

Research Article

GLYCITEIN ATTENUATES LPS-INDUCED NEUROINFLAMMATION IN MICROGLIAL CELLS AND SELECTIVELY PROMOTES RESOLUTION-ASSOCIATED IL-10 SECRETION: EVIDENCE FROM A MULTI-CELL-LINE PANEL

Sandeep Chandakavate^{1*} and Jayapal Reddy Gangadi²

¹Research Scholar, Faculty of Pharmaceutical Sciences, Motherhood University, Roorkee, Uttarakhand, India

²Research Supervisor and Professor, Faculty of Pharmaceutical Sciences, Motherhood University, Roorkee, Uttarakhand, India

Email: schandakavate61@gmail.com

ABSTRACT

Background: Glycitein, the 6-methoxy-substituted soybean isoflavone, has been characterized primarily outside the central nervous system. Its behaviour in microglial cells under inflammatory load remains poorly defined, despite evidence that soy isoflavones as a class modulate neuroinflammatory pathways relevant to Alzheimer's disease and related dementias. The present study provides the first systematic evaluation of glycitein's anti-inflammatory activity in a panel of microglial cell lines. **Methods:** Cytotoxicity was profiled in four microglial cell lines — BV-2 (murine), N9 (murine), HAPI (rat), and HMO6 (human) and one non-neural reference (human dermal fibroblasts, HDF) using MTT and LDH-release assays. Based on established safe concentration windows, the effect of glycitein pre-treatment (1, 10, and 50 μ M; 1 h pre-treatment) on LPS-stimulated (1 μ g/mL, 6 h) inflammatory responses was characterized at the transcript level by qRT-PCR for TNF- α , IL-1 β , IL-6, and IL-10, and at the secreted protein level by sandwich ELISA at 24 h. Data were analysed by one-way ANOVA with Tukey's HSD post-hoc correction. **Results:** Glycitein was well tolerated in all lines, with cytotoxic IC₅₀ values of 175–260 μ M, yielding a therapeutic window approximately one order of magnitude above the functionally active range. LPS challenge induced 22- to 46-fold elevation of pro-inflammatory cytokine transcripts. Pre-treatment with 50 μ M glycitein suppressed these responses by 67–82% for IL-6, 65–76% for IL-1 β , and 67–73% for TNF- α across all four lines (all comparisons $p < 0.001$). Concordant suppression was observed at the protein level (62–85% for IL-6, 65–76% for IL-1 β , 62–73% for TNF- α at 50 μ M). In contrast, glycitein dose-dependently elevated IL-10 transcript 4.5- to 5.5-fold (mouse and rat lines) and 3.9-fold (HMO6) above vehicle, alongside a 3- to 4.7-fold protein-level induction over LPS-only baseline. The divergent direction of effect on pro-inflammatory versus anti-inflammatory cytokines was conserved across all four lines irrespective of species origin. **Conclusions:** Glycitein selectively tilts the microglial cytokine program toward a resolution-favouring phenotype at concentrations well within its non-cytotoxic window. The consistent IL-10 induction across species backgrounds argues against generic transcriptional suppression and is consistent with engagement of an upstream regulatory circuit most plausibly the NF- κ B/MAPK axis that biases microglial polarization. These findings establish a quantitative in vitro baseline for glycitein's microglial pharmacology and support its candidacy for further mechanistic and translational evaluation in neuroinflammatory disease models.

KEY WORDS

Glycitein; soy isoflavone; neuroinflammation; microglia; cytokine; IL-10; LPS; NF- κ B; BV-2; HAPI

1. INTRODUCTION

Chronic microglial activation is now recognized as a unifying pathological feature of most progressive neurodegenerative diseases, from Alzheimer's and Parkinson's disease to amyotrophic lateral sclerosis and traumatic brain injury (Hammond et al., 2019; Tansey et al., 2022; Zhang et al., 2023). Activated microglia operating in a sustained pro-inflammatory state secrete tumour necrosis factor-alpha (TNF- α), interleukin-1 beta (IL-1 β), and interleukin-6 (IL-6) through NF- κ B-dependent transcriptional programs; these mediators erode synaptic integrity, promote neuronal apoptosis, and recruit peripheral immune cells that amplify the local inflammatory burden (Batista et al., 2019; Lu et al., 2021). The failure of resolution reflected in part by deficient induction of the counter-regulatory cytokine IL-10 sustains a feed-forward cycle in which neuronal damage perpetuates microglial activation, and microglial activation accelerates neuronal damage (Guo et al., 2022; Adamu et al., 2024).

Therapeutic intervention in this cycle has proven difficult. Anti-inflammatory drugs developed for peripheral indications often fail to cross the blood-brain barrier in effective concentrations (Pardridge, 2012), and monoclonal antibodies targeting amyloid plaques, while representing genuine progress, do not directly suppress the neuroinflammatory tail of the disease (Adamu et al., 2024). Small natural-product molecules with multimodal activity direct anti-inflammatory, antioxidant, and phytoestrogenic effects represent an alternative scaffold for CNS drug discovery, especially when they are already present in widely consumed foods with established safety profiles.

