable levels. However, upon insult or infection, proinflammatory cytokines and mediators lead to the expression of chemokines and adhesion molecules, recruitment of monocytes and other immune cells to the lesion site, and activation of resident microglia and/or astrocytes. Thus far, the role of monocytes and monocyte-derived cells in disease propagation has been under intense debate. Monocyte-derived macrophages may induce beneficial effects by their secretion of neuroprotective factors, phagocytosis of debris/apoptotic cells and initiation of repair processes (Schwartz and Shechter, 2010). Previous investigations indicate that microglia cause further disease aggravation through uncontrolled inflammation via release proinflammatory mediators and other neurotoxic factors (Lucas et al., 2006). Therefore, further studies are needed in order to better understand the phenotype and migratory dynamics of monocytes during inflammation of the CNS.

However, studies involving primary monocytes have proven difficult. In vitro static culturing methods using glass or plastic vessels quickly lead to monocyte adherence, activation, and differentiation into macrophages (Steiniger et al., 2001). Few studies have shown the successful maintenance of monocyte cultures in the absence of growth factors (M-CSF, GM-CSF) or differentiation into macrophages (Wirth et al., 1982; Mazo et al., 1986). Thus, we sought to establish an in vitro culturing system that provides more physiological conditions.

The aim of the present study is to characterize the inflammatory status of primary rat monocytes in a novel in vitro perfusion model. This model consists of two peristaltic pumps providing constant flow of medium and cell circulation into a glass chamber applying shear stress (Fig. 1).

Here, we compare the functional properties of monocytes after isolation, exposure to constant flow, and incubation under static conditions. In addition, we evaluate the interaction between monocytes...
and brain capillary endothelial cells (BCEC)/cortex brain slices when under the influence of constant flow including their release of inflammatory markers. Finally, we examine the effects of anti-inflammatory drugs (i.e., minocycline, indomethacin) on counteracting release of proinflammatory cytokines in cortical organotypic brain slices.

2. Methods

2.1. Isolation of primary rat monocytes

Primary rat monocytes were freshly isolated as previously described by us with some modifications (Humpel, 2008; Böttger et al., 2010; Hohsfield and Humpel, 2010). In brief, Sprague-Dawley rats (250 g, Himberg, Austria) were anesthetized by an intraperitoneal injection of 40 mg/kg body weight thiopental (Sandoz, Kundl, Austria) and perfused with 500 ml of 4 °C pre-chilled 10 mM phosphate-buffer saline (PBS)/2.7 mM EDTA/25 mg/ml heparin, pH 7.3 through the left ventricle. The collected effluent was centrifuged at 550 × g for 10 min at 4 °C. The perfusate pellet was resuspended in 50 ml of 10 mM PBS/1% bovine serum albumin (BSA; SERVA Electroporesis, Heidelberg, Germany)/2.7 mM EDTA, pH 7.3 and carefully overlaid on a Percoll working solution (Scriba et al., 1996). After centrifugation at 500 × g for 30 min at 4 °C, peripheral blood mononuclear cells (PBMC) were harvested from the interphase. PBMC were then washed once with 50 ml of PBS and -20 × 10^6 PBMC were resuspended in 100 μl of PBS/BSA/EDTA. Monocytes were purified from PBMC by negative magnetic selection: PBMC were incubated in a cocktail consisting of four different purified anti-rat monoclonal antibodies (20 μg of each: CD8α (clone OX-8), CD5 (clone OX-19), CD45RA (clone OX-33), PAN T (clone OX-52); all from Cedarlane Laboratories, Szabo, Austria) for 10 min at 4 °C shaking. PBMC were washed once with PBS and resuspended in 100 μl of PBS/BSA/EDTA and 40 μl of MACS Goat Anti-Mouse-IgG Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). PBMC were incubated for 15 min at 4 °C on a shaker and following incubation, were washed once with PBS. The cells were resuspended in 1000 ml of PBS/BSA/EDTA and then applied to a MS-MACS column fixed to a strong magnet. The purified monocytes were centrifuged and pooled for further experiments. Approximately 10 × 10^6 cells were isolated from one adult rat. The described isolation procedure yields approximately 90–95% CD68-positive monocytes (Moser and Humpel, 2007; Böttger et al., 2010). During this preparation monocytes were counted using the Cell Coulter Counter (COULTER®Z™ Series, Fischerlehner & Kucera, Innsbruck, Austria) in a range from 5.5 to 10 μm. All animal experiments were approved by the Austrian Ministry of Science and conformed to the
Austrian guidelines on animal welfare and experimentation. All possible steps were taken toward reducing the number of animals used and their suffering.

