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Yuhao Gao April 10, 2023

Streamlined Approach to Identify Novel Immunomodulatory Agents to Mitigate Ischemic Stroke in a Primary Microglia Model

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Abstract

Streamlined Approach to Identify Novel Immunomodulatory Agents to Mitigate Ischemic Stroke in a Primary Microglia Model

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Background

Microglia activation is associated with neuroinflammation, pathogenesis of secondary brain damage, as well as cerebral ischemia reperfusion injury post ischemic stroke. Baricitinib, a selective Janus kinase 1/2 inhibitor with potent anti-inflammation effects in treating rheumatoid arthritis, alopecia areata, and hospitalized COVID-19, may mitigate inflammation induced by microglia polarization, especially to classic pro-inflammatory M1 phenotype. This study aimed to investigate if baricitinib can prevent or reverse neuroinflammation in a primary cell model of ischemic stroke.

Methods

A primary cell model of ischemic stroke was established using differentiated monocyte-derived microglia-like cells (MDMG) from human blood buffy coat. The microglia activation marker expression profile was assessed by flow cytometry analysis. Further sub-gating with Flow Jo was performed to determine the microglia activation changes morphologically. The cytotoxicity of baricitinib and microglia viability inhibition were analyzed by MTS assay.

Results

The data revealed that baricitinib did not confer apparent toxicity in microglia at physiologically relevant concentrations in humans. Baricitinib significantly suppressed LPS-induced upregulation of microglia M1 phenotype-related markers CD40, CD32, CD86, CD16, HLA-DR, and activated state marker CD163 when administered as a post-treatment. Pre-treatment with baricitinib attenuated the expression of microglia activation markers CD40, CD16, CD163, and HLA-DR. Furthermore, the morphological activation was ameliorated by the baricitinib treatment at clinically relevant dosage.

Conclusion

Baricitinib could significantly reduce key markers of neuroinflammation and suppress polarization morphologically in a primary microglia model. Baricitinib can reduce ischemic stroke-like, LPS-induced inflammation in microglia, a proof of principle that baricitinib has therapeutic potential in mitigating ischemia-triggered neuronal inflammation.

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Table of Contents

Tables and Figures

Introduction

Stroke, a cerebrovascular accident, is a leading cause of mortality and disability worldwide. Ischemic stroke, characterized by arterial occlusion and subsequent interruption in cerebral blood flow, leads to brain infarction and accounts for 87% of strokes globally (Kuriakose et al., 2020). Brain ischemic injuries are caused by a cascade of events invoked when the availability of glucose and oxygen decreases due to a local reduction of blood flow. As a result, a series of downstream mechanistic cascades, including calcium overload, reactive oxygen species (ROS) production, mitochondrial dysfunction, apoptosis activation, and inflammation occur minutes after stroke onset (Qin et al., 2022).

Following an acute stroke, the release of damage-associated molecular patterns (DAMPs) from injured or dead cells initiates secondary neuroinflammation, which promotes further injury and apoptosis by facilitating the infiltration of inflammatory cells into the CNS compartment, ROS, and nitric oxidative species (NOS) into the penumbra (Stuckey et al., 2021). Inflammation plays a key role in ischemic stroke pathophysiology and hence represents an attractive target for safe, specific, and targeted therapeutic intervention to mitigate acute and chronic events of stroke (Figure 1 top panel). Further, persistent neuroinflammation responses have also been linked to secondary neurodegenerative process after ischemic stroke, which may lead to detrimental long-term outcomes including speech deficits and post-stroke cognitive impairment (Kliper et al., 2013, as cited in Stuckey et al., 2021).

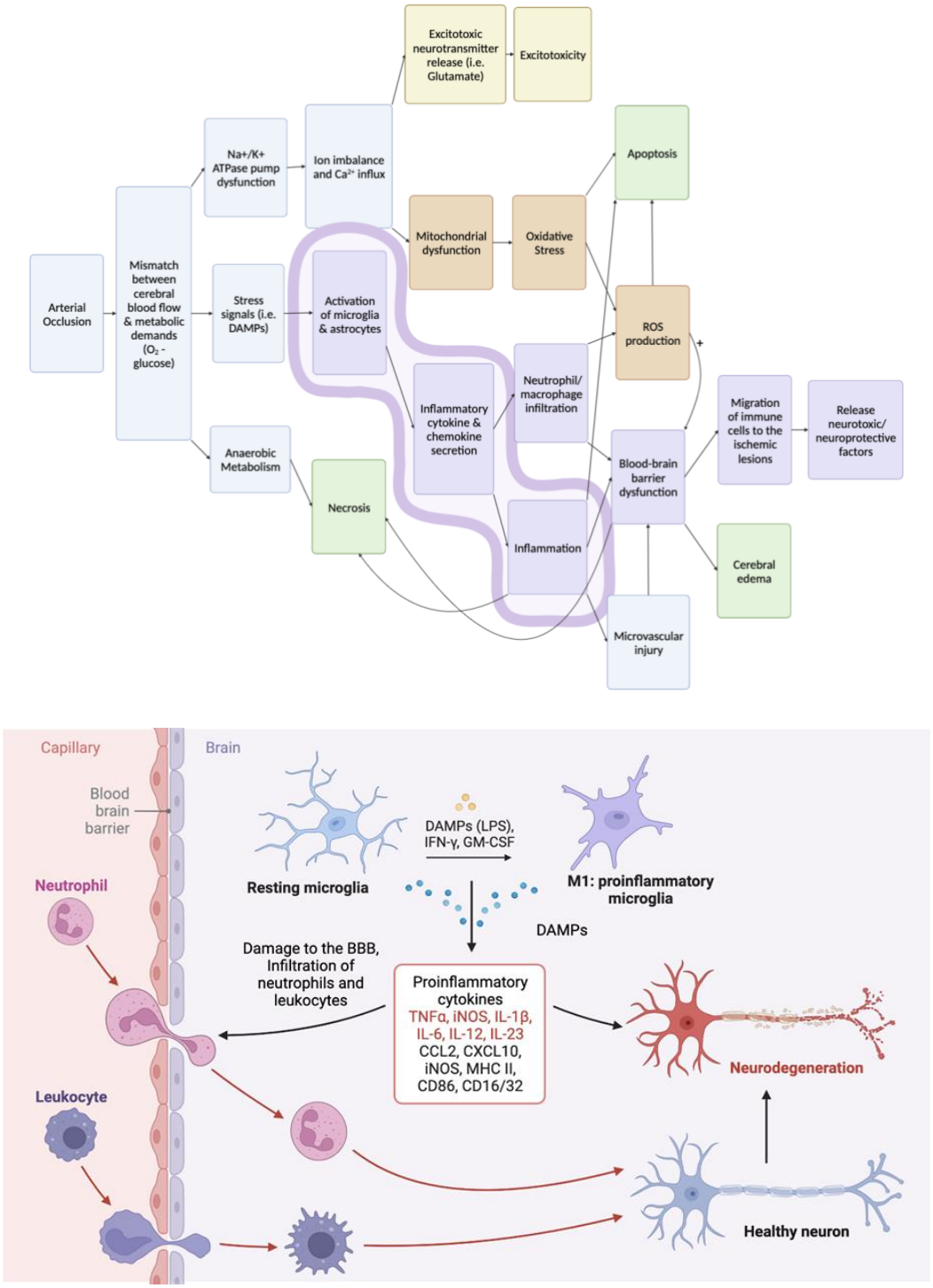


Figure 1. Schematic of proposed pathophysiological mechanism of stroke.

Specific pathway involving microglia-activation-induced neuroinflammation is highlighted with a purple outline (Top). Activated M1 phenotype microglia contribute to release of proinflammatory cytokines, BBB damage and neurodegeneration (Bottom). Adapted from "Activated Astrocytes and Microglia Release Proinflammatory Cytokines and Induce Neurodegeneration", by BioRender.com (2022).

