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Immune challenge by intraperitoneal administration of lipopolysaccharide directs gene expression in distinct blood–brain barrier cells toward enhanced prostaglandin E_2 signaling

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ABSTRACT

The cells constituting the blood–brain barrier are critical for the transduction of peripheral immune signals to the brain, but hitherto no comprehensive analysis of the signaling events that occur in these cells in response to a peripheral inflammatory stimulus has been performed. Here, we examined the inflammatory transcriptome in blood–brain barrier cells, including endothelial cells, pericytes, and perivascular macrophages, which were isolated by fluorescent-activated cell sorting, from non-immune-challenged mice and from mice stimulated by bacterial wall lipopolysaccharide. We show that endothelial cells and perivascular macrophages display distinct transcription profiles for inflammatory signaling and respond in distinct and often opposing ways to the immune stimulus. Thus, endothelial cells show induced PGE_2 synthesis and transport with attenuation of PGE_2 catabolism, increased expression of cytokine receptors and down-stream signaling molecules, and downregulation of adhesion molecules. In contrast, perivascular macrophages show downregulation of the synthesis of prostanoids other than PGE_2 and of prostaglandin catabolism, but upregulation of interleukin-6 synthesis. Pericytes were largely unresponsive to the immune stimulation, with the exception of downregulation of proteins involved in pericyte–endothelial cell communication. While the endothelial cells account for most of the immune-induced gene expression changes in the blood–brain barrier, the response of the endothelial cells occurs in a concerted manner with that of the perivascular cells to elevate intracerebral levels of PGE_2, hence emphasizing the critical role of PGE_2 in immune-induced signal transduction across the blood–brain barrier.

1. Introduction

The cells comprising the walls of the blood vessels in the brain parenchyma not only serve as a barrier that hinders cells and macromolecules from entering the brain (i.e., the blood–brain barrier), but also play a critical role in the transduction of inflammatory signals between the blood and brain. Thus, pro-inflammatory cytokines, which cannot pass through the blood–brain barrier, can induce a cascade of signaling events in the brain vessels, leading to the release of other inflammatory messengers into the brain, which in turn elicits various disease responses (Ek et al., 2001; Engblom et al., 2002b; Saper et al., 2012). Previous studies have provided important information about this signaling pathway through histochemical staining of mRNA or protein expression in brain sections in models of peripheral inflammation. For example, such studies have shown immune-induced expression of prostaglandin (PG) E_2 synthesizing enzymes in brain vascular cells (Ek et al., 2001; Engström et al., 2012; Rummel et al., 2005; Yamagata et al., 2001) and of the receptors and some associated down-stream signaling molecules for the pro-inflammatory cytokines interleukin (IL)-1 and IL-6 (LeBel et al., 2000; Matsuwaki et al., 2014; Rummel et al., 2005; Vallieres and Rivest, 1997). Furthermore, genetic deletion of interleukin (IL)-1 or IL-6 receptors or blockage of cyclooxygenase-2 induction specifically in brain endothelial cells have been shown to abolish or attenuate the febrile response to peripheral immune challenge (Ching et al., 2007; Eskilsson et al., 2014a,b; Ridder et al., 2011; Wilhelms et al., 2014), hence demonstrating the critical role of these cells in centrally elicited disease symptoms. However, no comprehensive

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analysis of how the blood–brain barrier cells respond to a peripheral immune stimulus has been performed, and in particular, knowledge of the response properties of the different cell types in the blood–brain barrier are lacking.

In this study, using a mouse model of peripheral inflammation, we addressed this issue by extensive gene expression analysis of dissociated blood–brain barrier cells that were simultaneously isolated by multi-color fluorescent activated cell sorting (FACS). Our data show that each of the examined cell types, i.e., endothelial cells (ECs), perivascular macrophages (PVCs), and pericytes, display distinct transcriptional profiles for inflammatory signaling and respond in distinct, but coherent ways to promote a central inflammatory response to the peripheral immune signal.

2. Materials and methods

2.1. Animals

Twelve adult male C57Bl/6 mice (9–12 weeks of age; Scanbur, Sollentuna, Sweden) were used. They were housed in a specific pathogen-free environment at 1–4 mice per cage on a 12-h light/dark cycle (lights on at 0700 h). Early during the light phase, the mice were injected intraperitoneally with 120 μg/kg LPS (Sigma, St. Louis, MO, USA; 0111:B4) in 100 μL saline ($n = 6$) or with saline only ($n = 6$), and killed 3 h later by asphyxiation with CO_2.

This dose of LPS evokes a centrally elicited sickness syndrome, with robust fever, pronounced anorexia (as observed during the dark, active period), and strong hypothalamic–pituitary–adrenal (HPA) axis activation, and the 3 h-time point coincides with the peak of these symptoms (Elander et al., 2007, 2009; Engblom et al., 2003; Nilsberth et al., 2009; Oka et al., 2003). The chosen LPS dose has also been shown in microarray studies to induce strong expression of a large number of genes in the hypothalamus at 3 h or adjacent time points (Hamzic et al., 2013; Vasilache et al., 2013). The experimental procedures were approved by the Animal Care and Use Committee at Linköping University and followed international guidelines.