2.2. In vitro perfusion system

The perfusion system consists of two peristaltic pumps (P-1 Pump, Pharmacia, London, UK) providing a constant flow rate of 5 ml/min (~3 dyn/cm² shear stress level) similar to the levels at post-capillary venules where leukocytes exit the blood (Resto et al., 2008). Prior to perfusion, the autoclaved glass container and the autoclaved glass Petri dish are connected to a 320 cm long tube system (silicone tubes, 3 mm inner diameter, 5 mm outer diameter, UniLab Politakis, Innsbruck Austria) and washed with 100 ml of 70% ethanol for 30 min, followed by 2 washes with sterile a.d. (100 ml each for 30 min). Then 37 °C pre-warmed sterile filtered culture medium is added to the system via a filter (0.4 μm) connected to a syringe and circulated for 30 min at 37 °C and 5% CO₂. One pump delivers the sterile culture medium to the glass chamber and the second pump carries the medium out of the chamber and back into the medium container allowing recirculation (Fig. 1). The medium is composed of 50% MEM/HEPES (GIBCO Invitrogen), 25% heat inactivated horse serum (GIBCO Invitrogen), 25% HBSS (GIBCO Invitrogen), 2 mM NaHCO₃ (Merck), 6.5 mg/ml glucose (Merck), and 2 mM glutamine (Merck) at pH 7.2. For some experiments brain slices or BCEC (grown on either 3 μm pore size membrane inserts allowing monocyte migration or 0.4 μm pore size inserts that do not permit transmigration) are inserted into the Petri dish glass chamber. Following this preparation, 5 × 10⁶ monocytes (Lebson et al., 2010) or different substances (e.g. lipopolysaccharide (LPS), minocycline, etc.) are added steriley to the perfusion system via an inlet in the medium container. Once the cells or substances have been added, they are also then pumped along with the medium resulting in continuous circulation. Following 24 h of incubation, the culture medium, monocytes, BCEC and brain slices are collected for further analysis. The perfusion system is then washed with ethanol and distilled water and disconnected and prepared for another experiment.

In order to visualize effective monocyte transmigration, monocytes were labeled with PKH67 Green Fluorescent Cell Linker (Sigma-Aldrich) according to manufacturer’s instructions as previously described (Böttger et al., 2010) prior to their addition into the perfusion system. LPS (1 μg/ml) was used to stimulate an inflammatory response in the perfusion model. As negative controls — monocytes and brain slices/BCEC were cultured alone or together without LPS. Minocycline (30 μM), ibuprofen (50 μM), and indomethacin (1 μM) were added to the system to observe the effects of anti-inflammatory drugs on counteracting inflammation.

2.3. Preparation of cortical organotypic brain slices

The use of organotypic brain slices has been well established and previously described by us in detail (Moser et al., 2003; Pirch et al., 2010). Briefly, cerebral cortices from postnatal day 8 (P8) rats were dissected under aseptic conditions and cut to a thickness of 400 μm using a McIlwain Tissue Chopper (Mickle Laboratory, Surrey, UK). Cortex slices were then placed on Millicell 0.4 μm pore size 30 mm diameter cell culture inserts or Millicell-PCF 3 μm pore size 12 mm diameter for (transmigration studies) cell culture inserts (2 slices per membrane, Millipore). Slices were cultured for approximately 2 weeks at 37 °C/5% CO₂ with 1.2 ml well of the following culture medium: 50% minimum essential medium (MEM)/4-(2-hydroxyethyl)-1-piperazinieethanesulfonic acid (HEPES) (GIBCO Invitrogen, Paisley, UK), 25% heat-inactivated horse serum (GIBCO Invitrogen), 25% Hanks’ Balanced Salt Solution (HBSS; GIBCO Invitrogen), 2 mM NaHCO₃ (Merck, Darmstadt, Germany), 6.5 mg/ml glucose (Merck), and 2 mM glutamine (Merck), pH 7.2. The medium was changed twice a week. After 2 weeks of incubation, the slices became attached to the membrane of the culture inserts and the inserts were placed into the glass chamber of the perfusion system (already containing medium) for 24 h. Following experiments, slices were washed with PBS and either collected as extracts for ELISA analysis or immediately investigated under the microscope.