To date, tissue plasminogen activator (tPA) is the only approved system of reperfusion therapy for acute ischemic stroke. For large vessel occlusion, endovascular treatment (EVT) or mechanical thrombectomy can be administered in combination with intravenous thrombolysis to recanalize cerebral blood vessels. Despite their efficacy, the treatments' short therapeutic window, high inclusion criteria, and risk of intracranial hemorrhage limited the scope of treatment delivery and outcomes in unqualified patient populations. Moreover, current revascularization strategies are unable to address the reperfusion injury (I/R) or promote neuronal regeneration (Mosconi et al., 2022). Although prompt revascularization after brain ischemia restores oxygen and glucose supply, the reestablished blood supply induces reperfusion injury which further exacerbates brain damage due to ROS production fueled by the reintroduction of oxygen. Pronounced inflammatory responses are present during I/R, indicating a potential therapeutic avenue with anti-inflammatory immunomodulators as a combined therapy to improve functional outcomes and reduce injury resulting from pharmacological thrombolysis or mechanical thrombectomy (Mizuma et al., 2017). Despite robust research in new therapeutic approaches, pharmacological interventions remain limited, suggesting the need for more research on discovering new neuroprotective compounds for the treatment of ischemic stroke.

Microglia are resident immune cells in the brain. In response to ischemic stroke, microglia migrate toward the ischemic lesion, become activated, and adopt an amoeboid phenotype which confers phagocytic capacity (as shown in Figure 1 bottom panel). Microglia cells that are polarized to M1 phenotype produce pro-inflammatory cytokines such as interleukin-1 (IL-1β) and tumor necrosis factor α (TNF-α), chemokines, and other signaling molecules like matrix metalloproteinases (MMPs) and reactive oxygen species (ROS). These inflammatory mediators consequently lead to the infiltration of neutrophils into the ischemic lesions, migration of

leukocytes from the blood to the brain, the disintegration of the blood-brain barrier (BBB), neuronal cell death, and enlargement of the infarct (Yenari et al., 2010, Qin et al., 2022).

While the acute and excessive inflammation during the acute phase of stroke exacerbates tissue damage, microglia is not exclusively cytotoxic. Microglia can also be neuroprotective and facilitate tissue repair by clearing debris and secreting neurotrophic factors. Additionally, there are multiple activation phenotypes of microglia, including classically activated (M1) and alternatively activated (M2), which release pro-inflammatory cytokines that intensify the neuronal damage, and anti-inflammation cytokines, which promote tissue repair, respectively. Different microglial polarization expression peaks at different points of stroke: typically, the number of M2-type microglia peaks 5 days after ischemic brain injury while M1 expression peaks during later stages of stroke (Jian et al., 2019). Hence, it is important to take into consideration of microglia's dual properties at different stages of stroke progression to develop drugs that not only adjust the M1/M2 polarization balance between beneficial and detrimental microglia responses but also specifically target microglia at the right time point (Pluta et al., 2021).

Baricitinib ($C_{16}H_{17}N_7O_2S$) is a FDA-approved selective Janus Kinases (JAKs) inhibitor (National Center for Biotechnology Information, 2023), across the indications of Rheumatoid Arthritis, Alopecia, and hospitalized COVID-19; the latter indication was invented by the Gavegnano Group. By selective inhibition of JAK1 and JAK2, baricitinib attenuates JAK-modulated pro-inflammatory signaling pathways and immune responses. Multiple studies have reported the marked immunomodulatory properties of baricitinib for the treatment of rheumatoid arthritis (Taylor et al., 2017; Al-Salama&Scott, 2018) and COVID-19 (Stebbing et al., 2020), anti-inflammatory activities in inflammatory disease, and HIV-1 reservoir reducing functions including decrease in HIV-induced CNS inflammation across microglia, macrophages, astrocytes and

neurons in a murine model of HIV-1 in the CNS (Gavegnano et al., 2019). However, it has not yet been determined whether baricitinib also possesses the ability to attenuate neuroinflammation in cerebrovascular diseases. To examine the anti-inflammatory efficacy and safety of baricitinib for inhibiting stroke-induced microglial neuroinflammation, we generated human monocyte-derived microglia (MDMG) and studied their response to lipopolysaccharide (LPS)-induced activation, their expression of proinflammatory activation marker, and their morphological alterations with the addition of various concentrations of baricitinib. LPS, a potent immunogenic agent, is commonly used to trigger neuroinflammation in microglia models *in vitro* (Skrzypczak-Wiercioch & Sałat, 2022). Since elevated LPS levels have been linked to worse stroke outcomes in multiple studies, we chose LPS to simulate stroke-induced inflammatory environment (Hakoupian et al., 2021). We observed that microglia activated by LPS stimulation had lower levels of proinflammatory cellular marker expression and demonstrated a resting microglia-typical morphology characterized by motile, fine processes and small cell soma when treated by baricitinib. Our results provide the first *in vitro* evidence demonstrating baricitinib's anti-inflammatory efficacy in suppressing inflammatory responses evoked by microglia activation following stroke-like stimulation. This study offers valuable insights into drug discovery for baricitinib as a promising candidate for treating ischemic brain injury, particularly in the subacute and reperfusion phase when neuroinflammation is the main driver of pathogenesis, by targeting microglial activation states and suppressing their deleterious pro-inflammatory neurotoxicity.

Methods

Blood Collection and PBMC isolation

Buffy Coat blood product (Lifesouth, Dunwoody, GA) was obtained from healthy human donors. Each sample was processed within 24 hr of the collection time to further isolate peripheral blood

mononuclear cells (PBMCs) from each donor using SepMate tubes with Lymphoprep (STEMCELL Technologies) according to the manufacturer's instructions for density gradient isolation and cell isolation. In preparation for PBMC isolation, SepMate tubes were pre-filled with 15 mL of Accu-Prep Lymphoprep in the bottom chamber and centrifuged at 1800 rpm for 5 minutes at 21°C to ensure no bubbles form below the filter. Approximately 25-30 mL of buffy coats were transferred to sterile 50 mL conical tubes before ~1:1 diluted by PBS-EDTA (1 mM) to 50 mL. Twenty-five mL of diluted blood was then decanted on top of the Lymphoprep layer in the SepMate tubes. The tubes were centrifuged at 2500 rpm for 15 minutes at 21°C with no brake. After centrifugation, the white ring (buffy coat layer) of PBMCs was carefully collected using a sterile transfer pipette into a 50 mL conical tube. Each tube containing the supernatant was filled to 40 mL with PBS-EDTA (1 mM) and then centrifuged at 300 x g for 10 minutes at 21°C with no brake. The supernatant was aspirated, and the cell pellet was resuspended in 40 mL of PBS-EDTA (1 mM). Following the wash step, the cell pellets were pooled in 20 mL PBS-EDTA (1 mM). Cell viability and density were determined using Countess (Thermo Fisher) with trypan blue exclusion dye (ThermoFisher) after the cell suspension was diluted 1:100.

Monocyte Isolation and Monocyte-Derived Microglia-like cells differentiation

Reagents: CD14 Microbeads - human (Miltenyi Biotec Cat # 130-050-201), Bovine Serum Albumin Stock Solution (BSA; Miltenyi, Cat# 130-091-376), AutoMacs Running Buffer Solution (500 mL 1X PBS plus 25 mL BSA and 2 mL 0.5 M EDTA), Macrophage Media (RPMI (500 mL), neonatal calf serum (neonatal calf serum, 50 mL), Penicillin-streptomycin (P/S, 10 mL)), MDMG Media (Macrophage media, 0.3% GM-CSF stock solution, 0.005% IL-34 stock solution, 0.01% FGF-2 stock solution, 0.025% dorsomorphin stock solution).

$CD14^+$ monocyte positive selection was performed according to manufacturer's protocol (Miltenyi Biotec Cat# 130-050-201). PBMC suspension was centrifuged at 300 x *g* for 10 minutes. The cell pellet was resuspended in 80 µL of buffer per 10^7 total cells and 20 µL of CD14 MicroBeads per 10^7 total cells. The cell mixture was then incubated for 15 minutes at 4°C, washed with 1-2 mL of buffer per 10^7 total cells, and centrifuged at 300 x *g* for 10 minutes at 21°C. After centrifugation, the cell pellet was resuspended up to 10^8 cells in 500 µL of buffer for magnetic separation. The MACS columns were situated in the magnetic field of a MACS Separator and rinsed with 3 mL buffer. After applying the cell suspension through the columns, 3 mL of buffer was added three times to wash the column reservoir. To obtain the magnetically labeled $CD14^+$ cells, the column was removed from the separator and placed on a collection tube. Five mL of buffer was added to the column followed by an immediate plunging to flush all the magnetically labeled $CD14^+$ cells. $CD14^+$ monocyte suspension was eluted to 12 mL with Macrophage media and centrifuged at 1500 rpm for 10 minutes at 21°C. The pellet was resuspended in 10 mL of MDMG media and centrifuged at 1500 rpm for 8 minutes. The pellet was then resuspended in 10 mL of MDMG media and plated in 96 well flat bottom plates at the concentration of $1e^6$ cells/mL. The MDMGs were cultured at 37 °C with 95% or greater humidity and 5% CO_2. The method used to putatively differentiate MDMG ($CD45^{int}CD11b^+$) has been validated by the Gavegnano group to ensure that these cells are no longer expressing monocyte marker CD14 and are expressing cell marker similar to resident CNS microglia (unpublished, Gavegnano Group validated method ongoing in the laboratory).