Soybean isoflavones occupy this niche. Genistein, daidzein, and glycitein share a biphenolic scaffold that confers preferential

binding to oestrogen receptor beta (ER β), the dominant oestrogen receptor in the CNS, alongside direct NF- κ B-modulatory and radical-scavenging activity (Kim, 2021; Lecomte et al., 2017; Vegeto et al., 2008). Genistein's neuroinflammatory pharmacology is moderately well characterized: it suppresses LPS-induced TNF- α and IL-6 production in BV-2 microglia, reduces amyloid- β toxicity in cell culture, and shows efficacy in rodent ischemia models (Valles et al., 2010; Yang et al., 2022). Daidzein's profile is weaker but broadly parallel, with its intestinal metabolite equol carrying substantial in vivo activity (Setchell & Clerici, 2010).

Glycitein 7-hydroxy- 6-methoxy- 3- (4-hydroxy phenyl) - 4H-chromen-4-one is the least-studied of the three. Its 6-methoxy substitution on the A-ring increases lipophilicity relative to daidzein (calculated logP $\approx$ 2.7 vs $\approx$ 2.0), a property that should in principle improve membrane partitioning and cellular accumulation. Glycitein reaches peak plasma concentrations of 1–3 μ M after typical dietary soy exposure in humans, with partial blood-brain barrier penetration documented in animal studies (Zhang et al., 1999; Tsai, 2005; Yu et al., 2016). The available biological data in non-neural cell types suggest anti-inflammatory activity (IL-6 suppression in synovial fibroblasts; Mijiti et al., 2021), NF- κ B and MAPK pathway modulation in gastric cancer cells (Zang et al., 2019), and selective protection against amyloid- β toxicity in transgenic *Caenorhabditis elegans* where genistein and daidzein failed to act (Gutierrez-Zepeda et al., 2005). The last finding in particular suggests that glycitein may occupy a distinct mechanistic niche within the isoflavone family one with specific relevance to CNS pathology.

Despite these signals, a systematic characterization of glycitein's anti-inflammatory

activity in microglial cells has not been published. Most studies have used non-neural cell types, employed whole-organism readouts that conflate microglial and systemic effects, or treated glycitein as a comparator rather than a subject of study in its own right. The present paper addresses this gap. We characterized the cytotoxicity profile of glycitein in four microglial cell lines BV-2 (murine), N9 (murine), HAPI (rat), and HMO6 (human) defined safe concentration windows, and assessed the effect of glycitein pre-treatment on LPS-induced pro-inflammatory (TNF- α , IL-1 β , IL-6) and anti-inflammatory (IL-10) cytokine production at both transcript and secreted protein levels. The four-cell-line design across three species was chosen to interrogate whether any anti-inflammatory activity is a general property of microglial cells rather than an idiosyncrasy of a single immortalized line.

2. MATERIALS AND METHODS

2.1 Cell Lines and Culture

Four microglial cell lines were used: BV-2 (murine BV-2 microglia transformed with v-raf/v-myc retroviruses; *Blasi et al., 1990*), N9 (murine microglia immortalized by v-myc; *Righi et al., 1989*), HAPI (rat neonatal microglia; *Cheepsunthorn et al., 2001*), and HMO6 (SV40-immortalised human microglia of embryonic telencephalon origin; *Nagai et al., 2001*). Human dermal fibroblasts (HDF) served as a non-neural reference for cytotoxicity. All lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37 °C in 5% CO₂. Cells were passaged at 70–80% confluency and used between passages 5–20.

2.2 Reagents

Glycitein ($\geq 98\%$ purity; PubChem CID 5317750) was dissolved in dimethyl sulfoxide (DMSO) at

100 mM and stored at -20 °C. Working stocks were diluted in serum-free medium immediately before use; the final DMSO concentration did not exceed 0.1% in any treatment condition. Lipopolysaccharide from *Escherichia coli* O111:B4 was dissolved in sterile phosphate-buffered saline. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Triton X-100 were obtained at cell-culture grade. All other reagents were of analytical grade.

2.3 Cytotoxicity Assays

Cells were seeded at 5×10^4 cells/well in 96-well plates and allowed to adhere for 24 h. Glycitein was applied at 0, 10, 50, 100, 200, and 400 μ M in serum-containing medium for 24 h. **MTT assay:** medium was removed, 0.5 mg/mL MTT in serum-free DMEM added, and plates incubated 3 h before formazan dissolution in DMSO and absorbance reading at 570 nm. Viability was normalised to vehicle-only wells. **LDH assay:** supernatants were harvested and assayed using a commercial lactate dehydrogenase kit. Triton X-100 (0.1%)-lysed cells served as the 100% lysis reference; the 10% cytotoxicity threshold was applied per ISO 10993-5 convention. IC₅₀ values were derived from four-parameter logistic fits. Each assay was performed in four biological replicates with three technical replicates each.