2.4. Rat brain capillary endothelial cell culture

The culturing of rat brain capillary endothelial cell (BCEC)-monolayers was performed as described previously (Moser et al., 2004). Immortalized brain capillary endothelial cells (rBCEC4; Blasig et al., 2001) were cultured on collagen-coated wells (5 μg/cm² rat tail collagen, Roche Diagnostics, Mannheim, Germany) in full BCEC medium (13.5 mg/ml Dulbecco’s modified Eagle’s medium (DMEM), Sigma-Aldrich, Steinheim, Germany; 10% heat-inactivated fetal bovine serum (FBS), PAA Laboratories, Pasching, Austria; 100 μg/ml heparin, Sigma) at 37 °C/5% CO₂. After 2–3 days, the cells were then scraped and placed onto the outer membrane of inverted collagen-coated Millicell 0.4 μm pore size 30 mm diameter cell culture inserts or Millicell-PCF 3 μm pore size 12 mm diameter (for transmigration studies) cell culture inserts (Millipore, Carrigtwohill, Ireland). Following 3 h incubation, BCEC adhered to the membrane inserts and were thus turned right side up and maintained in full BCEC medium for 1 day. This plating technique ensured that BCEC came in direct contact with perfused medium and cells (similar to their formation of a capillary wall) thereby providing an in vitro blood–brain barrier. Following incubation, the culture medium was changed and cells were incubated in full BCEC medium containing hydrocortisone (500 ng/ml, Sigma) for 1 day. Following incubation, cells were placed in BCEC medium without FBS and maintained for 1–2 weeks. After 1–2 weeks BCEC displayed a fully confluent monolayer and were used for experimental purposes. To verify cell confluency, some cells (plated in parallel) were stained for nuclear DAPI distribution (30 min, 1:10,000, Sigma) one day prior to the experiment. Only cells displaying a fully confluent monolayer were used for perfusion studies. Following perfusion experiments, BCEC were washed with PBS and either collected as extracts for ELISA and Western blot analysis or stained for immunohistochemical evaluation. To evaluate cell integrity following perfusion, some BCEC were fixed and stained for tight junction (ZO-1, 1:100), F-actin (phallolidin-TRITC, 45 min, 1:2000, Fluka/Sigma-Aldrich), and DAPI (20 min, 1:10,000, Sigma) distribution.

2.5. Immunohistochemistry

Immunohistochemistry was performed as previously described (Moser et al., 2006; Humphel, 2008). For evaluation of surface marker expression, monocytes were spotted on collagen-coated glass slides and fixed in cold 4% paraformaldehyde/10 mM PBS (4 °C, 30 min). After fixation, cells were washed with PBS and then incubated in PBS/0.1% Triton (T-PBS) for 30 min at 20 °C shaking. After incubation, the cells were then blocked in T-PBS/20% horse serum (GIBCO Invitrogen)/0.2% BSA (SERVA) for 30 min at 20 °C shaking. Following blocking, monocytes were incubated with primary antibody: ED-1 (CD68, 1:750, Chemicon Millipore, Temecula, CA, USA), Ox-42 (CD11b, 1:500, Chemicon Millipore), ionized calcium binding adaptor molecule-1 (Iba-1; 1:1000, Wako Pure Chemical, Osaka, Japan) in T-PBS/0.2% BSA overnight at 20 °C. The cells were then washed and incubated with secondary antibody Alexa Fluor 488 anti-mouse (ED-1, Ox-42) or anti-rabbit (Iba-1) (1:200, Molecular Probes Invitrogen, Eugene, CA, USA) in T-PBS/0.2% BSA for 1 h at 20 °C shaking. Cells were then mounted with a glass coverslip using Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA) and visualized under the microscope. Alternatively, immunoperoxidase staining was performed using Vectastain Elite ABC kit (Vector Laboratories). Monocytes were fixed and washed in T-PBS as usual. To quench endogenous peroxidase, cells were treated with PBS/1%H2O2/5% methanol. Cells were then incubated and washed in T-PBS/0.2% BSA (Sigma) for 30 min at 20 °C shaking. Following incubation, the cells were then blocked in T-PBS/20% horse serum (GIBCO Invitrogen)/0.2% BSA (SERVA) for 30 min at 20 °C shaking. Following blocking, monocytes were incubated with primary antibody: Ed1 (CD68, 1:750, Chemicon Millipore, Temecula, CA, USA), Ox-42 (CD11b, 1:500, Chemicon Millipore), ionized calcium binding adaptor molecule-1 (Iba-1; 1:1000, Wako Pure Chemical, Osaka, Japan) in T-PBS/0.2% BSA for 1 h at 20 °C shaking. Cells were then mounted with a glass coverslip using Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA) and visualized under the microscope. Alternatively, immunoperoxidase staining was performed using Vectastain Elite ABC kit (Vector Laboratories). Monocytes were fixed and washed in T-PBS as usual. To quench endogenous peroxidase, cells were treated with PBS/1%H2O2/5% methanol. Cells were then washed, blocked, and incubated with primary antibody as usual. After washes, cells were incubated with biotinylated secondary antibody (1:200, Vector Laboratories). After rinsing with PBS, cells were...
incubated in avidin–biotin complex solution (Elite ABC kit, Vector Laboratories) for 1 h at 20 °C shaking. Finally, the cells were washed with 50 mM Tris-buffered saline (TBS) and then incubated in 0.5 mg/ml 3,3′-diaminobenzidine (DAB, Sigma)/TBS/0.003% H2O2 at 20 °C in the dark until signal was detected. Once DAB staining was visible, the reaction was stopped by adding TBS to cells. Cells were rinsed with TBS and then evaluated by microscopy. Images were captured with a Leica DM IRB (Leica DFC350FX camera) or Olympus BX61 (ProgRes C14 camera) microscope equipped with Openlab 4.0.4 or Openlab 5.5.0 imaging software, respectively.