In vitro treatment of MDMG with baricitinib and LPS for activation markers

For activation marker studies, fully differentiated MDMGs were used. The mature MDMG cell cultures were divided into two groups: pre-treatment and treatment group. For the pre-treatment

group, the cells were incubated with baricitinib (0.01μM, 0.1μM, and 1μM) for 2 hr in standard culture conditions and then stimulated with LPS (100 ng/mL; Escherichia coli 055: B5) for 24 hr. For the treatment group, LPS was added to all samples except for controls. baricitinib (0.01μM, 0.1μM, and 1μM) was added one hour after the addition of LPS stimulating media. The cells were cultured for an additional 24 hours.

Assessment of cell viability

Microglial viability was assessed using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethyl phenyl)-2-(4-sulfophenyl)-2H-tetrazolium) Cell Proliferation Colorimetric Assay Kit (BioVision). Fully differentiated MDMGs were plated in 96-well, flat bottom plates at 100,000 cells per well and maintained in the following conditions: (1) drug-free media, (2) media containing 0.1, 1, 10, or 100 μM baricitinib, (3) media containing 6.25%, 12.5%, 25%, 50% DMSO for 7 days prior to the assessment of cytotoxicity (positive control for cell death as described below). The cells were incubated with MTS reagents for an additional 2 hours in standard culture conditions before the absorbance was measured using a microplate multimode absorbance reader (Synergy H1, BioTek) at 490 nm. The threshold of cytotoxicity was 50% viability inhibition. DMSO, an amphipathic solvent that increases cell permeability, was used as a cytotoxic positive control (Notman et al., 2006).

Cell survival rates were calculated as the mean percentage of the optical density (OD) value of baricitinib-treated cells over that of cells without any treatment. Background OD was corrected and adjusted for by subtracting the culture medium background from the assay reading during data processing.

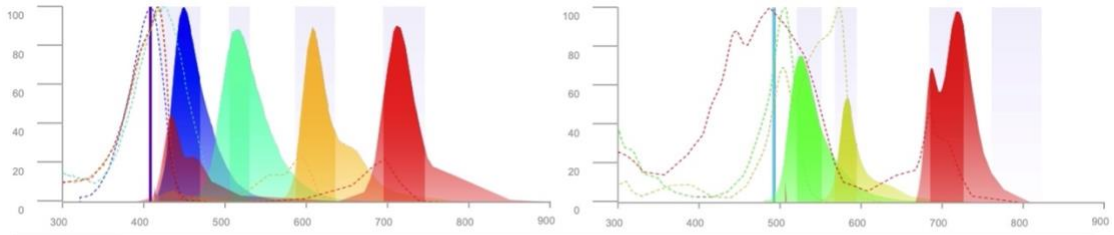
Flow cytometry

The treated cells were first washed with PBS 1X to remove FBS and then transferred to a V-bottom, 96-well plate by washing the original plates with MDMG media and cell scraping with cell lifters. After washing with FACS Buffer (PBS 1X and 2% FBS), the cells were centrifuged at 4000 g for 2 minutes. To maintain the integrity of the sample, 100 µL 4 % paraformaldehyde (PFA) was added to the plate and the cells were incubated for 30 minutes at room temperature. The samples were then washed with FACS buffer, centrifuged at 4000 g for 2 minutes and resuspended in 100 µL FACS buffer.

Pretreated and LPS-activated MDMGs were washed in 100 µL PBS per sample and centrifuged at 1800 rpm for 3.5 minutes. For surface stain, the cells were resuspended in 7.5/10 µL/well of antibody cocktail (see Table 1, Figure 2) in the staining buffer (PBS 1X and FBS 2%) and incubated for 20 minutes at 4°C in the dark. After incubation, the samples were washed in 150 µL FACS buffer per sample and centrifuged at 1800 rpm for 3.5 minutes. After removing all the supernatant, the cells were resuspended and fixed with 4% PFA for 15 minutes at room temperature. Then, the cells were washed and resuspended in FACS Buffer. Untreated media were transferred from the V-bottom 96-well plates into polystyrene round-bottom falcon tubes to determine voltage setting and optimal gating for wavelength compensation.

To examine microglia activation, the cells were analyzed using an acoustic focusing flow cytometer (Attune™ NxT, invitrogen). Total events were collected using forward scatter (FSC) and side scatter (SSC) followed by live/dead discrimination with a primary FSC/SSC gate (Figure 3 A) and doublet discrimination (SSC-area versus SSC-height and FSC-area versus FSC-height, Figure 3 B&C). The voltages and gates were established using untreated controls. The expression of activation markers of interest (CD206, CD40, CD32, CD86, CD163, CD16, HLA-DR) were

quantified based on channel specific gates established with events greater than one-log shift from negative populations from unstained controls (Figure 3D-J). Percentages, total events, and mean fluorescent intensity were collected.



Antigen	Fluorochrome	Channel	Emission	eFluor 450	eFluor 506	Super Bright 600 (SB600)	Super Bright 702 (SB702)	Alexa Fluor 488	PE
CD32	eFluor 450	440/50	445	66.7%	1%	4.4%	29.5%	-	-
CD16	eFluor 506	512/25	508	4.3%	38.1%	0.3%	1.7%	0.9%	-
CD86	Super Bright 600 (SB600)	603/48	601	-	5.8%	59.1%	0.4%	0.2%	1.7%
HLA-DR	Super Bright 702 (SB702)	710/50	702	-	-	3.2%	47.8%	-	-
CD206 (MMR)	Alexa Fluor 488	530/30	520	48.7%	0.2%	-	-	2.8%	-
CD163	PE	574/26	576	20.5%	53.7%	-	-	1.2%	0.3%
CD40	PerCP-eFluor 710	695/40	708	-	0.8%	66%	-	-	-

Figure 2. Flow cytometry antigen and fluorochrome panel design on Thermal Fisher's FCM panel builder. The spillover threshold is based on an average of 40%. Panel was designed based on the available lasers, Violet (405 nm) and Blue (488 nm), as well as target antigens. The principle behind panel design is balancing minimizing spillover between different channels and maximizing signal at specific channels.

Table 1. Antibody cocktail composition for MDMGs activation marker analysis using flow cytometry

	Target	Conjugate	Emissions
Cocktail 1	CD206 (MMR)	FITC	B488/525
	CD40	PerCP-eFluor 710	B488/708
	CD32	eFluor 450	V405/445
	CD86	Super Bright 600 (SB 600)	V405/601
Cocktail 2	CD163	PE	B488/574
	CD16	eFluor 506	V405/508
	HLA-DR	Super Bright (SB702)	V405/702