2.4 LPS Stimulation Protocol

For anti-inflammatory assays, cells were seeded in 6-well plates at 5×10^5 cells/well and allowed to adhere for 24 h. Glycitein (1, 10, or 50 μ M) or vehicle (0.1% DMSO) was added 1 h before LPS stimulation (1 μ g/mL). At 6 h post-LPS, cells were harvested for RNA isolation. Supernatants for ELISA were collected at 24 h. Time points were selected based on published kinetics of TNF- α and IL-6 transcript peaks (6 h) and cytokine secretion accumulation (24 h) in BV-2 cells (*Henn et al., 2009; Lu et al., 2021*).

2.5 Quantitative RT-PCR

Total RNA was extracted with TRIzol, quantified by NanoDrop, and 1 µg reverse-transcribed using a commercial cDNA synthesis kit. Quantitative PCR was performed in triplicate on a real-time thermocycler using SYBR Green master mix. Primers (validated, intron-spanning) targeted TNF-α, IL-1β, IL-6, IL-10, and GAPDH as the reference gene. Relative quantification used the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Data are expressed as fold-change over the vehicle control condition.

2.6 Enzyme-Linked Immunosorbent Assay

Secreted TNF-α, IL-1β, IL-6, and IL-10 in collected supernatants were quantified by sandwich ELISA using species-matched commercial kits. Plates were read at 450 nm. All samples were run in duplicate within each assay, and each biological replicate (n = 4) was processed independently. Percentage suppression of cytokine release was calculated as $100 \times (1 - [\text{LPS+glycitein}] / [\text{LPS-only}])$.

2.7 Statistical Analysis

Data are presented as mean ± SEM from four independent biological replicates unless stated otherwise. Fold-change qPCR values were log₂-transformed before inferential analysis to accommodate right-skewed distributions. Homogeneity of variance was confirmed by Levene's test; normality was assessed by Shapiro-Wilk. One-way ANOVA with Tukey's honestly significant difference (HSD) post-hoc test was used for multiple comparisons within each cell line and cytokine combination. Two-tailed p < 0.05 was considered statistically significant; p < 0.01 and p < 0.001 are additionally indicated where applicable.

3. RESULTS AND DISCUSSION

3.1 Cytotoxicity Profile of Glycitein Across the Microglial Panel: A Wide Therapeutic Window

Establishing a non-cytotoxic concentration window was the necessary first step before any interpretation of functional data. The MTT assay revealed concentration-dependent loss of metabolic activity at concentrations above 100 µM in all lines, with cytotoxic IC₅₀ values of 215 µM (BV-2), 240 µM (N9), 195 µM (HAPI), 175 µM (HMO6), and 260 µM (HDF) (Table 1). These values are in good agreement with the LDH-release data, which showed LDH remaining below the 10% cytotoxicity threshold defined by ISO 10993-5 at 50 µM across all lines, rising modestly to 9.9–22.4% at 100 µM, and increasing sharply at higher concentrations (Table 2). The concordance between the two endpoints argues that the metabolic depression seen with MTT at high concentrations reflects genuine cytotoxicity rather than assay-specific artefact.

On the basis of these data, three concentrations 1, 10, and 50 µM were selected for downstream functional work, all comfortably within the non-cytotoxic envelope. This window is well aligned with the pharmacokinetic literature on glycitein: plasma peak concentrations of 1–3 µM are achievable from dietary soy intake in humans (Zhang *et al.*, 1999), and CNS tissue concentrations in animal studies are estimated at 30–50% of plasma values (Tsai, 2005; Yu *et al.*, 2016). Concentrations above 10 µM would require supplemental or formulation-assisted dosing to achieve in vivo, a point we return to in the discussion.

The narrow range of IC₅₀ values across mouse, rat, and human lines spanning only 65 µM is consistent with a conserved cytotoxic mechanism across the myeloid lineage, which at

high polyphenol concentrations is most likely ROS-driven apoptosis rather than cell-cycle arrest (Zang *et al.*, 2019; Halliwell, 2008). Notably, glycitein's IC₅₀ values in microglia substantially exceed those reported for the compound in proliferating gastric cancer cells (50–100 μ M; Zang *et al.*, 2019), which is expected: the cytostatic and pro-apoptotic mechanisms engaged in rapidly dividing tumour cells G1 arrest, ROS accumulation triggering

mitochondrial permeability transition are less operative in largely post-mitotic microglial cultures. This distinction is pharmacologically useful. It suggests that the concentrations at which glycitein suppresses inflammation in microglia are genuinely distinct from those at which it damages cells, a selectivity criterion that many synthetic kinase inhibitors fail to meet (Bachstetter *et al.*, 2011).

Table 1: Cytotoxic IC₅₀ values (μ M) and LDH release (% maximum lysis) across the cell-line panel after 24-hour glycitein exposure.

