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RAPID COMMUNICATIONS

HCAR1-Mediated L-Lactate Signaling Suppresses Microglial Phagocytosis

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Abstract

Microglia, the primary brain-resident immune cells, protect the brain from various harmful pathogens, insulting and maintaining its homeostasis by phagocytosing extracellular particles. How microglia are metabolically regulated by their microenvironment remains largely elusive. Here, we investigated how extracellular lactate, which is abundant in the brain and dynamically changes in pathological states, affects microglial phagocytotic ability. We show that L-lactate reduces microglia phagocytic capacity in a Hydroxycarboxylic Acid Receptor 1 but not Monocarboxylate transporter 1-dependent manner. Our findings point to a potential role for extracellular lactate in suppressing the phagocytic activity of microglial cells in homeostasis and inflammatory conditions.

Keywords Lactate · HCAR1 · Phagocytosis · Amyloid-beta · Microglia

Introduction

Microglial cells help maintain tissue homeostasis mostly via surveillance of their microenvironment. Upon receptor-mediated activation by pathogen- or tissue damage-derived cues, they engage in active phagocytosis (Bar & Barak, 2019; Li & Barres, 2018), which can be dysregulated under chronic inflammatory states, including neurodegeneration, leading to microglia hyperactivated state with detrimental effects on the brain (Fu et al., 2014; Hickman et al., 2018). During activation, microglial cells dynamically adapt their metabolic flux to meet requirements dictated by their phenotypic and functional states. Specifically, homeostatic microglia mostly rely on oxidative phosphorylation as an ATP source, while pro-inflammatory activated microglia shift their metabolism towards glycolysis and increased lactate production (Baik et al., 2019).

Lactate is a pleiotropic molecule that serves as a substrate for oxidizing pyruvate, gluconeogenic precursor, signaling molecule, or epigenetic regulator (Monsorno et al., 2022). In the brain, lactate is shuttled between different cell types: from astrocytes to neurons to sustain neuronal activity, and from endothelial cells to newly formed neurons during neurogenesis (Nicola & Okun, 2021). Lactate can also affect myelin synthesis in oligodendrocytes and vascular endothelial growth factor (VEGF) production in pial fibroblast-like cells (Nicola & Okun, 2021). Microglial cells can utilize extracellular lactate or produce it internally in response to inflammatory signals and increased glycolysis rate, as lactate stimulates basal respiration of microglia in vitro (Nagy et al., 2018). Moreover, MCTs (MCT1, 2, and 4) are expressed on microglia alongside lactate dehydrogenase B (LDHB), which preferentially converts lactate into pyruvate. These expression patterns indicate that lactate can be imported and metabolized inside microglia (Monsorno et al., 2022). In the present work, we addressed whether increasing extracellular lactate levels affect the phagocytotic abilities of microglia under homeostasis and in an activated pro-inflammatory state as well as the underlying mechanism.