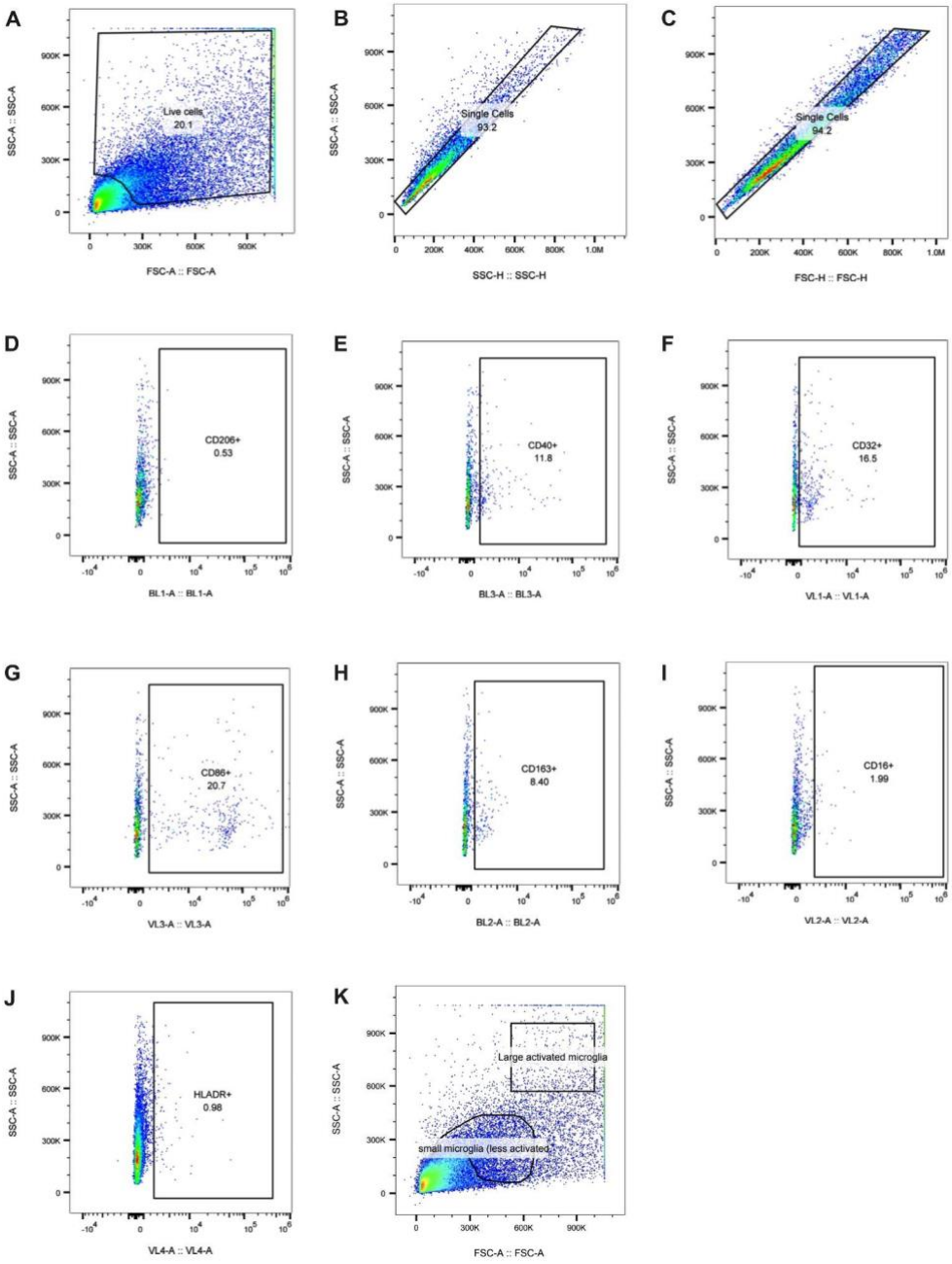


Figure 3. Gating for flow cytometry analysis using FlowJo 10.8.

Cells are first gated for live/dead cells differentiated by excluding debris characterized by low granularity and small size (A), for doublets removal (B & C), and then for each individual cell activation and lineage markers (D-J). Gates were drawn based on unstained and untreated cell population: cell marker positive populations were established by gating populations right to the events in unstained samples (<1% of the unstained cells). Additional gating for activated and inactive microglia separation is shown in (K).

Immunofluorescence Staining

Differentiated MDMG cultured in 24-well plates were washed with FACS Buffer to remove the media. Then, 4% PFA was applied to fix the cells for 30 minutes at 4℃. Before staining, PFA was removed and FACS buffer was applied. The cells were then incubated for 20 minutes at 4℃ with primary antibodies against Iba-1 (1002-5) conjugated with FITC (1:150, Santa Cruz Biotechnology Cat # sc-32725 FITC, read at excitation 488 nm, emission 525 nm) and DAPI (1:100, 4',6-diamidino-2-phenylindole, 5 g/mL, read at excitation 405 nm, emission 450 nm). Subsequently, the cells were fixed again with 4% PFA for 15 minutes at 4℃. A fluorescence microscope (EVOS M5000, Invitrogen, Thermo Fisher Scientific) was used to acquire images.

Statistical analysis

All statistical comparisons were performed using GraphPad Prism 8 software. Data were presented as the mean ± SEM. To account for the small sample size and non-normally distributed data, non-parametric Kruskal-Wallis tests were performed to evaluate the statistical differences across different treatment groups. Post-hoc analyses with uncorrected Dunn's test were conducted to determine which baricitinib dosage performs differently. Differences were considered statistically significant at $P<0.05$.

Results

Modified Gavegnano differentiation method yields MDMG with microglia morphological phenotypes

Microglia are implicated in neurodegenerative diseases and stroke pathophysiology. In this study, a novel modified Gavegnano differentiation protocol was used to differentiate monocyte-derived microglia-like cells (MDMG) from human whole blood. Media changes with MDMG base media containing IL-34, GM-CSF, and dorsomorphin were performed every 5 days to differentiate

plated monocytes to MDMGs. The primary microglia culture was further used to investigate microglial-related neuroinflammation post ischemic stroke. MDMG differentiated for 12-15 days using Gavegnano group modified MDMG base media exhibited small soma and branching structures (Figure 4C), which are morphological characteristics resembling that in published reports for MDMi and brain derived primary microglial cells (Quek et al., 2022).

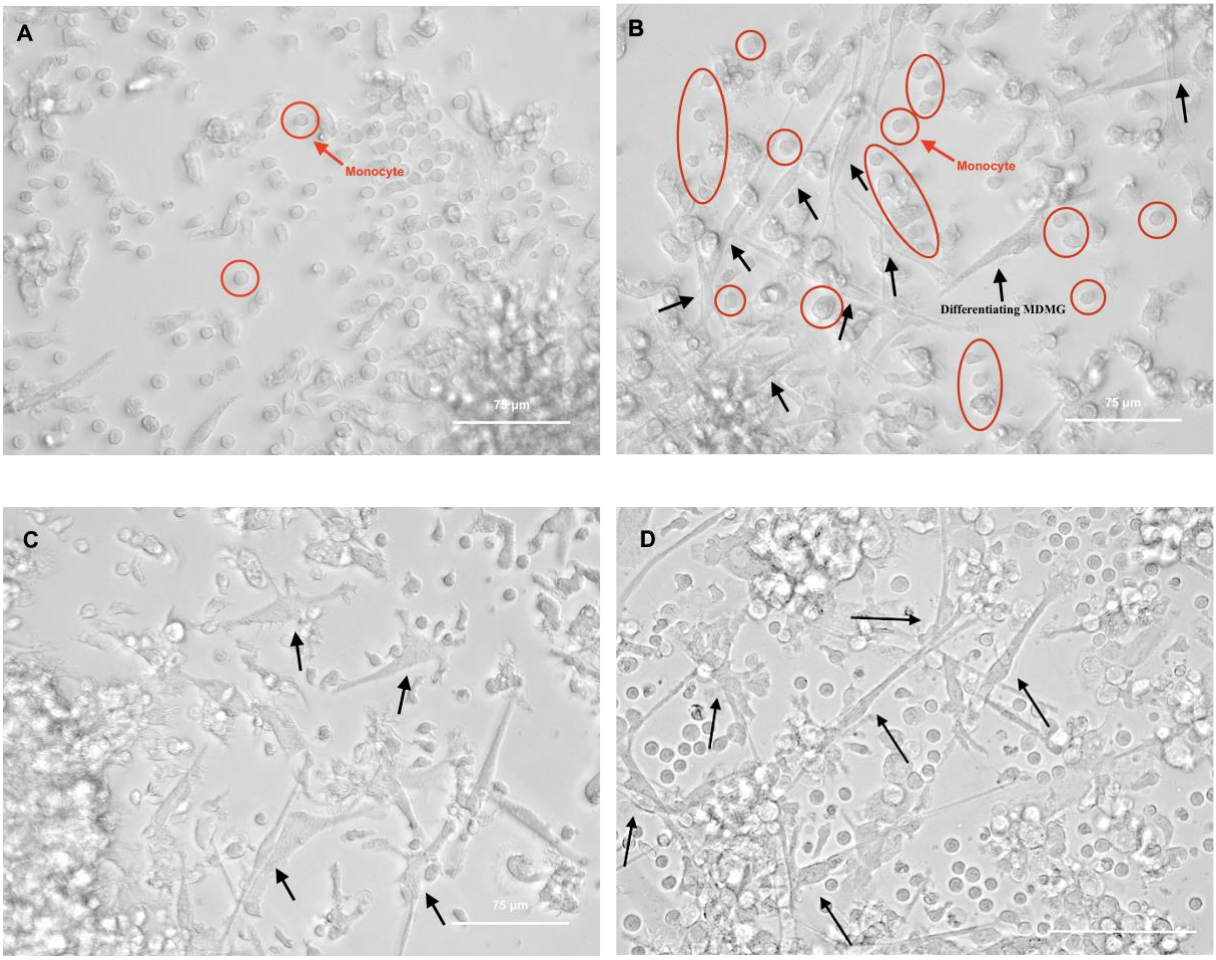


Figure 4. Light micrograph of monocyte-derived microglia through 15 days of differentiation. MDMGs were cultured in MDMG media for 15-25 days before use. **A.** Culture of monocytes on day 2. **B.** Monocytes adopt an elongated shape while some remains rounded at Day 6. **C.** On Day 11, cells displayed an increase in ramification. **D.** Fully ramified MDMGs. Black arrow, differentiating or differentiated microglia. Red arrow and circle, monocyte. Scale bar, 75 µm.