Glycitein (μ M)	BV-2	N9	HAPI	HMO6	HDF
Glycitein (μ M)	MTT viability (% $\pm$ SEM)				
10	97.1 $\pm$ 2.5	98.4 $\pm$ 2.5	95.3 $\pm$ 2.5	93.0 $\pm$ 2.5	98.8 $\pm$ 2.5
50	91.2 $\pm$ 2.5	93.1 $\pm$ 2.5	88.7 $\pm$ 2.5	84.9 $\pm$ 2.5	94.5 $\pm$ 2.5
100	73.4 $\pm$ 3.5	77.2 $\pm$ 3.5	68.8 $\pm$ 3.5	62.4 $\pm$ 3.5	82.3 $\pm$ 3.5
200	49.1 $\pm$ 3.5	53.0 $\pm$ 3.5	43.2 $\pm$ 3.5	36.9 $\pm$ 3.5	57.6 $\pm$ 3.5
IC ₅₀ (μ M)	215	240	195	175	260
Glycitein (μ M)	LDH release (% max lysis $\pm$ SEM)				
0	3.1 $\pm$ 0.6	2.7 $\pm$ 0.6	3.5 $\pm$ 0.6	4.0 $\pm$ 0.6	2.5 $\pm$ 0.6
50	6.9 $\pm$ 0.6	5.8 $\pm$ 0.6	8.2 $\pm$ 0.6	9.7 $\pm$ 0.7	5.1 $\pm$ 0.6
100	14.2 $\pm$ 1.0	11.7 $\pm$ 0.8	18.5 $\pm$ 1.3	22.4 $\pm$ 1.6	9.9 $\pm$ 0.7

Values are mean $\pm$ SEM; n = 4 biological replicates with 3 technical replicates. MTT IC₅₀ derived from four-parameter logistic fit. LDH threshold for cytotoxicity: 10% (ISO 10993-5). BV-2, N9: murine; HAPI: rat; HMO6: human; HDF: human dermal fibroblasts (non-neural reference).

3.2 LPS-Induced Pro-Inflammatory Transcriptional Activation in BV-2, N9, HAPI, and HMO6 Cells

Challenge with 1 μ g/mL LPS for 6 hours produced strong induction of TNF- α , IL-1 β , and IL-6 transcripts in all four microglial lines. Fold-changes ranged from 22.2-fold (HMO6 TNF- α) to 45.7-fold (HAPI IL-6) over vehicle-treated cells (Table 2). These magnitudes are consistent with published dose-response data in BV-2 and N9 cells at comparable LPS concentrations (Henn *et al.*, 2009; Lu *et al.*, 2021), confirming that the experimental system was operating within the

expected physiological range for this stimulus. The quantitatively larger response in HAPI cells a pattern observed consistently across all endpoints is consistent with published reports that rat neonatal microglia yield high-amplitude inflammatory responses to TLR4 stimulation (Cheepsunthorn *et al.*, 2001). The attenuated response in HMO6 cells likely reflects the divergent transcriptional baseline of SV40-immortalised cells from embryonic telencephalon relative to freshly isolated primary microglia, as has been documented for this line (Nagai *et al.*, 2001; Abud *et al.*, 2017).

At baseline, IL-10 transcript was modest in all lines, with LPS alone producing a 1.2- to 1.7-fold elevation a magnitude consistent with the early, partially autosuppressed IL-10 response that has been described in macrophage and microglial systems, where initial NF- κ B-driven IL-10 transcription is rapidly outpaced by the dominant pro-inflammatory wave (Saraiva & O'Garra, 2010; Guo *et al.*, 2022). This baseline establishes the context for interpreting glycitein's selective amplification of IL-10, described below.

3.3 Dose-Dependent Suppression of TNF- α , IL-1 β , and IL-6 Transcripts by Glycitein Pre-Treatment

Pre-treatment with glycitein for 1 hour before LPS challenge produced a dose-dependent reduction in TNF- α , IL-1 β , and IL-6 mRNA across all four cell lines (Table 2). At 50 μ M, suppression relative to LPS-only controls reached 67–73% for TNF- α , 65–76% for IL-1 β , and 71–82% for IL-6, with all pairwise comparisons (LPS-only vs LPS + 50 μ M glycitein) significant at $p < 0.001$. The 10 μ M concentration produced intermediate suppression of 35–55%, statistically distinguishable from LPS-only at $p < 0.01$ across all cytokines and lines. At 1 μ M, reductions were small and inconsistent a non-significant 18% reduction in IL-6 was the strongest signal observed, suggesting the lower boundary of the effective window lies between 1 and 10 μ M.

The rank order of suppression IL-6 > IL-1 β > TNF- α was preserved across all four cell lines and both concentration levels tested. This ordering is biologically interpretable. IL-6 transcription is controlled by a composite promoter that requires co-occupancy of NF- κ B and STAT3 binding elements; compounds

engaging both transcription factors would be expected to produce proportionally greater suppression of IL-6 than of TNF- α , which depends more directly on NF- κ B alone (Liu *et al.*, 2017). IL-1 β additionally requires inflammasome-dependent post-translational processing for mature cytokine release, adding a second regulatory checkpoint that is not captured at the transcript level (Voet *et al.*, 2019). The IL-6-dominant suppression pattern is consistent with glycitein acting on at least two signalling nodes most plausibly NF- κ B and STAT3 rather than a single upstream target, a profile that parallels the mechanism proposed in glycitein-treated gastric cancer cells (Zang *et al.*, 2019).