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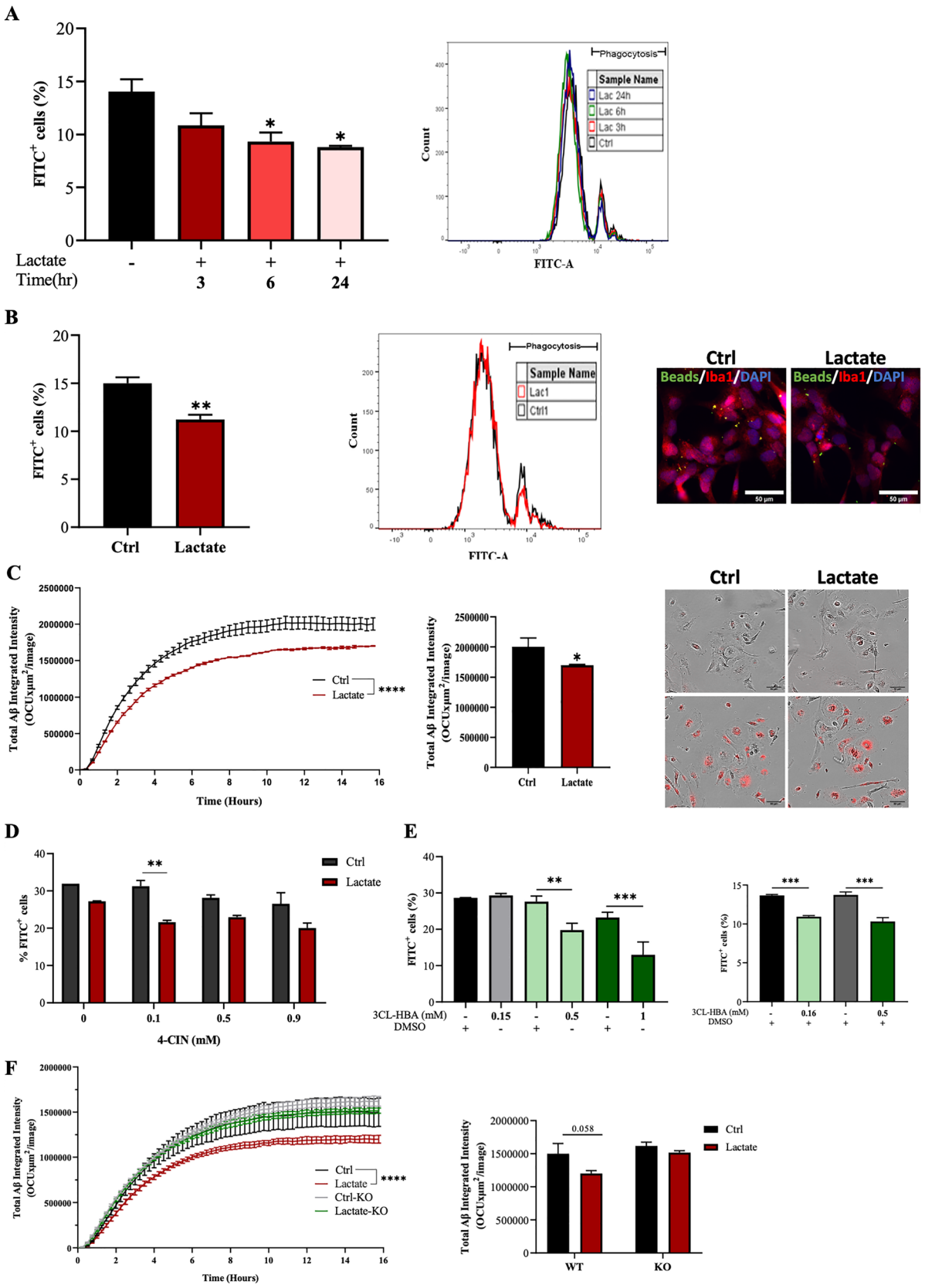
A
FITC+ cells (%)
Lactate
Time(hr)
Phagocytosis
Sample Name
Lac 24h
Lac 6h
Lac 3h
Ctrl
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FITC-A
B
Ctrl
Lactate
Lac1
Ctrl1
Beads/Iba1/DAPI
C
Total Aβ Integrated Intensity (OCU×μm²/image)
Time (Hours)
Ctrl
Lactate
D
% FITC+ cells
4-CIN (mM)
E
3CL-HBA (mM)
DMSO
F
Ctrl-KO
Lactate-KO
WT
KO
0.058