Effects of baricitinib on the viability of microglia

To determine the safety profile of baricitinib in microglia *in vitro*, its cytotoxicity was tested using MTS assay. MDMGs were incubated with different concentrations of baricitinib (0.1 μM, 1 μM, 10 μM, and 100 μM) or dimethyl sulfoxide (DMSO) (6.25%, 12.5%, 25%, and 50%) for 7 days. A one-way ANOVA revealed that there was no significant statistical difference in cell viability inhibition across different dosages of baricitinib treatment ($F(3,12) = 0.532$, $P = 0.669$). As shown in Figure 5A, the inhibition of microglia viability by baricitinib at selected concentrations is notably lower than 50%. We also calculated IC_{50}, the half maximal inhibitory concentration, which is a prevalently used pharmacological parameter of cytotoxicity *in vitro*. The data revealed that the concentration of baricitinib needed to inhibit microglia viability by 50% is higher than 100 ($IC_{50} > 100$, $r = 0.022$). In sum, these results demonstrated that baricitinib conferred no apparent toxic effect to microglia *in vitro* within the range of 0.1 μM, 1 μM, 10 μM, and 100 μM.

Whereas in the positive control groups, there was a statistically significant difference in percent cell viability inhibition between groups with difference dosages of DMSO treatment ($F(3,14) = 12.66$, $P = 0.0003$). IC50 calculation result showed that the concentration of DMSO required for 50% inhibition is 44.558% ($r = 0.994$). Overall, the data revealed that microglia viability inhibition changes is associated with the dosage of DMSO administration and DMSO indeed confer apparent toxicity at 50% concentration (Figure 5B), indicating the efficacy of the MTS assay.

Notably, clinically and physiologically relevant concentrations of baricitinib were chosen based on previous clinical trial data in HIV and COVID-19 indications (Stebbing et al., 2020) and published results (Gavegnano et al., 2019). Baricitinib is not toxic at concentrations more than 2

logs above the concentrations we administered in the *in vitro* model (0.01 μM, 0.1 μM, and 1 μM), underscoring that data observed are due to specific effects of baricitinib, not non-specific toxicity in the human microglia model.

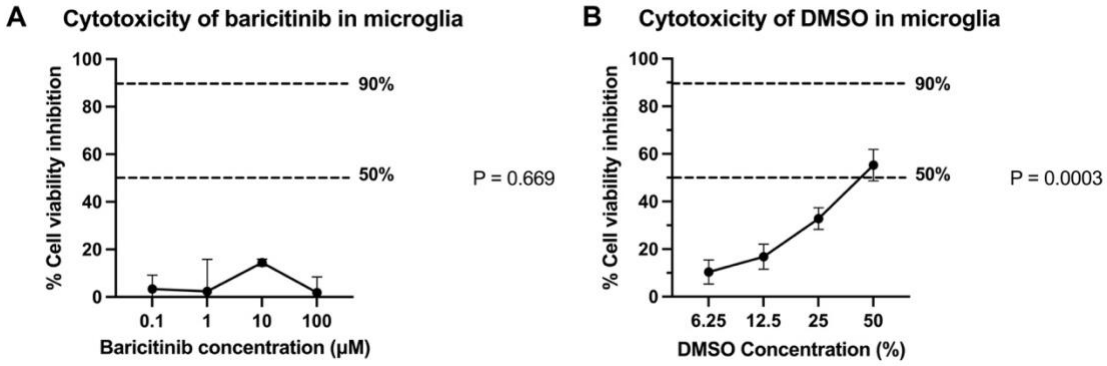


Figure 5. MTS toxicity assay assessing microglia viability.

The result of a 7-day incubation with increasing dosage of baricitinib (**A**) and DMSO (**B**) as shown. All data is presented as mean with SEM.

Baricitinib attenuates the activation of LPS stimulated microglia

We sought to measure the effect of baricitinib on LPS-induced activation of microglia, a key cell type involved in stroke pathophysiology in the CNS. Results show that baricitinib blocked the LPS-induced upregulation of activation markers CD206, CD86, CD163, CD16, and HLA-DR in monocyte-derived microglia (Figure 6).

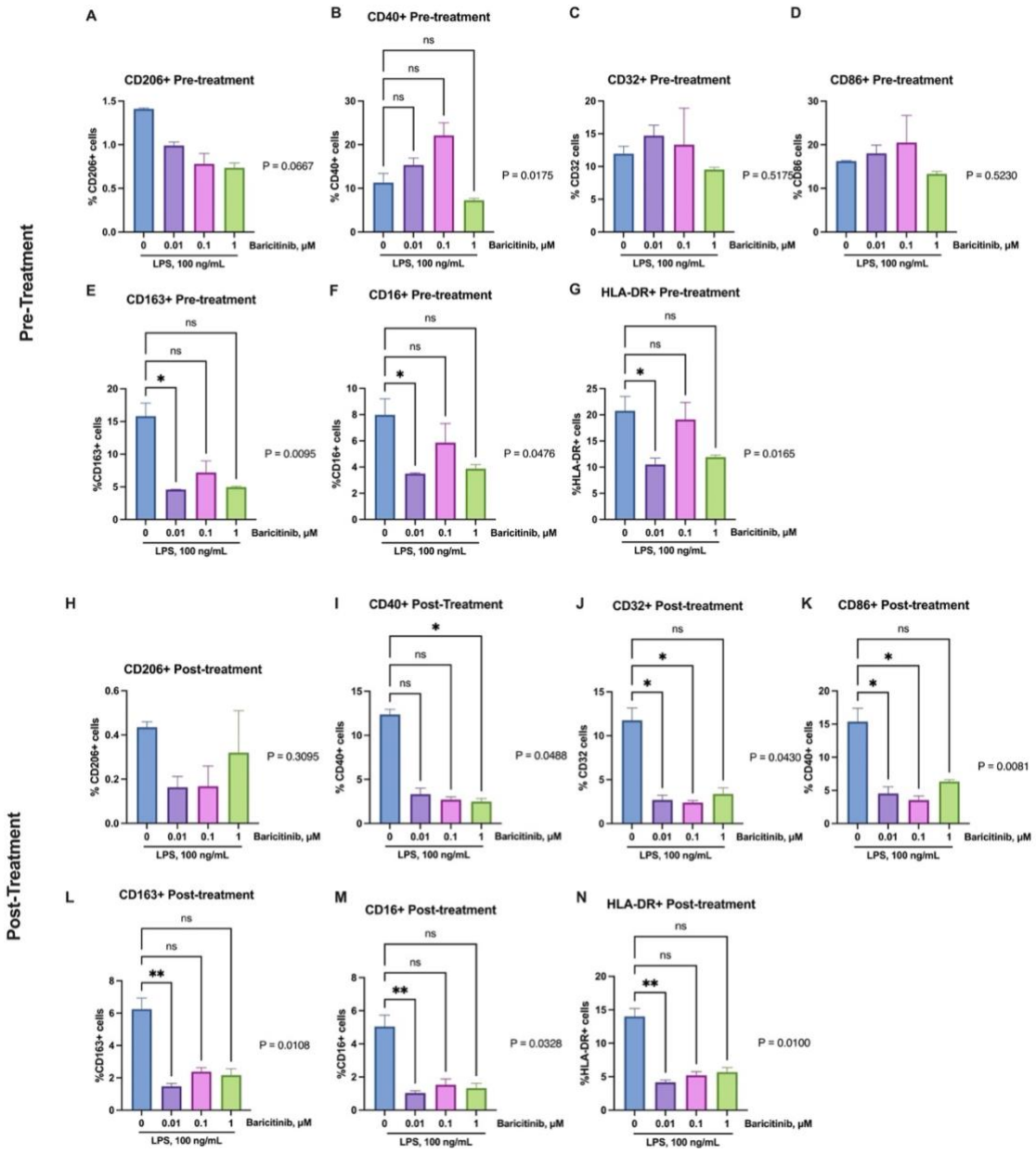


Figure 6. Baricitinib attenuates LPS-induced expression of microglial proinflammatory activation markers.