2.6. Flow cytometry

Monocyte cell death was evaluated by in situ staining of non-fixed cells with the nuclear dye propidium iodide (PI). Cell death was determined by analyzing the number of PI-positive cells using flow cytometry (BD FACS Calibur). In brief, cells were washed with PBS and incubated in 2 μg/ml PI (Moser et al., 2006) immediately prior to evaluation.

2.7. Searchlight Multiplex ELISA

The detection of inflammatory proteins (monocyte chemotactic protein-1, MCP-1; macrophage inflammatory protein-2, MIP-2; tumor necrosis factor-α, TNFα; interleukin-1β, IL-1β) was performed using the Thermo Scientific SearchLight Protein Array Technology (THP Medical Products, Vienna) according to the manufacturer’s recommendations (Bio-Rad) and as previously described by us (Hohsfield and Humpel, 2010; Hochstrasser et al., 2011). Briefly, collected culture medium, cell extracts (diluted 1:2 in diluent) or calibrated standards were added to coated wells of the provided plate and incubated for 3 h. After washing, the biotinylated antibodies were added and following 30 min incubation the wells were washed again and incubated with streptavidin-horseradish peroxidase conjugate. After the final washing step the SuperSignal Chemiluminescent Substrate was added. All incubation steps were carried out on a shaker at 20 °C. The luminescent signal was detected using a compatible CCD imaging and analysis system and the absorbance was measured at 450 nm. The concentration of each sample was quantified by comparing the spot intensities with the corresponding standard curves calculated from the standard sample results using the SearchLight Array Analyst Software. Integrated density values were proportional to the concentrations of bound proteins. Standard curves, raw data and final pg/ml concentrations for each analyte and each sample were reviewed in the array software and exported to Microsoft Excel Software for further statistical analysis. Sample values were calculated from the standard curve in a linear range.

2.8. Quantitative analysis and statistics

All data are depicted as mean ± SEM (n = independent experiments). Statistical analysis was obtained by one-way ANOVA or Independent T-test with Fisher LSD post hoc test, comparing controls against respective treatments in which p < 0.05 represents the minimum level of statistical significance.

3. Results

3.1. Cell phenotype and viability of primary rat monocytes

Primary rat monocytes were stained for monocyte/macrophage markers: ED-1 (CD68), OX-42 (CD11b), and Iba-1 (Fig. 2). ED-1 staining was mostly restricted to the cytoplasm with occasional intense intracellular vesicle staining (Fig. 2B). OX-42 labeling was mostly localized on the cell surface (Fig. 2C). Iba-1 staining was distributed throughout the entire cell (Fig. 2D). Previous studies by us have shown that these cells display strong immunostaining (90%) for CD172a (ED-9) (Zassler and Humpel, 2006). Monocytes cultured under static conditions (i.e. in an eppendorf tube or 24-well plate) and flow conditions displayed similar immunostaining of monocyte/macrophage specific markers to freshly isolated cells by microscopy evaluation (Fig. 2). Confirming the data by flow cytometry analysis, we observed that the majority (70–95%) of freshly monocytes expressed typical surface markers ED-1 and OX-42 (data not shown). Directly following isolation, monocytes were also stained with the membrane linker dye PKH67. This labeling did not result in an altered cell size or morphology. Naïve primary rat monocytes displayed a diameter of approx. 6 μm following isolation and staining (Figs. 2F and 3A). This cell size and morphology appeared unaltered in monocytes incubated under static conditions (Fig. 2). The majority of freshly isolated cells and cells incubated for 24 h under static conditions exhibited small and round morphologies, whereas perfused cells displayed multiple forms (Fig. 3B). These varying sizes and morphologies consisted of three major groups: small and round (Fig. 3C), small and elongated, and large and round/elongated (Fig. 3D). We observed that the number of large and elongated cell forms was significantly enhanced (p < 0.0001) when cells were subjected to constant flow (Table 1).

Flow cytometry analysis was also carried out in order to evaluate monocyte survival. Immediately following isolation or cultivation, monocytes were incubated with propidium iodide (PI) and analyzed. Approximately 13 ± 2% of freshly isolated cells were PI-positive (n = 7). Cell death was not significantly enhanced in monocytes incubated for 24 h under static conditions compared to freshly isolated cells. Approximately 19 ± 2% of cells displayed PI-positive staining (n = 6).

In addition, under static conditions we observed a population of cells displaying a more granular phenotype. No significant difference was found in cell death between cells subjected to 24 h constant flow and cells incubated under static conditions. Specifically, 29 ± 5% of cells subjected to constant flow were PI-positive (n = 5). Under these conditions we did not observe a defined population of cells exhibiting higher granularity as was seen in cells under static incubation (data not shown). The effects of PKH67 labeling on monocyte cell death were also evaluated by flow cytometry. Approximately 9 ± 3 (n = 4) % of cells were PI-positive and 99 ± 1 (n = 4) % were positive in the FL-1 channel detecting green dyes.