◂**Fig. 1** Lactate reduces phagocytosis in microglia through HCAR1 signaling. L-lactate significantly reduces phagocytosis of fluorescent beads by N9 cell line after 6 h and 24 h (**A**) and after 6 h in HMC3 (**B**) as quantified (Left panel) via flow cytometry and shown in the representative histogram and immunofluorescence images (Right panel). Cells were deprived of FBS and incubated with 15 mM of L-lactate (867-56-1, Sigma) for 3, 6, 24 h (**A**), or 6 h (**B**). L-lactate reduces phagocytosis of pHrodo Aβ in mouse primary microglia, as measured in live-cell imaging over 16 h (left panel); comparison between lactate and control at the last timepoint (middle panel), and representative images from live-cell imaging (right panel). Primary microglia were treated with 15 mM L-Lactate for 5 h, then 1 µM of pHrodo Red-labeled Aβ was added. pHrodo is an amine-reactive pH-sensitive dye that has a low fluorescence intensity at neutral pH and increases its fluorescence intensity upon acidification in the lysosome. $A\beta_{42}$ peptide (ab120301, Abcam, Cambridge, UK) was labeled with the pHrodo Red SE (P366o, ThermoFisher) using a protocol adapted from Fujifilm/Cellular Dynamics Labeling (**C**). Different concentrations of 4-CIN (C2020, Sigma) did not block the lactate effect on phagocytosis in N9 as measured via flow cytometry (**D**). 3CL-HBA (53984-36-4, Cayman) reduced phagocytosis of fluorescent latex beads in N9 as measured via flow cytometry (left panel) at a concentration of 0.5 mM and 1 mM, and in HMC3 (right panel) at a concentration of 0.16 mM and 0.5 mM. Control cells were treated with the same percentage of DMSO as in the 3CL-HBA treatment (**E**). L-lactate reduces Phagocytosis of pHrodo Aβ in WT mouse primary microglia, but not in HCAR1 knockout primary microglia, as measured in live-cell imaging over 16 h (left panel); comparison between lactate and control in the WT and knockout at the last timepoint (right panel) (**F**). Experiments were performed at least two times. Data presented as mean ± SEM. Asterisks mark significance between the control group and the group represented by that bar. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$

Results

Extracellular Lactate Reduces Phagocytosis in Human and Mouse Microglial Cell Lines and Primary Mouse Microglia

To assess the effect of increasing extracellular lactate levels on microglial phagocytosis, we treated N9 cells with L-lactate for 3, 6, or 24 h and added fluorescent latex beads at the last hour of incubation. We noticed a gradual reduction in the number of cells that phagocytosed at least one bead, at 6 h (33%, $p < 0.05$) and 24 h (37%, $p < 0.05$) (Fig. 1A). A 25% reduction in phagocytosis was induced by L-lactate in the human microglial cell line HMC3 ($p < 0.01$) after 6 h of treatment (Fig. 1B). To determine whether L-lactate negatively affects phagocytosis of Alzheimer's disease-related Amyloid-β (Aβ), we pre-treated primary mouse microglia with L-lactate for 5 h, followed by treatment with Aβ prelabeled with pHrodo (see Methods) for 16 h. Using a live-cell imaging and analysis platform (IncuCyte), we found a significant reduction in internalized Aβ ($p < 0.0001$) as early as after 2 h of incubation, which lasted until the end of the incubation time ($p < 0.05$, Fig. 1C).