MDMGs were divided into pre-treatment (A-G) and post-treatment (H-N) groups and were either pretreated with baricitinib for 2 hours before activated with LPS for 24 hours or post-treated with baricitinib one hour after LPS administration. MDMGs were further separated into five groups: control (omitted, see Discussion), LPS-activated, LPS with 0.01 µM baricitinib, LPS with 0.01 µM baricitinib, LPS with 0.01 µM baricitinib. Microglia were stained and fixed at day 20 and assessed for proinflammatory activation markers using flow cytometry. (A, H) %CD206 $^{+}$ cells, (B, I) CD40^{+} cells, (C, J) CD32^{+} cells, (D, K) CD86^{+} cells, (E, L) CD163^{+} cells, (F, M) CD16^{+} cells, and (G, N) HLA-DR^{+} cells. n=2-4 per group. *P<0.05, **P<0.01, ***P<0.001. Baricitinib treated groups were compared to groups only stimulated with LPS. All bars present means with SEM.

Activation of microglia, the resident immune cells in the CNS, is involved in neuroinflammation during both the acute phase of ischemic attack and the subacute, chronic phase of ischemic stroke. Cellular activation of microglia to M1 proinflammatory phenotype results in inflammatory factor production, blood-brain-barrier disruption, and, ultimately, exacerbation of tissue injury and reduction of functional recovery post stroke. Hence, flow cytometry on LPS-stimulated MDMG pre-treated or post-treated with baricitinib of different concentrations was conducted to measure the effect of baricitinib on LPS-induced activation of human MDMG. The total events as well as specific activation marker positive events were collected with gates established from untreated controls. Selected markers of interest are activated state markers and membrane proteins found in microglia.

Using the Kruskal-Wallis test, we founds significant inhibitory effect of baricitinib on LPS-stimulated upregulation of activation markers CD40 ($H = 6.978$, $P = 0.0175$), CD163 ($H = 6.667$, $P = 0.0095$), CD16 ($H = 5.946$, $P = 0.0476$), and HLA-DR ($H = 7.712$, $P = 0.0165$) on primary human microglia when administered 2 hours prior to 24-hour LPS stimulation as a pre-treatment (Figure 6B, 6E-G). Post-hoc analysis with uncorrected Dunn's test showed that the percent of MDMGs expressing $CD163^+$ ($P = 0.0143$), $CD16^+$ ($P = 0.0311$), and $HLA\text{-}DR^+$ ($P = 0.0193$) were significantly different between the LPS-treated control group and the group pre-treated with 0.01 μM baricitinib. Additionally, we also observed marginally significant difference between the CD206 expression among the four treatment groups ($H = 6.000$, $P = 0.0667$) as well as significant difference between 0.1 μM, 1 μM baricitinib and LPS treated groups ($P = 0.0412$), indicating similar downregulation of microglia membrane protein expression when pre-treated with baricitinib (Figure 6A). These results indicate that baricitinib at specific concentrations selected in this study inhibited the expression of microglial proinflammatory markers, exhibiting anti-

inflammatory properties in MDMGs. Interestingly, there were no statistically significant differences in CD32, CD16 expression between baricitinib-treated microglia and the control groups with only LPS stimulation, suggesting that baricitinib has no effect on expression of these markers in human primary microglia when given as a pre-treatment prior to LPS administration.

The suppression effect of baricitinib on microglial activation markers' expression in the post-treatment group is consistent with that revealed in the pre-treatment group. Flow cytometry analysis results showed that the percentage of cells expressing CD40 ($H = 6.741$, $P = 0.0488$), CD32 ($H = 6.709$, $P = 0.0430$), CD86 ($H = 7.727$, $P = 0.0081$), CD163 ($H = 8.436$, $P = 0.0108$), CD16 ($H = 7.308$, $P = 0.0328$), and HLA-DR ($H = 8.465$, $P = 0.0100$) were significantly decreased in microglia treated with baricitinib (Figure 6I-N). Post-hoc pairwise uncorrected Dunn's test revealed that at 0.01 μM, baricitinib significantly reduced the percentage of $CD40^+$ MDMG ($P = 0.0138$, Figure 6I). The expression of $CD32^+$ and $CD86^+$ were significantly downregulated by baricitinib at concentrations of 0.01 μM and 0.1 μM (Figure 6J, K), and the percent of $CD163^+$, $CD16^+$, and $HLA\text{-}DR^+$ expressing microglia is significantly decreased by baricitinib at 0.01 μM (Figure 6L-N). These findings suggested that baricitinib suppressed microglia-specific activation marker upregulation induced by LPS, indicating baricitinib's potential immunomodulatory capacities in suppressing inflammation at the cell marker level even after the onset of LPS-stimulated activation.

Further, to evaluate the effects of baricitinib on microglia-specific activation markers, microglia previously stimulated with LPS or treated with baricitinib were stained with DAPI and Iba-1. As shown in Figure 7, LPS administration altered the morphological characteristics of the primary microglia, which exhibited enlarged cell bodies and shorter processes compared to control. Whereas baricitinib treatment suppressed morphological changes that signified microglia

activation and restored control like morphology in treated MDMGs (Figure 7). The immunofluorescent analysis results are consistent with the cell marker analysis, providing additional evidence for the activation suppression effect of baricitinib in microglia.

Consistent with visual inspection, results derived from the sub gate for larger, more granular (activated) microglia and the smaller, less granular (resting) microglia demonstrated that the percentage of activated microglia, which are characterized by larger and more granular cells located in the top right corner of the FSC-SSC graph (Figure 8A), was increased with LPS stimulation but significantly reduced by baricitinib at 0.01 μM ($p<0.05$) (Figure 8B). Again, this result is in consensus with the previous results, supporting the notion that baricitinib can reduce microglia activation conferred by LPS as quantified by morphological phenotypes.