3.2. Cytokine and chemokine secretion of primary rat monocytes

We were also interested in the effects of different culturing methods on monocyte functional inflammatory behavior, specifically on their cytokine and chemokine secretion. We observed that monocytes incubated under static conditions released high amounts of inflammatory markers. Overall, cells incubated for 24 h in the presence of LPS under static conditions displayed decreased release of MCP-1, MIP-2, and TNF-α. Statistical analysis showed that MCP-1 secretion was slightly reduced (p = 0.08) and MIP-2 secretion was significantly reduced (p < 0.01) by LPS treatment under static conditions compared to control static conditions (Fig. 4). On the other hand, IL-1β release was slightly, but not significantly enhanced. Cells under constant flow illustrated markedly decreased inflammatory mediator release compared to cells under static incubation. The release of these inflammatory markers was slightly, however not significantly increased with the addition of LPS (Fig. 4).

3.3. Interaction of monocytes with BCEC under constant flow

To evaluate the interaction of monocytes with BCEC, we incubated monocytes in the presence of rat brain capillary epithelial cells (BCEC) for 24 h under constant flow. BCEC alone under constant flow showed high release of MCP-1 measured in the culture medium. This high secretion was significantly elevated by LPS treatment and the addition of circulating monocytes (p < 0.001) under static conditions, however not under constant flow (Fig. 4). All other cytokines (MIP-2, TNF-α, IL-1β) exhibited low level release or were below
assay detection limits. BCEC release of MIP-2 was significantly enhanced in the presence of monocytes \( (p < 0.05) \).

We also evaluated the number of monocytes in contact with the BCEC-monolayer. On average, \( 11 \pm 3 \) (\( n = 8 \)) monocytes adhered to a BCEC-monolayer as visualized in a 113 mm\(^2\) field (Fig. 5E–5G).

### 3.4. Interaction of primary rat monocytes with cortex slices under constant flow

After observing monocyte cytokine and chemokine secretion alone and in the presence of LPS, we were also interested in the effects of cortical organotypic brain slice and primary monocyte co-cultures on inflammatory marker output. Slices alone showed little to no inflammatory marker release, with the exception of MCP-1 (Fig. 4). Slice release in the presence of LPS was significantly elevated compared to slices alone \( (p < 0.05) \). Slices incubated in the presence of monocytes under constant flow and static conditions resulted in markedly enhanced secretion of inflammatory markers into the culture medium.

In addition to measuring cytokine and chemokine secretion, we were also interested in evaluating whether monocytes could come in contact with the cortical brain slices and whether this interaction resembled transmigration into or adhesion to slices. In order to visualize monocyte transmigration and/or adhesion, monocytes were labeled with membrane dye PKH67 prior to addition to the perfusion system (Fig. 5A). On average, \( 163 \pm 60 \) (\( n = 11 \)) monocytes migrated into a cortical brain slice as visualized in a 113 mm\(^2\) field (Fig. 5B). Furthermore, \( 71 \pm 1\% \) of total migrated monocytes displayed small and round morphology (Fig. 5C) whereas the other \( 29 \pm 1\% \) displayed small and elongated morphology (\( n = 4 \), Fig. 5D).

In addition, we evaluated inflammatory marker release of brain slices in the presence of circulating monocytes following treatment with anti-inflammatory drugs. All three anti-inflammatory drugs (minocycline, ibuprofen, and indomethacin) slightly reduced cytokine

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**Fig. 2.** Immunohistochemical analysis of monocyte surface marker expression. Primary monocytes were isolated, spotted onto glass slides and immunohistochemically stained for various surface markers: ED1 (B, E), cluster of differentiation 11b (CD11b) (C), ionized calcium binding adaptor molecule-1 (Iba-1) (D). As a control, cells were incubated without primary antibody (A). Monocytes were also fluorescently stained using PKH67 (F). Nuclei were counterstained with nuclear DAPI (A–D). Scale bar = 15 µm (A–D), 60 µm (E) and 20 µm (F).
release from cortical slices in the presence of circulating monocytes compared to slices incubated with monocytes alone (Table 1). However, this reduction in MCP-1, MIP-2, and TNF-α secretion was not significant. IL-1β levels were below assay detection limits.

3.5. Effects of anti-inflammatory drugs on monocytes under constant flow

In order to evaluate the effects of anti-inflammatory drugs on the profile and behavior of monocytes under constant flow, we added the anti-inflammatory drugs minocycline, ibuprofen, and indomethacin to our perfusion system (containing monocytes and cortical brain slices). We observed that minocycline in perfusion slightly enhanced (p = 0.09) the amount of small round cell forms compared to perfusion alone (Table 1). Indomethacin treatment significantly reduced (p < .01) the number of large cells observed compared to non-treated samples.