Blocking Lactate Transportation Through MCTs Does Not Affect Phagocytosis

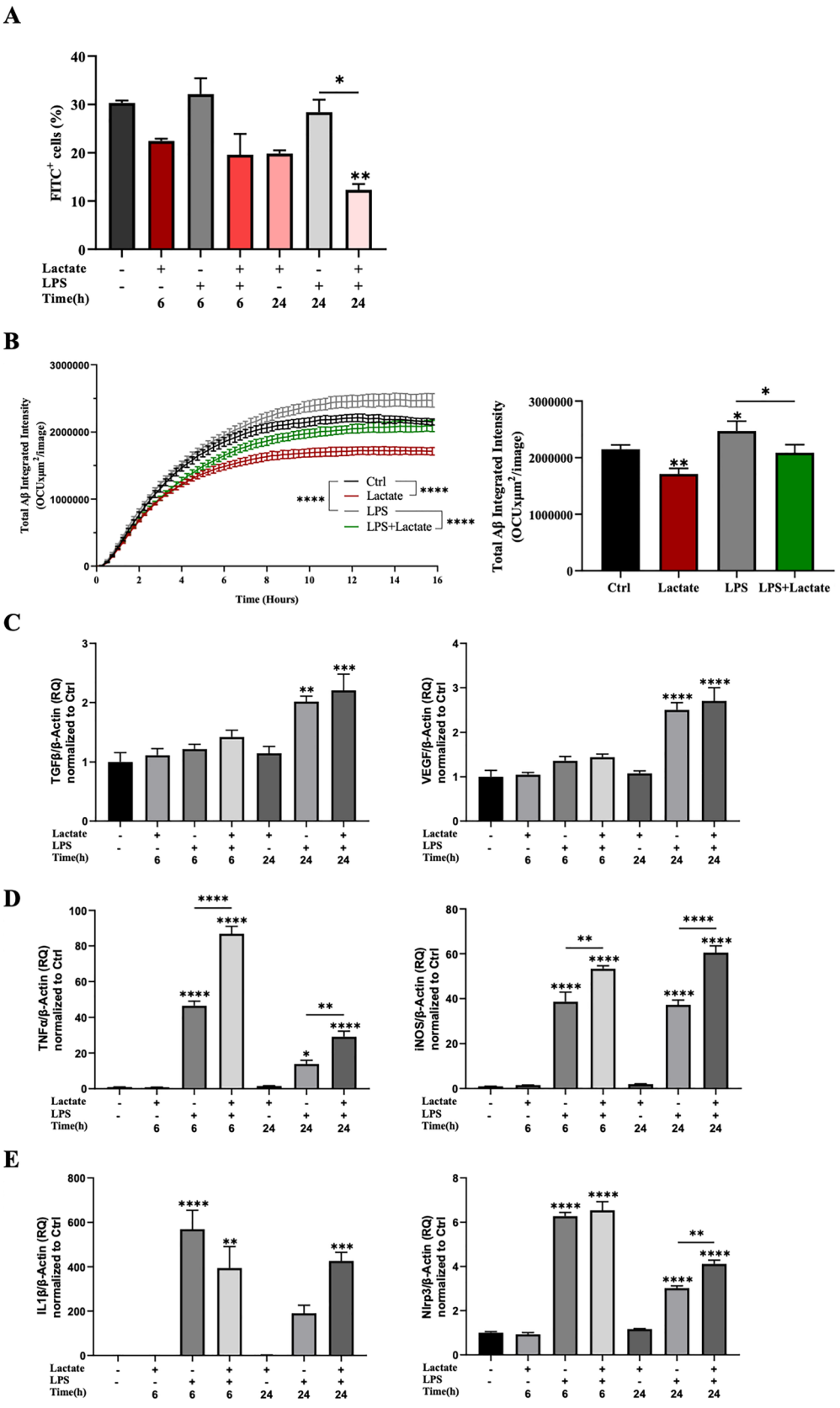
To test whether lactate entry via monocarboxylate transporters (MCTs) mediated the effects of L-lactate on phagocytosis, we applied the competitive α-cyano-4 hydroxycinnamate (4-CIN), MCT inhibitor, at different concentrations, prior to the lactate treatment in N9 cells (Matsui et al., 2017). Blocking lactate entry to the cells did not abolish the effect on phagocytosis, as a consistent reduction in phagocytosis due to lactate treatment was found in all tested 4-CIN concentrations (Fig. 1D).

HCAR1 Mediates Lactate Effect on Phagocytosis

Lactate is a natural ligand of Hydroxycarboxylic Acid Receptor 1 (HCAR1) and can activate it in its physiological concentration range of 1–20 mM (Liu et al., 2009). In both N9 and HMC3 cells, we found that treating the cells with the HCAR1 agonist 3-chloro-5-hydroxybenzoic acid (3CL-HBA) for 6 h reduced the phagocytosis of fluorescent beads. While in HMC3, a reduction of 20% occurs at a low concentration of 0.16 mM ($p < 0.001$), in N9, a higher concentration (0.5 mM, $p < 0.01$) is needed to lead to a reduction of 28% (Fig. 1E). Similarly, while in primary microglia from WT, lactate reduced the phagocytosis of pHrodo Aβ ($p < 0.0001$), it did not affect the phagocytosis in microglia derived from HCAR1 knockout mice (Fig. 1F).