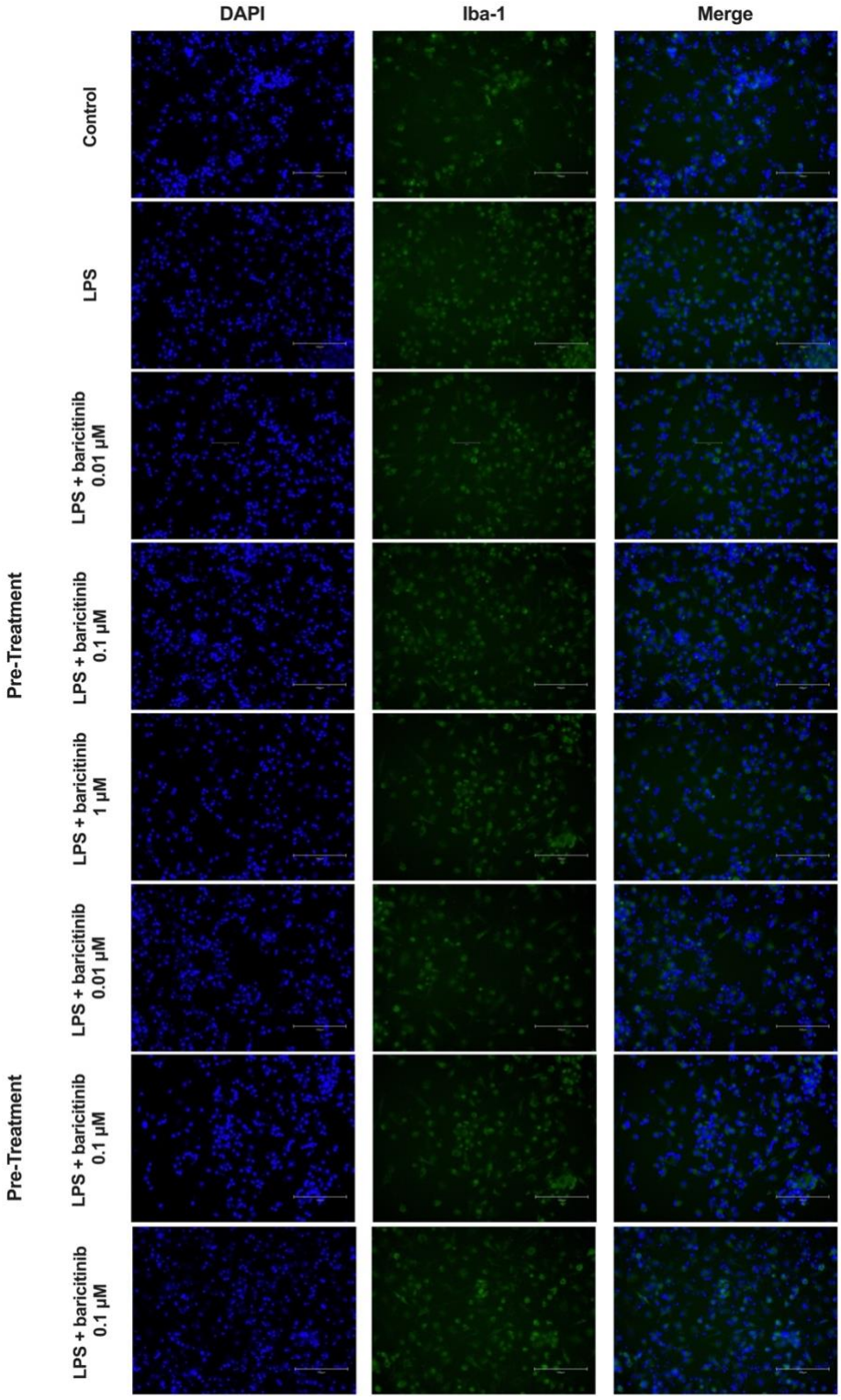


Figure 7. Baricitinib reduces LPS-stimulated microglia activation morphologically.
MDMGs were treated with 100 ng/mL LPS or LPS and baricitinib (0.01 µM, 0.1 µM, 1µM) and then stained with DAPI and Iba-1. Scale bars: 150 µm. LPS, lipopolysaccharide; DAPI, 4',6-diamidino-2-phenylindole.

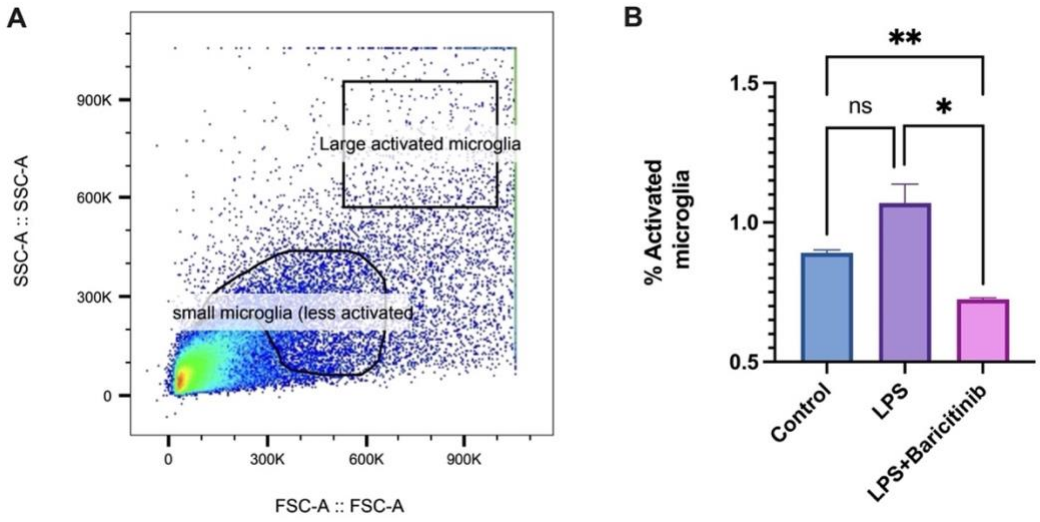


Figure 8. Flow cytometry of MDMGs with activated and resting (surveilling) microglia gating.

A. Gating method for activated and resting microglia differentiation. The percentage of activated microglia was quantified by FlowJo gating for high granularity (550K < SSC < 950K) and large size (500K< FSC <1000K) cells of the population. The percentage of resting (inactive) microglia was established by gating for low granularity (50K < SSC < 400K) and small size (50K <FSC <650K) cells. **B**. Percentage of activated microglia in the population in control, LPS-treated, and LPS + baricitinib 0.01 μM treated group. Unpaired student t test, * $p < 0.05$, ** $p < 0.01$. All data presents mean with SEM.

Discussion

In this study, we showed that clinically relevant doses of baricitinib suppressed excessive morphological microglia activation and inhibited microglial proinflammatory activation marker expression induced by LPS, demonstrating that this immunomodulator has anti-inflammatory properties and therapeutic potential for immune-related disease. Our flow cytometry data revealed that baricitinib exerted an anti-inflammatory effect by decreasing the expression of activated state markers found on the membrane of microglia.

Specifically, the expression of CD163 and CD206, key phenotypic markers commonly used to distinguish resident microglia (CD206low/−/CD163−) (Böttcher et al., 2019), were significantly downregulated by baricitinib, indicating baricitinib's ability to reverse activation of microglia. We also found significant inhibition of CD16, CD32, CD40, CD86, and HLA-DR expression. These surface markers are typically present on pro-inflammatory M1 phenotype microglia, indicating that baricitinib can reverse the microglia polarization phenotype on the M1/M2 spectrum, which decreases pathological inflammation and acute immune responses (Jurga et al., 2020). Further, as established in previous studies, the classical M1 polarization of microglia as a response to pathological stimulation attribute to not only general cell marker expression but also pro-inflammatory cytokine and chemokine production. Proinflammatory mediators including IL-1, IL-6, TNFα, iNOS, and NO are secreted by microglia after being stimulated with LPS (Deng et al., 2022, Biswas and Mantovani, 2010; Kalkman and Feuerbach, 2016). Additional multiplex immunoassays are needed to better understand how baricitinib affects the cytokine, chemokine, and oxidative metabolite production post ischemic injury *in vitro*. In the context of the current data, baricitinib successfully decreased the expression of immunoglobulin gamma Fc region receptor, CD16 and CD32, which are involved in inflammatory signal induction (Jurga et al., 2020; Kigerl et al., 2009), are membrane receptors for the Fc region of IgG. Levels of CD86, a membrane

co-stimulatory receptor responsible for IL-2 production, was also downregulated by baricitinib. Since activation of membrane proteins pertaining to M1-microglia increases the synthesis and secretion of inflammatory factors, by analyzing these markers we provide perspectives, although indirectly, into how baricitinib attenuates neuroinflammation in a disease model.

Correspondingly, we observed similar suppression of morphological and functional changes with our flow cytometry data with additional gating and microscopy immunofluorescence analysis. Unstimulated MDMGs have small, circular cell bodies with extensive branching processes (Nimmerjahn et al., 2005) underwent drastic changes when stimulated with LPS and adopted an amoeboid shape with larger cells body and shorter, thicker pseudopodia (Das Sarma et al., 2013; Figure 4). Baricitinib reversed the phenotypic changes and restored cell morphology of MDMGs to a resting state (Figure 7). Collectively, these results demonstrate that baricitinib has the capacity to mitigate inflammation mediated by microglia and the therapeutic potential to be applied to attenuate ischemic brain injury by exerting anti-inflammatory effect.

To our surprise, although we did not run a statistical analysis (i.e., 2 way ANOVA) on the difference between pre- and post-treatment results, we observed that baricitinib, when administered one hour post the LPS stimulation, more significantly inhibited the expression of microglial activation marker expression than the pre-treatment group in which baricitinib was added to the cell culture two hours in advance. Replication of the flow cytometry assay is necessary to confirm this observation. Pre-treatment is clinically equivalent to preventive prophylactic care, whereas post-treatment represents therapies given following an injury with the goal of reversing diseases after the initiation of pathology. In the context of stroke, baricitinib as a pretreatment agent can target the healthy brain tissues near the infarct or the penumbra of a patient with ischemic brain injury. We hypothesize that its anti-inflammatory properties may be less potent when the

microglia are unstimulated and inactive, hence the less marked inhibitory effect on marker expression observed in our current flow cytometry data (Figure 6A-N). On the other hand, baricitinib added after the onset of disease had a more robust suppressing effect on microglia activation likely due to its higher deviance from baseline marker expression levels, implying that baricitinib may exert stronger immunomodulatory effect as a post-treatment therapeutic.