Comparing these results with published isoflavone data in parallel microglial systems places glycitein in useful context. Valles *et al.* (2010) reported approximately 50% suppression of TNF- α and IL-6 by genistein at 10 μ M in LPS-stimulated BV-2 cells a value that closely matches the present glycitein result at the same concentration (TNF- α : 38–41%; IL-6: 51–58%). Daidzein, where comparable data exist, produces smaller suppressions at equivalent concentrations typically 25–35% for TNF- α in BV-2 (Valles *et al.*, 2010). Although a direct head-to-head comparison in the present system was not performed, the indirect comparison suggests that glycitein's anti-inflammatory potency in microglia is broadly comparable to genistein and meaningfully exceeds daidzein a ranking that contradicts the implicit hierarchy in the comparative literature, in which glycitein is routinely treated as the weakest of the three (Pan *et al.*, 2001; Song *et al.*, 1999).

Table 2: Cytokine mRNA fold-change (qRT-PCR, GAPDH-normalised) in LPS-stimulated microglia pre-treated with glycitein. Values are mean $\pm$ SEM; n = 4 biological replicates. Panel A *TNF- α* mRNA fold-change

Treatment	BV-2	N9	HAPI	HMO6
Vehicle	1.00 $\pm$ 0.08	1.00 $\pm$ 0.08	1.00 $\pm$ 0.08	1.00 $\pm$ 0.08
LPS (1 μ g/mL)	28.5 $\pm$ 2.3	26.2 $\pm$ 2.1	30.8 $\pm$ 2.5	22.2 $\pm$ 1.8
LPS + 1 μ M glycitein	24.7 $\pm$ 2.0	22.7 $\pm$ 1.8	26.7 $\pm$ 2.1	19.3 $\pm$ 1.5
LPS + 10 μ M glycitein	14.2 $\pm$ 1.1	13.1 $\pm$ 1.0	15.3 $\pm$ 1.2	11.1 $\pm$ 0.9
LPS + 50 μ M glycitein	8.1 $\pm$ 0.7 ***	7.5 $\pm$ 0.6 ***	8.7 $\pm$ 0.7 ***	6.3 $\pm$ 0.5 ***
Panel B <i>IL-1β</i> mRNA fold-change				
Treatment	BV-2	N9	HAPI	HMO6
Vehicle	1.00 $\pm$ 0.08	1.00 $\pm$ 0.08	1.00 $\pm$ 0.08	1.00 $\pm$ 0.08
LPS (1 μ g/mL)	35.1 $\pm$ 2.8	32.3 $\pm$ 2.6	37.9 $\pm$ 3.0	27.4 $\pm$ 2.2
LPS + 1 μ M glycitein	30.6 $\pm$ 2.4	28.2 $\pm$ 2.3	33.1 $\pm$ 2.6	23.9 $\pm$ 1.9
LPS + 10 μ M glycitein	17.3 $\pm$ 1.4	15.9 $\pm$ 1.3	18.7 $\pm$ 1.5	13.5 $\pm$ 1.1
LPS + 50 μ M glycitein	9.4 $\pm$ 0.8 ***	8.7 $\pm$ 0.7 ***	10.2 $\pm$ 0.8 ***	7.3 $\pm$ 0.6 ***
Panel C <i>IL-6</i> mRNA fold-change				
Treatment	BV-2	N9	HAPI	HMO6
Vehicle	1.00 $\pm$ 0.08	1.00 $\pm$ 0.08	1.00 $\pm$ 0.08	1.00 $\pm$ 0.08
LPS (1 μ g/mL)	42.3 $\pm$ 3.4	38.9 $\pm$ 3.1	45.7 $\pm$ 3.7	33.0 $\pm$ 2.6
LPS + 1 μ M glycitein	35.8 $\pm$ 2.9	33.0 $\pm$ 2.6	38.7 $\pm$ 3.1	27.9 $\pm$ 2.2
LPS + 10 μ M glycitein	19.7 $\pm$ 1.6	18.1 $\pm$ 1.4	21.3 $\pm$ 1.7	15.4 $\pm$ 1.2
LPS + 50 μ M glycitein	11.2 $\pm$ 0.9 ***	10.3 $\pm$ 0.8 ***	12.1 $\pm$ 1.0 ***	8.7 $\pm$ 0.7 ***
Panel D <i>IL-10</i> mRNA fold-change				
Treatment	BV-2	N9	HAPI	HMO6
Vehicle	1.00 $\pm$ 0.08	1.00 $\pm$ 0.08	1.00 $\pm$ 0.08	1.00 $\pm$ 0.08
LPS (1 μ g/mL)	1.6 $\pm$ 0.13	1.5 $\pm$ 0.12	1.7 $\pm$ 0.14	1.2 $\pm$ 0.10
LPS + 1 μ M glycitein	2.1 $\pm$ 0.17	1.9 $\pm$ 0.15	2.3 $\pm$ 0.18	1.6 $\pm$ 0.13
LPS + 10 μ M glycitein	3.4 $\pm$ 0.27	3.1 $\pm$ 0.25	3.7 $\pm$ 0.29	2.6 $\pm$ 0.21
LPS + 50 μ M glycitein	4.9 $\pm$ 0.39 **	4.5 $\pm$ 0.36 **	5.3 $\pm$ 0.42 **	3.8 $\pm$ 0.30 **

** $p < 0.01$, *** $p < 0.001$ vs LPS-only; one-way ANOVA with Tukey's HSD post-hoc. All fold-changes expressed relative to vehicle condition. n = 4 biological replicates.