4. Discussion

In the present study, we evaluated the inflammatory status of primary rat monocytes under constant flow in an in vitro perfusion system. In addition, we examined the effects of anti-inflammatory drugs

Table 1

|                  | (−)               | Minocycline      | Ibuprofen       | Indomethacin     |
|------------------|-------------------|------------------|-----------------|-----------------|
| MCP-1            | 199 ± 56 (10)     | 147 ± 105 (4) ns | 165 ± 66 (4) ns | 145 ± 26 (6) ns |
| MIP-2            | 18 ± 5 (10)       | 12 ± 10 (4) ns   | 6 ± 4 (4) ns    | 6 ± 2 (6) ns    |
| TNF-α            | 20 ± 5 (10)       | BDL (4) ns p = .09 | 12 ± 7 (4) ns  | BDL (6) p = .09 |
| % small, round cells | 43 ± 2 (4)       | 59 ± 4 (4) ns p = .09 | 37 ± 13 (3) ns | 40 ± 6 (4) ns  |
| % large cells    | 13 ± 1 (4)        | 10 ± 1 (4) ns    | 12 ± 2 (3) ns  | 6 ± 1 (4) **    |
| % small, elongated cells | 45 ± 2 (4)      | 32 ± 5 (4) ns    | 50 ± 15 (3) ns | 55 ± 5 (4) ns  |

Inflammation was evaluated by the measurement of inflammatory markers using Searchlight Multiplex ELISA (monocyte chemotactic protein-1, MCP-1; macrophage inflammatory protein-2, MIP-2; tumor necrosis factor-α, TNF-α). 5 × 10⁶ monocytes were added to the perfusion system containing cortical slices. Minocycline (30 μM), ibuprofen (50 μM), and indomethacin (1 μM) were added to the system to observe the effects of anti-inflammatory drugs in counteracting inflammation. (−) indicates control conditions i.e. monocytes and slices cultured in the absence of drugs. IL-1β levels were also measured, however, all samples were below assay detection limits. Values = mean ± SEM, pg/ml × 24 h. Statistical analysis was performed using one-way ANOVA with Fisher’s LSD posthoc test (**p < 0.01; ns not significant). Monocyte profiles were evaluated by fluorescent microscopy for varying cell sizes and morphologies. For these experiments, monocytes were labeled with PKH67 membrane dye immediately prior to addition to perfusion system. Values = mean ± SEM % of cells. Data not shown: Slices alone in perfusion secrete 48 ± 19 (8) pg/ml MCP-1, 0.9 ± 0.2 (8) pg/ml MIP-2, however, TNF-α was below assay detection limits. Freshly isolated monocytes display 98 ± 0.5 (4) % small, round morphology, 1.8 ± 0.5 (4) % large morphology, and no small, elongated morphology.
on monocyte profiles and proinflammatory cytokine release. Ultimately, we show the effects of perfusion on the monocyte inflammatory profile and demonstrate the usefulness of such a model in evaluating monocyte-induced inflammation.

4.1. Surface marker expression in monocytes

Rat monocytes are characterized morphologically as mononuclear cells with bean-shaped nuclei and phenotypically by their expression of surface markers: ED-1, ED-9 and OX-42 (CD11b; Grau et al., 2000). ED-1 is a monoclonal antibody directed against a lysosomal membrane (CD68-like) antigen. The monoclonal antibody ED-9 recognizes a member of the signal-regulatory protein-α (SIRP-α) family. Here, we report that isolated monocytes demonstrate strong immunoreactivity with ED-1 and OX-42, as well as Iba-1, a monocyte/macrophage lineage marker that can be an indicator for microglia activation (Imai and Kohsaka, 2002). Several factors have been shown to change the properties of monocytes (Strauss-Ayali et al., 2007), thus, it is important to consider that different culturing methods could also have varying effects on the phenotypic and functional properties (Buckner et al., 2011).

4.2. Culturing monocytes

Previous investigations involving the in vitro culture of monocytes have proven difficult and lead to variable results. Cells under serum-free conditions are less adherent and differentiate into macrophages more slowly (Bennett and Breit, 1994), whereas cells incubated with serum show low survival rates, induced activation, and clumping (Vincent et al., 1992). In this study, we cultivated primary rat monocytes under serum-free conditions and compared their survival and cytokine production in adherent and non-adherent conditions. Preliminary FACS analysis showed that cells under static conditions express typical surface markers and are more granular. For further analysis, we evaluated the inflammatory profile of these monocytes. These cells secreted high levels of MCP-1, MIP-2, and TNF-α indicative of cell activation. In support of these findings, others have reported that monocytes incubated in untreated plastic Petri dishes under serum-free conditions secrete high