Due to time constraints, some previously planned experiments, including bulk RNA sequencing, mesoscale discovery multiplex immunoassays, and neuron-microglia co-culture, were not conducted. Further investigation on the specific cell signaling pathway and immune factors involved in microglia-induced neuroinflammation are needed and will be conducted in the future by the Gavegnano group to inform the pathophysiological mechanism behind ischemic stroke. Additionally, to understand how the neuron and neurological functions are impacted by ischemic injury and inflammation, we will co-culture microglia with astrocytes and neurons (MAP-2) and evaluate neuronal morphological changes, such as neuronal dendritic synaptic shortening. This experiment will provide key insight in how baricitinib affects inflammation-driven CNS dysfunction which in turn drives clinical manifestation of symptoms and disease severity.

Presented flow cytometry data only represents a subset of analyses we have conducted in the given timeframe. Further data processing is needed for more precise gating methods to differentiate distinct microglia phenotypes. Additionally, replication of our flow cytometry analysis is recommended. We noticed that at our current FSC and SSC voltage setting, more than 15% of the microglia population were not captured within the plot. Future replications should refine the voltage setting by adding a back-gating step on activated microglia to determine the appropriate FSC and SSC for the cell population.

In-vitro models for ischemic stroke often employ the oxygen and glucose deprivation (OGD) model by replacing oxygen with N2 and omitting glucose in the media. In this study, however, lipopolysaccharide was utilized to activate microglia and mimic the microglia-mediated neuroinflammation post-stroke (Deng et al., 2022, Yang et al., 2020). LPS are bacterial cell wall endotoxins known to induce neuroinflammation and evoke proinflammatory mediator release. This is a common stimulation used to polarize primary microglia cells and induce neuroinflammation (Bowyer et al., 2020). Therefore, although LPS stimulation cannot mimic the multifaceted pathophysiology of stroke, it induces microglial neuroinflammation and simulates the inflammatory environment in the CNS after ischemic injury onset, allowing us to investigate how inflammatory responses are attenuated by baricitinib. In vivo studies with baricitinib needs to be conducted to validate our findings derived from the primary human microglia model in more dynamic and complicated systems like co-culture models or in living organisms.

Numerous studies have established PI3K/Akt/NF-κB signaling pathways responsible for inflammation mediation in cerebral ischemic injury. A study by Deng et al. (2022) demonstrated that fraxetin inhibited neuroinflammation via the PI3K/Akt/NF-κB pathway. This finding is supported by many studies, validating the important role downstream factors NF-κB, STAT3, and PI3K/Akt play in regulating neuroinflammation (Wu et al., 2018; Liu et al., 2021). The immunomodulating agent of this study, baricitinib, is a FDA-approved selective Janus Kinases (JAKs) inhibitor. JAK modulates cytokine signaling and is involved in immune cell function by phosphorylating transcription proteins, STATs, and initiating intracellular cascades of inflammatory factors. Previous studies have extensively investigated baricitinib's mechanism of action, particularly in the indication for rheumatoid arthritis (Taylor et al., 2017; Al-Salama & Scott, 2018) and COVID-19 (Stebbing et al., 2020). This JAK 1/2 inhibitor prevents

phosphorylation and activation of STAT3, which subsequently suppresses secretion of inflammatory mediators like IL-1β, TNF-α, and IL-6. We hypothesize that NF-κB and JAK/STAT pathway is inhibited by baricitinib, which ultimately leads to suppressed neuroinflammation and brain damage caused by ischemic stroke, especially in the subacute phase when inflammation acts as the main driver of pathology (Figure 9).

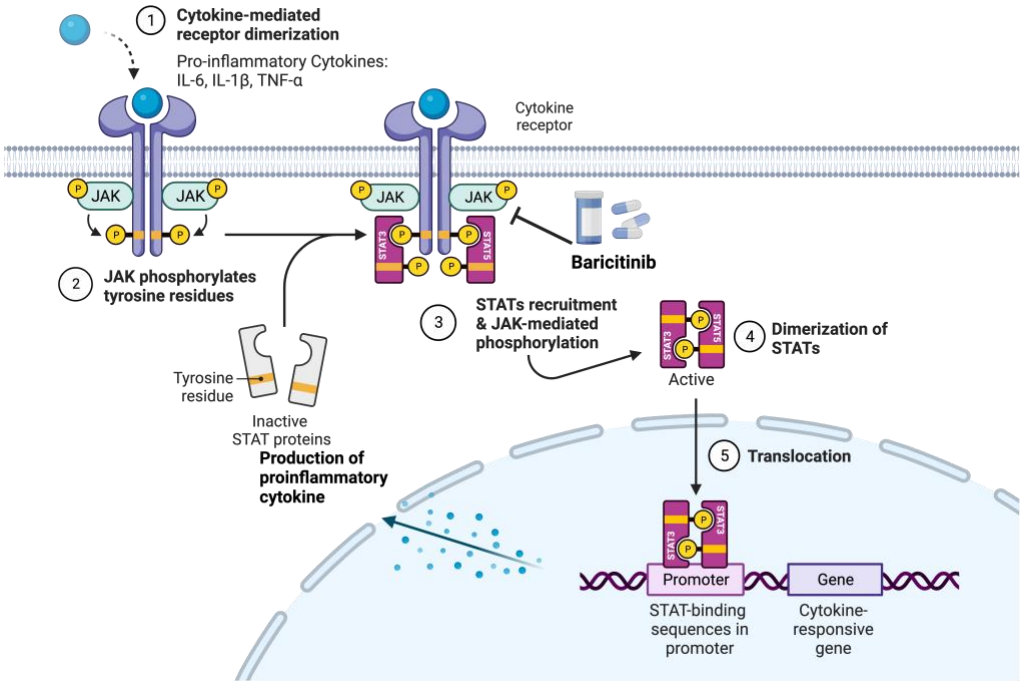


Figure 9. Proposed mechanism of action of baricitinib: Baricitinib, selective JAK 1/2 inhibitor suppresses cytokine signaling through the JAK-STAT Pathway.
Adapted from "Cytokine Signaling through the JAK-STAT Pathway", by BioRender.com (2022).

Excitingly, our group and collaborators have demonstrated that baricitinib can penetrate the BBB, reduce neuroinflammatory markers like IL-6, and mitigate neuronal degradations (Gavegnano et al., 2019), highlighting that baricitinib may also confer similar effects in the stroke model. The long-term goal of this work is to gather preclinical data necessary to launch a human trial with baricitinib, additionally leveraging the existing Phase 2 human trials in our group with

baricitinib as an immunomodulator based intervention in the CNS (National Library of Medicine [NLM], NCT05452564, 2023).

Despite the pharmacological value of baricitinib being investigated in many diseases, few studies have reported its effects in cerebrovascular diseases. To our knowledge, we are the first group to show that baricitinib treatment leads to reduced activation of proinflammatory phenotype microglia. Our data provides strong evidence for baricitinib's neuroprotective effects in microglia and its potential in ameliorating ischemic brain injury. In terms of theoretical significance, elucidating microglial markers regulated by baricitinib may lead to novel discoveries in therapeutic targets for ischemic stroke and other neurological diseases driven by inflammation.

This study also validated a novel protocol for microglia differentiation from human peripheral blood mononuclear cells (PBMC)-derived monocytes. The Gavegnano group modified method yields cell marker expression and fully ramified microglia-like morphology on day 15 and continues to differentiate until day 21. Mature MDMGs can be used for various downstream analysis including PCR, flow cytometry, immunohistochemistry, etc. as well as establishing co-cultured models with other brain cell types. Additionally, while the yield of MDMG cells is dependent on the number of monocytes and PBMCs harvested, this method has a consistent large yield of differentiated cells of approximately 100 million cells per donor, allowing large scale evaluation of druggable candidate agents as well as investigations on the molecular mechanisms of immune-related neurological disorders *in vitro*.

In summary, this model represents a proof of principle that clinically relevant concentrations of baricitinib can significantly reduce key markers of neuroinflammation in a primary microglial model. As microglia interact with neurons and astrocytes in the human brain,

a critical role of their function is to confer inflammatory signals to these cells. The data herein demonstrate that baricitinib can reduce the stroke-like induction of inflammation in microglia, a proof of principle that baricitinib may have clinical impact in mitigating ischemic stroke-induced neuroinflammation in relevant human cells.

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