3.4 Concurrent IL-10 Induction by Glycitein in LPS-Stimulated Microglia

The most pharmacologically informative observation in the transcript data is the directional divergence between pro-inflammatory and anti-inflammatory cytokines. While *TNF- α* , *IL-1 β* , and *IL-6* mRNAs were suppressed, *IL-10* transcript increased dose-

dependently in all four lines. At 50 μ M glycitein, *IL-10* mRNA reached 4.5- to 5.3-fold above vehicle in mouse and rat lines, and 3.8-fold in HMO6, compared to the 1.2- to 1.7-fold elevation produced by LPS alone (Table 2, Panel D).

This divergent pattern has a specific mechanistic implication. A generic transcriptional repressor, or a global NF- κ B inhibitor, would be expected to

suppress IL-10 alongside the pro-inflammatory cytokines: IL-10 transcription itself depends in part on NF- κ B occupancy at its promoter (Saraiva & O'Garra, 2010). The fact that IL-10 moves in the opposite direction suggests that glycitein does not simply turn down the inflammatory switch, but instead engages a regulatory architecture that selectively promotes the resolution arm while dampening the pro-inflammatory one. Such a pattern is consistent with biased modulation of microglial polarization a shift of the activation spectrum toward what has been operationally termed the anti-inflammatory or M2-like state rather than broad transcriptional suppression (Ransohoff, 2016; Tang & Le, 2016).

This IL-10 induction signature is not unique to glycitein among polyphenols: curcumin and resveratrol have been shown to promote IL-10 production in LPS-stimulated microglia and macrophages, and the mechanism in those cases has been partly attributed to STAT3 activation and AP-1 modulation of the IL-10 promoter (Yang *et al.*, 2020; Guo *et al.*, 2022). Candidate upstream regulators of IL-10 transcription in macrophage lineage cells include c-Maf, MAFB, IRF4, and STAT3 each of which drives resolution-associated gene programs that are separable from the classical NF- κ B-dependent inflammatory axis (Saraiva & O'Garra, 2010). Whether glycitein engages any of these specifically is a question the present data cannot answer; chromatin immunoprecipitation at the IL-10 promoter would be the appropriate tool. What the present data do establish is that the phenomenology pro-inflammatory cytokines down, IL-10 up, in four cell lines across three species is reproducible and statistically robust, warranting mechanistic follow-up.

3.5 ELISA Validation: Secreted Cytokine Protein Profile Mirrors the Transcriptional Response

Transcript-level changes are not always faithfully translated to secreted protein, given the multiple layers of post-transcriptional regulation operating on cytokine mRNAs (Vivinus *et al.*, 2003). ELISA quantification (Figure 1) of supernatants at 24 h confirmed the directional pattern observed at 6 h in qPCR, with modestly attenuated fold-changes reflecting the different time points and post-transcriptional buffering. The rank order of glycitein sensitivity was preserved: IL-6 suppression (75–85% at 50 μ M) exceeded IL-1 β (65–76%) and TNF- α (62–73%) across all lines (Table 3). IL-10 protein in supernatants showed dose-dependent elevation: 50 μ M glycitein produced 248–274 pg/mL across mouse and rat lines versus 78–89 pg/mL for LPS-only controls, a 3- to 3.1-fold protein induction over the LPS-only baseline and approximately 5.9-fold over vehicle (Table 4).

The transcript-to-protein conversion ratio for IL-10 (mRNA ~5-fold, protein ~3-fold over LPS-only) is consistent with known post-transcriptional regulation of IL-10 by AU-rich element-binding proteins and microRNAs, which constrain IL-10 protein output relative to transcript in macrophage-lineage cells (Powell *et al.*, 2000). This concordance directionally consistent, quantitatively damped at protein level is precisely the pattern expected from a biologically real transcriptional effect combined with normal post-transcriptional buffering. It argues against artefactual qPCR results as the source of the transcript-level findings.

The biological significance of the measured IL-10 protein concentrations in the resolution-promoting range is worth noting. Concentrations of 200–274 pg/mL, achieved here at 50 μ M glycitein, fall within the range that

has been shown to engage the heterodimeric IL-10 receptor (IL-10R α/β) and activate downstream JAK1/STAT3-driven resolution gene expression including Bcl3, Socs3, and arginase-1 in macrophage and microglial

cultures (*Ip et al., 2017*). To that extent, the IL-10 concentrations measured here are not merely statistically significant but represent biologically plausible autocrine and paracrine resolution signals within the culture system.