Fig. 4. Inflammatory profile of primary rat monocytes. The inflammatory profile of primary rat monocytes was evaluated by measurement of inflammatory markers by Searchlight Multiplex ELISA (monocyte chemotactic protein-1, MCP-1; macrophage inflammatory protein-2, MIP-2; tumor necrosis factor-α, TNF-α; interleukin-1β, IL-1β). Primary rat monocytes (10^5 cells/ml) were cultured under static or flow conditions in the presence or absence of LPS, a brain capillary endothelial cell (BCEC)-monolayer or cortical brain slices, lipopolysaccharide (LPS) was added to the cultured cells to stimulate an inflammatory response. As controls, BCEC and slices were also cultivated under static or flow conditions in the presence or absence of LPS. Values = mean ± SEM, pg/ml × 24 h × 10^5 cells; pM static (n = 22), pM + LPS static (n = 22), pM + LPS perfusion (n = 8), pM + slice (n = 10), pM + BCEC (n = 9), slice perfusion (n = 7), and for all others (n = 6). Statistical analysis was performed using one-way ANOVA or Independent T-test with Fisher’s LSD posthoc test (*p < 0.05; **p < 0.01, ***p < 0.001). Detection limits: MCP-1: 3.13 pg/ml, MIP-2: 0.78 pg/ml, TNF-α: 6.3 pg/ml, IL-1β: 12.5 pg/ml.
levels of TNF-α and IL-6 during the first week of incubation (Bennett et al., 1992). Interestingly, we observed that cells treated with LPS show significantly reduced levels of MCP-1 and MIP-2 secretion. IL-1β secretion is slightly, but not significantly elevated. This could be due to high variability between different ELISAs and the relatively low detection limit of this marker. Previous reports have demonstrated that LPS treatment stimulates cytokine production in monocytes (Gessani et al., 1993; Bennahmed et al., 1997). It is possible that differences in isolation procedure, incubation period, and/or culture conditions lead to these contradictory results. However, it is also possible that incubating monocytes in polypropylene tubes leads to an altered response toward LPS.

4.3. Monocytes in perfusion

In this study, we were interested in generating a more in vivo-like culturing method that applies shear stress and simulates constant blood flow. We demonstrate that perfusion causes cells to undergo a change in their size and morphology. Cells under constant flow become significantly elongated and larger compared to their freshly isolated counterparts. In addition, we show that these culturing conditions have no significant impact on cell death. In order to evaluate the function and inflammatory profile of these cells we also measured cytokine levels present in the circulating medium. We found that under perfusion conditions, monocytes show significantly reduced levels of MCP-1, MIP-2, and TNF-α. These findings suggest that culturing monocytes under constant flow leads to less cell activation compared to those incubated under static conditions. Furthermore, monocytes demonstrate a slight increase in cytokine production in response to LPS. This data indicates that the cells are capable of generating a proper cellular response to LPS and suggests that perfusion is a more physiological culturing method compared to static conditions. Taken together, these findings indicate that the in vitro perfusion system serves as a useful culturing method in studying the phenotype and function of circulating monocytes.

4.4. Monocytes and inflammation

Monocytes play an important role during the immune response (Ziegler-Heitbrock, 2000; Tacke and Randolph, 2006). Upon pathological stimulation (including neurodegeneration or inflammation in the brain), monocytes are recruited from the blood flow to adhere to the BBB (Ley et al., 2007; D’Mello et al., 2009). Following activation from chemotactic stimuli (e.g. MCP-1 (CCL-2) or MIP-2 (CXCL2)), monocytes transmigrate across the BBB to the lesion sites where they exert their effector functions (Tanimoto et al., 2007; Khandelwal et al., 2011). They can produce TNF-α and IL-1β as important immune mediators in response to inflammatory stimuli (Conroy et al., 2009). In this study, we show that primary rat monocytes are capable of secreting these inflammatory cytokines and chemokines and describe a culturing system that incorporates elements involved in the trafficking of monocytes into the inflamed or diseased brain.

4.5. Monocytes and the blood–brain barrier (BBB)

Previous studies using rat brain endothelial cells under stationary culture conditions detect basal levels of MCP-1 at 2000–2700 pg/ml and 5500 pg/ml upon TNF-α stimulation (Harkness et al., 2003). In our model, using a simple artificial rat BCEC monolayer incubated under perfusion conditions, we measured MCP-1 release at approx. 1800 pg/ml. We are the first to report constitutive levels of MIP-2 in rat BCEC. Interestingly, our results indicate that the presence of circulating monocytes has a more significant effect on cytokine release than LPS administration. However, further experiments are needed in order to understand the mechanisms by which these cells lead to enhanced cytokine release. It is important to note that our in vitro BBB does not contain astrocytes. This may explain the limited secretion of the proinflammatory cytokines MIP-2, TNF-α, and IL-1β as well as the absent response from LPS treatment. In previous investigations, we have shown that primary rat monocytes can adhere and
transmigrate across this in vitro BBB and subsequently display microglia/macrophage-like phenotypes (Moser and Humpel, 2007; Böttger et al., 2010). In the present study we show that monocytes can adhere to an in vitro BBB when cultured under simulated blood flow. However, this adhesion is very limited (approximately 11 cells/113 mm²) compared to the adhesion we have previously observed under static conditions (1200 cells/113 mm²; Moser and Humpel, 2007). Others have previously demonstrated that following 6 h incubation 200–300 monocyte-derived macrophages adhere/migrate through an artificial BBB (Chung et al., 2002). These results could differ from our findings due to differences in incubation time, the mechanical forces that constant flow puts on cells, cell type, and/or lack of astrocytes and MCP-1 (as chemotactic stimuli).