Extracellular Lactate Represses Phagocytosis Under Inflammatory Conditions

To test whether extracellular L-lactate reduced phagocytosis also under inflammatory conditions, we stimulated microglia with lipopolysaccharides (LPS) in combination with L-lactate. We found that phagocytosis of fluorescent beads was further downregulated in cells co-treated with L-lactate and LPS. In N9 cells, we found that the combination of L-lactate and LPS for 6 h reduced phagocytosis by 40%, while L-lactate alone reduced phagocytosis by 25%. Moreover, prolonged treatment of L-lactate and LPS for 24 h further downregulated phagocytosis by 56% ($p < 0.01$) (Fig. 2A). We next pre-treated primary microglia with LPS for 16 h and then replaced it with L-lactate for 5 h. Following this, pHrodo-Aβ was added for 16 h to measure phagocytosis using IncuCyte. As previously shown, lactate decreased phagocytosis, whereas LPS slightly increased phagocytosis ($p < 0.0001$). The combined treatment, however, lowered phagocytosis in comparison to LPS ($p < 0.0001$) but not to the untreated group (Fig. 2B).


A
FITC+ cells (%)
Lactate
LPS
Time(h)
B
Total Aβ Integrated Intensity (OCUxμm2/image)
Time (Hours)
Ctrl
Lactate
LPS
LPS+Lactate
C
TGFβ/β-Actin (RQ) normalized to Ctrl
VEGF/β-Actin (RQ) normalized to Ctrl
D
TNFα/β-Actin (RQ) normalized to Ctrl
iNOS/β-Actin (RQ) normalized to Ctrl
E
IL1β/β-Actin (RQ) normalized to Ctrl
Nlrp3/β-Actin (RQ) normalized to Ctrl

◂**Fig. 2** Lactate represses phagocytosis under inflammatory conditions and increases pro-inflammatory M1 state. L-lactate reduces phagocytosis of fluorescent latex beads in N9, and its combination with LPS for 24 h further reduces the phagocytosis, as measured via flow cytometry (**A**). L-lactate reduces phagocytosis of pHrodo Aβ in mouse primary microglia. Pre-activation with 1 mg/ml LPS for 16 h increased phagocytosis, while lactate diminished the effect, as quantified using live-cell imaging over 16 h (left panel); comparison between different treatments at the last timepoint (right panel) (**B**). RT-PCR analysis of M2 markers: TGFβ (left panel) and VEGF (right panel) (**C**). RT-PCR analysis of M1 markers: TNFα (left panel) and iNOS (right panel) (**D**). RT-PCR analysis of Il-1β (left panel), Nlrp3 (right panel) (**E**). Data presented as mean ± SEM. Asterisks mark significance between the control group and the group represented by that bar. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. RT-PCR

Extracellular Lactate Induces a Dysfunctional Hyperactivated M1 State

As L-lactate treatment resulted in decreased phagocytosis, we sought to assess whether genes associated with microglial cell polarization could be correlated with this effect. Interestingly, while M2-related genes (e.g., VEGF and TGF-beta) were not affected by lactate co-treatment with LPS (Fig. 2C) in N9 microglia, some key pro-inflammatory genes were significantly upregulated. Namely, the expressions of TNFα ($p < 0.01$) and iNOS ($p < 0.01$) were upregulated after 6 h and 24 h of LPS and lactate co-treatment (Fig. 2D), while the expression of Il-1β was slightly increased after 24 h of co-treatment of LPS with lactate in comparison to LPS ($p < 0.07$, Fig. 2E). Nlrp3 expression was increased after 24 h of co-treatment of LPS with lactate compared to LPS ($p < 0.01$, Fig. 2E).