2.2. Preparation of single-cell suspensions from brain tissue

Forebrains, including the leptomeninges, choroid plexus, and circumventricular organs (organum vasculosum of the lamina terminalis, median eminence and the subfornical organ), were dissected and collected into ice-cold phosphate-buffered saline (PBS) containing 10% fetal bovine serum (FBS; PAA Laboratories, Pasching, Austria). Tissues were cut into small pieces and treated with 0.1% collagenase II (Worthington Biochemical, Lakewood, NJ, USA) in 10% FBS for 30 min and then minced in a 70-μm cell strainer (BD Biosciences, Franklin Lakes, NJ, USA) using a 5-mL syringe and an 18-G needle. Remaining tissue pieces were treated with 0.05% trypsin–EDTA (Gibco; Life Technologies, Carlsbad, CA, USA) for 10 min at 37 °C, after which ice-cold FBS was added to a final concentration of 20%. Tissues were then filtered again using 70-μm cell strainers. The cells were centrifuged at 300×*g* for 5–10 min and then resuspended in PBS containing 10% FBS and 0.5 μg/mL propidium iodide (PI) for FACS.

2.3. FACS isolation and analysis of ECs, PVCs, and pericytes

Live cells (propidium iodide (PI) negative) from each sample were first sorted in PBS with 10% FBS using the yield-sorting mode on a FACS Aria III or II Sorp (BD) instrument, as described in detail elsewhere (Engström et al., 2012). The sorted cells were resuspended in PBS with 10% FBS and incubated with rat anti-mouse Fc Receptor Block (CD32/16; clone 93; eBioscience, San Diego, CA, USA) for 10 min on ice in order to block unspecific binding. Cells were then incubated with rat anti-mouse CD45-PECY5 (clone 30-F11; eBioscience), CD31-PECY7 (clone 390; eBioscience), CD206-Alexa 647 (clone 5R3MD; AbD Serotec, Kidlington, UK), and PDGFRb/CD140b-PE (BioLegend, San Diego, CA, USA) for 15–20 min on ice. The cells were washed and resuspended in PBS with 10% FBS. Dead cells were again excluded by PI staining. $CD45^-CD31^+$, $CD45^-CD31^-PDGFRb^+$, and $CD45^+CD206^+$ cells were gated based on fluorescent minus one (FMO) controls (Figs. 1 and 2). The whole sorting procedure was performed under cooling system (4 °C) in both sample chamber and collection tube holder. After purity analysis, the cell populations were sorted directly into 350 μL of RLT buffer (Qiagen, Hilden, Germany) containing 143 mM β-mercaptoethanol (Sigma–Aldrich) and stored at −80 °C until further use. The numbers of sorted cells per mouse were similar between LPS-treated animals and control animals, with ranges of 2000–14,000 for ECs (mean = 5636 cells/mouse), 1294–2880 for PVCs (mean = 2072 cells/mouse), and 273–1570 for pericytes (mean = 765 cells/mouse).

2.4. Analysis of gene expression in ECs, PVCs, and pericytes using quantitative reverse transcription polymerase chain reaction

Total RNA was extracted using an RNeasy Micro Kit (Qiagen), and reverse transcription was performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). The cDNA was first amplified for 14 cycles using a Preamplification Master Mix (Applied Biosystems) and pooled 0.2× assays (Custom TaqMan PreAmp pool, Applied Biosystems). Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was then performed using a Gene Expression Master Mix (Applied Biosystems), with samples run in duplicates, on a 48-format TaqMan Low Density array (TLDA, Applied Biosystems). The analyzed genes with the corresponding inventoried TaqMan assays used on the TLDAs are shown in Table 1. The genes of interest were grouped as control genes, cell-type specific genes, cytokines/chemokines and lipocalin-2 with their corresponding receptors, intracellular cytokine signaling molecules, prostaglandin synthases, prostaglandin and lipocalin transporters, and peroxisome proliferator activated receptors. Data were analyzed using the ΔΔCq method (Cq, quantification cycle, according to the MIQE guidelines), with *Gapdh* as the reference gene. Each gene was normalized against the reference gene (ΔCq) both in the stimulated and control groups as $Cq_{\text{target gene}} - Cq_{\text{reference gene}}$. The relative expression values for each gene in a cell sample were calculated as $2^{-\Delta Cq}$. The gene expression changes between the stimulated and control cell samples were calculated as FC values: $2^{-\Delta\Delta Cq}$, with ΔΔCq being the difference between the $\Delta Cq_{\text{stimulated}}$ and the $\Delta Cq_{\text{control}}$. The cDNA loading concentrations were controlled by using similar numbers of cells in each sample (2.2 ± 0.5 cells/μL in the final cDNA volume) to yield a $Cq_{\text{reference gene}}$ in the range of 18–26 cycles in the final RT-qPCR. Cqs for the target gene were considered acceptable if under 34 cycles.

2.5. Statistics

Statistical significance of the' RT-qPCR data was determined using the *t*-distribution. The standard error of the mean (SEM) for fold differences in the RT-qPCR data was obtained by first calculating the SD for each of the two groups that were compared (s_1 and s_2 with $[n_1 - 1]$ and $[n_2 - 1]$ degrees of freedom) and then applying these values in the following formula: $[(s_p^2 \times (1/n_1 + 1/n_2)]^{0.5}$ in which s_p is $s_p^2 = [s_1^2 \times (n_1 - 1) + s_2^2 \times (n_2 - 1)]/(n_1 + n_2 - 2)$.

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