In this study we also show that monocytes can interact with organotypic brain slices (approx. 163 cells/113 mm²). This has also been confirmed by others that have shown monocyte-derived macrophages migrate into hippocampal brain slices to a depth of approximately 40 μm after 24 h and approximately 80 μm after 72 h (Chung et al., 2002). Here, we also report changes in cell morphology under perfusion. Further studies are needed to characterize the differences between these phenotypes and their meaning on a physiological and functional level. Future studies involving this model could also evaluate the differences in monocyte transmigration into diseased vs. non-diseased brain slices.

4.6. Monocytes and anti-inflammatory drugs

We used our in vitro perfusion model in order to screen anti-inflammatory drugs that may counteract the observed elevation in proinflammatory cytokines in organotypic brain slices incubated with circulating monocytes. Inflammation has been implicated in the pathogenesis of a number of neurodegenerative diseases and epidemiological studies indicate that the use of non-steroidal anti-inflammatory drugs (NSAID) may decrease their risk (Akiyama et al., 2000; Glass et al., 2010; Khandelwal et al., 2011). In one study, ibuprofen treatment reduced Aβ accumulation, proinflammatory cytokine production, and microglia activation in AD transgenic mice (Wyss-Coray and Mucke, 2002). Minocycline is a broad-spectrum tetracycline antibiotic, but is also known for its neuroprotective and anti-inflammatory properties in neurodegenerative diseases (Blum et al., 2004; Malm et al., 2008; Luzi et al., 2009). It has been shown to inhibit macrophage/microglia activation, inflammation, and apoptosis by downregulation of pro-inflammatory cytokine secretion (Zhang et al., 2009). Our results indicate that these anti-inflammatory drugs alone cannot counteract proinflammatory cytokine production seen in cortical brain slices in the presence of circulating monocytes. However, it could be possible that the drug dose, incubation time, or inflammatory stimuli (i.e. lack of a diseased brain) were not optimal for observing an anti-inflammatory response. It may also be possible that a more optimal anti-inflammatory therapy requires a combination of drugs rather than a single pharmaceutical treatment.

4.7. Limitations of this perfusion model

Although this perfusion model allows for the investigation of circulating monocytes, simulating blood flow and shear stress, it also presents several limitations. First, as with any other plastic culture system, the monocytes adhere to the plastic and glass components in our perfusion system. However, it is likely that the adhered monocytes represent differentiated macrophages and that these cells (although adhered) still undergo shear stress and constant flow. We also observed that the majority of the adhered cells were trypsin insensitive (data not shown). Unfortunately, these adhered cells cannot be studied in the system due to the inaccessibility of tubes and glass vessels for immunohistochemical techniques. In addition, we cannot completely exclude that mediators are sticking to tubes and glass. However, when we evaluated the levels of MCP-1, MIP-2 and TNF-α during perfusion we only found a very slight decrease in these cytokine levels after 24 h (data not shown). On the other hand, we cannot exclude sticking of other non-tested mediators, such as LPS. Furthermore, due to the decreased number of circulating cells following perfusion for 24 h, it is not possible to perform a detailed cellular analysis (e.g. by FACS, Western Blot etc.) on these cells. Finally, we cultured monocytes under serum-free conditions to exclude any exogenous influences of serum-derived components. However, in future studies, one may think of using serum or monocyte-specific cytokines, to enhance survival or differentiation. In order to overcome most of these mentioned problems, it may prove useful to decrease the perfusion time of circulating monocytes to 1–3 h. In addition, pre-treatment of tubes/glasses with serum or blocking substrates may also help reduce cell adhesion.

Taken together, this novel perfusion model may allow for the improved study of monocytes under shear stress and simulated blood flow as well as further exploration of their transmigration and adhesion to an in vitro BBB or organotypic brain slices. Our model also provides a unique system for screening the use of potential pharmaceutical agents on the effects of cell trafficking into the brain. Since the role of monocytes and monocyte migration in neurological disorders is still controversial, this model could provide answers to this ongoing debate and ultimately help in finding new ways of interfering with inflammation of diseased brains.

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