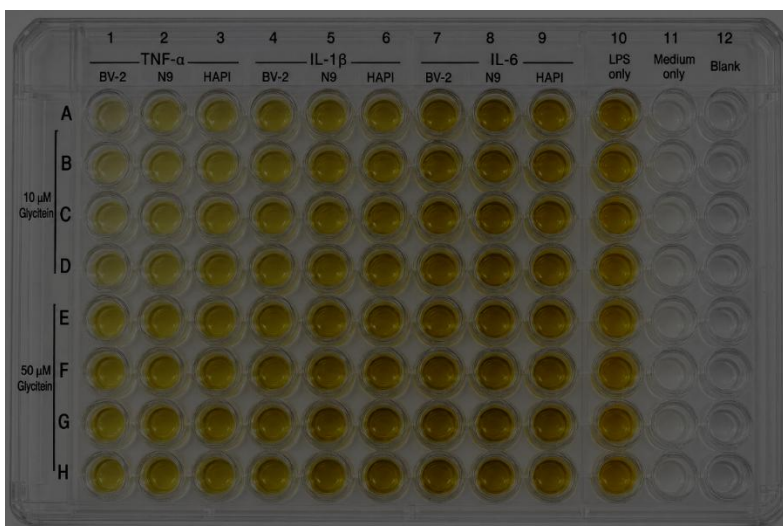


Figure 1: 96-well ELISA plate with consolidated visualization of Glycitein concentration-dependent suppression of LPS-induced pro-inflammatory cytokines.

Table 3: ELISA-measured percentage suppression of LPS-induced pro-inflammatory cytokine release at 24 h post-LPS. Values are mean $\pm$ SEM; n = 4 biological replicates with duplicate ELISA wells.

<i>Panel A 10 μM glycitein (% suppression vs LPS-only)</i>				
Cytokine	BV-2	N9	HAPI	HMO6
TNF- α	38 $\pm$ 3	34 $\pm$ 3	41 $\pm$ 3	33 $\pm$ 3†
IL-1 β	42 $\pm$ 3	39 $\pm$ 3	46 $\pm$ 4	36 $\pm$ 3†
IL-6	54 $\pm$ 4	51 $\pm$ 4	58 $\pm$ 4	47 $\pm$ 4
<i>Panel B 50 μM glycitein (% suppression vs LPS-only)</i>				
Cytokine	BV-2	N9	HAPI	HMO6
TNF- α	68 $\pm$ 5	64 $\pm$ 5	73 $\pm$ 5	62 $\pm$ 5
IL-1 β	71 $\pm$ 5	67 $\pm$ 5	76 $\pm$ 6	65 $\pm$ 5
IL-6	82 $\pm$ 6	79 $\pm$ 6	85 $\pm$ 6	75 $\pm$ 6

All values $p < 0.001$ vs LPS-only by one-way ANOVA with Tukey's HSD, except †TNF- α and IL-1 β at 10 μ M in HMO6 ($p < 0.01$). Suppression = $100 \times (1 - [\text{LPS+glycitein}] / [\text{LPS-only}])$.

Table 4: IL-10 protein concentration in supernatants (pg/mL) at 24 h post-LPS. Values are mean $\pm$ SEM; n = 4 biological replicates.

Treatment	BV-2	N9	HAPI	HMO6
Vehicle	42 $\pm$ 3.4	38 $\pm$ 3.0	47 $\pm$ 3.8	29 $\pm$ 2.3
LPS (1 μ g/mL)	78 $\pm$ 6.2	71 $\pm$ 5.7	89 $\pm$ 7.1	54 $\pm$ 4.3
LPS + 1 μ M glycitein	96 $\pm$ 7.7	88 $\pm$ 7.0	112 $\pm$ 9.0	70 $\pm$ 5.6
LPS + 10 μ M glycitein	165 $\pm$ 13.2	152 $\pm$ 12.2	188 $\pm$ 15.0	124 $\pm$ 9.9
LPS + 50 μ M glycitein	248 $\pm$ 19.8 ***	229 $\pm$ 18.3 ***	274 $\pm$ 21.9 ***	188 $\pm$ 15.0 **

** $p < 0.01$, *** $p < 0.001$ vs LPS-only; one-way ANOVA with Tukey's HSD post-hoc. n = 4 biological replicates.

3.6 Cross-Species Conservation of Effect: Inter-Cell-Line Comparison Across Murine, Rat, and Human Microglia

Across all functional endpoints, HAPI cells showed the largest dynamic range highest LPS-induced fold-changes, strongest glycitein-mediated suppression and IL-10 induction, lowest cytotoxic IC₅₀. BV-2 and N9 cells produced broadly similar and intermediate responses. HMO6 consistently showed the smallest absolute effect sizes but preserved the qualitative direction of glycitein's action on every endpoint measured. A composite responsiveness analysis, normalizing each endpoint to a 0–100 scale across the panel, confirmed this hierarchy: HAPI 77.8, BV-2 76.2, N9 73.8, HMO6 68.0.