Discussion

Herein, we provide new evidence to support lactate as a repressor of microglial phagocytosis in both human and mouse microglial cells in an HCAR1-mediated mechanism during both homeostatic and inflammatory conditions. The roles of lactate in inflammation are still not entirely understood. Some studies report an anti-inflammatory effect of lactate with enhanced release of VEGF and ARG1 (Kong et al., 2019), while others reported a pro-inflammatory role of lactate with increased release of TNF-α, IL-6, and Il-1β (Samuvel et al., 2009). These discrepancies may partly stem from the use of different cell types and experimental settings. Our findings implicate lactate as a signaling molecule that signals microglial cells to reduce phagocytosis, potentially as a counter-balancing modality to prevent excessive microglial phagocytosis and synapse pruning. Alternatively, bacterial cells might induce lactate secretion as a modality to impair the phagocytosis capabilities of the host's immune system. These effects can be particularly instated in pathologies characterized by increased immune activity in the brain during chronic inflammation.

Methods

Microglial Cell Cultures

N9 microglial cell line and Human microglia clone 3 cell line (HMC3) were grown in Dulbecco's modified Eagle's medium (DMEM) and Eagle's Minimum Essential Medium (EMEM), respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, and 1% Penicillin–Streptomycin. Primary mouse microglial cells were harvested from 2-month-old C57BL/6 or HCAR1$^{-/-}$ mice (kindly provided by S. Offermanns, Max-Planck-Institute for Heart and Lung Research, Germany) (Ahmed et al., 2010), cultured as previously described (Moussaud & Draheim, 2010).

Fluorescent-Beads Phagocytosis Assay

Fluorescent latex beads (1 µm-L1030, Sigma, St. Louis, MO) were pre-coated with FBS for 1 h at 37 °C and then applied to cells at a dilution of 1:1000 for 1 h at 37 °C. Cells were then washed, trypsinized, and analyzed with BD-LSRFortessa Cell Analyzer (BD bioscience, East Rutherford, NJ).

Phagocytosis Assay with Live Microglia

Plates with microglial cells were placed in an IncuCyte S3 Live-Cell Analysis System (Sartorius), and nine images per well were captured every 15 min for 16 h. The total integrated intensity, defined as the total sum of Aβ fluorescent intensity in the entire image, was analyzed.

Quantitative PCR

Total RNA was extracted from cells using TRIzol (Ambion, Life Technologies, CA). 1ug of extracted RNA was reverse transcribed using RevertAid H minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA). q-PCR reactions were performed using Fast SYBE Green Master MIX (Applied Biosystems, CA) with primers indicated in Table 1, in StepOnePlus instrument (Applied Biosystems, CA). For relative quantification, we used the double delta Ct method.

Statistical Analysis

The data, presented as mean ± SEM, were tested for significance in the unpaired t test for samples of equal variance,

Table 1 Primers used for quantitative PCR

Gene	Forward primer	Reverse primer	Annealing temperature	Product size (bp)
TGFβ	GTGTGGAGCAACATGTGGAACTCTA	CGCTGAATCGAAAGCCCTGTA	56	174
VEGF	TGTACCTCCACCATGCCAAGT	GTCCACCAGGGTCTCAATCG	57	131
TNFα	TGCCTATGTCTCAGCCTCTTC	GAGGCCATTTGGGAACTTCT	54	117
iNOS	CCCTTCAATGGTTGGTACATGG	ACATTGATCTCCGTGACAGCC	60	158
Il1β	CATCCAGCTTCAAATCTCGCAG	CACACACCAGCAGGTTATCATC	57	215
βActin	TTCTTTGCAGCTCCTTCGTT	ATGGAGGGGAATACAGCCC	56	149

one-way ANOVA, two-way ANOVA, and two-sample Kolmogorov–Smirnov test. Post hoc tests were conducted using Tukey or Sidak's corrections. All statistical analyses were carried out using GraphPad Prism Software. Significant results were marked according to conventional critical p values: $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$.

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