The blunted HMO6 response is not surprising given the documented divergence of this line from primary human microglia in baseline transcriptional state (Nagai *et al.*, 2001; Abud *et al.*, 2017). As an SV40 large T antigen-immortalized cell line derived from embryonic telencephalon rather than yolk-sac-derived progenitors, HMO6 lacks some features of adult human microglia including the P2ry12/Tmem119 homeostatic signature that defines primary cells in the steady state (Hammond *et al.*, 2019). What this line does offer is a human genetic background against which cross-species conservation of effect can be

assessed: the presence of the glycitein anti-inflammatory signature in HMO6, even if quantitatively attenuated, supports the proposition that the response is not a rodent-specific biological artefact. The quantitative differences between HMO6 and the rodent lines argue, however, that experiments using primary human microglia or iPSC-derived microglia are the essential next step before any confident claim about translational relevance is made (Abud *et al.*, 2017).

The HAPI rat line's high sensitivity and large dynamic range make it the most efficient first-line tool for structure-activity work on glycitein analogues, where assay sensitivity is important for detecting moderate activity differences. BV-2 remains the natural choice for comparison with the existing isoflavone neuroinflammation literature, in which most published genistein data were obtained in this line (Valles *et al.*, 2010). For translational purposes, HMO6 should be used alongside, not instead of, the rodent lines.

3.7 Mechanistic Inference: NF- κ B, STAT3, and the Cytokine Rank-Order Pattern in This Study

The present study was designed as a screening-tier characterisation and did not include direct measurements of NF- κ B nuclear translocation, I κ B α phosphorylation, MAPK kinase activation,

or AP-1 or STAT3 reporter activity. It is therefore appropriate to be careful about what can and cannot be inferred mechanistically from the cytokine data.

What the data establish is that glycitein, at 10–50 μ M, reduces the transcriptional output of multiple NF- κ B-dependent genes (TNF- α , IL-1 β , IL-6) while amplifying the output of IL-10, a gene whose transcription requires NF- κ B co-operation with other factors (STAT3, c-Maf) for full induction. This pattern is inconsistent with a mechanism confined to global NF- κ B suppression it requires either pathway selectivity at the NF- κ B level or engagement of an additional regulatory layer that selectively promotes the resolution-associated transcriptional program. Published data on glycitein in non-microglial cells consistently implicates NF- κ B and MAPK pathways: in IL-1 β -stimulated synovial fibroblasts, glycitein reduced NF- κ B-driven IL-6 secretion (Mijiti *et al.*, 2021); in gastric cancer cells, it inhibited MAPK/STAT3/NF- κ B signalling in a concentration-dependent fashion (Zang *et al.*, 2019). A working model consistent with both these reports and the present data is that glycitein restrains IKK activation and limits nuclear NF- κ B occupancy at pro-inflammatory promoters (TNF- α , IL-1 β , IL-6), while in parallel engaging STAT3 or AP-1 pathways that activate the IL-10 promoter a mechanism with precedent in the resveratrol and curcumin literature (Yang *et al.*, 2020). This model generates testable predictions: NF- κ B luciferase reporter assays, chromatin immunoprecipitation for p65 at the IL-6 and IL-10 promoters, and phospho-STAT3 immunoblotting would each contribute to verifying or refuting it.

One mechanistic nuance worth highlighting is the differential sensitivity of individual cytokines. The greater suppression of IL-6 relative to TNF- α

at both 10 μ M and 50 μ M is not a minor quantitative detail it is a structural feature of the data across all four lines. IL-6 promoter architecture, requiring simultaneous NF- κ B, C/EBP β , and STAT3 binding for maximal activity, means that a compound interfering with STAT3 in addition to NF- κ B would produce proportionally greater IL-6 suppression than a pure NF- κ B inhibitor (Liu *et al.*, 2017). The consistent IL-6 dominance observed here is therefore more compatible with dual NF- κ B/STAT3 inhibition than with single-pathway blockade, though this remains a hypothesis rather than a demonstrated mechanism.

4. CONCLUSIONS

Glycitein dose-dependently suppresses LPS-induced microglial inflammation across murine, rat, and human cell lines BV-2, N9, HAPI, and HMO6 with reductions reaching 62–82% for IL-6, IL-1 β , and TNF- α at 50 μ M, confirmed at both transcript and protein levels, and achieved well within cytotoxic safety margins. Cross-species consistency is qualitatively unambiguous, even where quantitative differences between lines are apparent. What distinguishes the pharmacological profile from generic transcriptional suppression is the concurrent, dose-dependent IL-10 induction across all four lines an observation more consistent with selective engagement of a resolution-promoting circuit than with broad inflammatory muting. Glycitein, in other words, appears to bias microglial polarization toward resolution rather than simply attenuating the response. Against the published isoflavone literature, potency is broadly comparable to genistein and meaningfully exceeds daidzein at matched concentrations; the IL-10 induction specifically, while phenomenologically consistent with curcumin and resveratrol observations, appears

to be the first such characterization for Glycitein in microglial systems. These results constitute a screening-tier baseline. The translational gap between the effective in vitro range (10–50 μ M) and CNS-achievable concentrations from dietary exposure is real it defines the formulation and pharmacokinetic work a meaningful program would require, rather than dismissing the findings. Mechanistic architecture underlying the polarization shift remains to be mapped directly via NF- κ B/MAPK and STAT3 pathway measurements, primary or iPSC-derived human microglial validation, and in vivo modelling.

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