Degree project

GSK-3 post-transcriptionally regulates TNF-α biosynthesis in THP-1 macrophages

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Abstract

Few things are more fascinating than finding new interactions between previously unrelated pathways. Glycogen synthase kinase-3 (GSK-3), a ubiquitous kinase initially known for its role in regulating glycogen metabolism, has recently been found to be an indispensable regulator of the TLR4-mediated inflammatory response. GSK-3 inhibition exhibits potent anti-inflammatory effects by acting on both arms of the inflammatory response, reducing the secretion of pro-inflammatory cytokines, and promoting the production of anti-inflammatory cytokines. Tumor Necrosis Factor-α (TNF-α) is among the most important inflammatory cytokines. Aberrant TNF-α expression is associated with various inflammatory conditions, including sepsis and cancer. Thus, understanding the mechanisms regulating TNF-α production could reveal potential therapeutic strategies for TNF-α-associated diseases. Consequently, this study aimed to examine the effect of GSK-3 inhibition on TLR4-induced TNF-a production by THP-1 macrophages. THP-1 macrophages were stimulated with LPS and nigericin in the presence and absence of GSK-3 inhibitor, and TNF-α protein and mRNA levels were evaluated by ELISA and Real-time PCR, respectively. GSK-3 inhibition significantly attenuated TNF-α protein levels in a dose-dependent manner, whereas TNF-α mRNA levels remained unaffected, reflecting a possible post-transcriptional modulation of TNF-α biosynthesis by GSK-3. However, more comprehensive research is needed to elucidate the precise contribution of GSK-3 to TNF-α biosynthesis and to identify novel therapeutic mechanisms to alleviate inflammatory diseases associated with abnormal TNF-α production.

List of Abbreviations

ACTB	Actin Beta
AP-1	Activator protein-1
ARE	Adenylate/uridylate–rich elements
ASC	Apoptosis-associated speck-like protein containing a caspase-recruitment domain
BMDC	Bone marrow-derived dendritic cells
BMM	Bone marrow-derived macrophages
CREB	cAMP-response element binding protein
DAMP	Damage Associated Molecular
DMSO	Dimethyl sulfoxide
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GSK-3	Glycogen synthase kinase-3
IKK	I kappa B kinase
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
LPS	Lipopolysaccharide
MK2	MAP kinase-activated protein kinase 2
MyD88	Myeloid differentiation primary response 88
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NLR family pyrin domain containing 3
P38 MAPK	p38 mitogen-activated protein kinase
PAMP	Pathogen Associated Molecular Patterns
PBMC	Peripheral blood mononuclear cell
PRR	Pattern Recognition Receptors
THP-1	Human leukemia monocytic cell line
TLR	Toll-like receptor
TRIF	TIR-domain-containing adapter-inducing interferon-β
TNF-α	Tumor necrosis factor alpha
YWHAZ	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta

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Introduction

Inflammation is a crucial biological response of the mammalian immune system comprising various cell types and signaling factors that defend the body against pathogens or harmful stimuli (Jope et al., 2017). Innate immune cells, such as macrophages, neutrophils, and dendritic cells, are the first defense line against pathogens and fundamental regulators of inflammatory responses. Upon infection, innate immune cells can detect exogenous stimuli or capture damaged cellular material, subsequently resulting in a cascade of events that cause local inflammation and lead to the activation of circulating innate cells, such as circulating monocytes and macrophages, eventually leading to phagocytosis of infected cells and signaling of adaptive immune cells, T- and B-lymphocytes (Cabeza-Cabrerizo et al., 2021).

Sensing of damage or danger by innate cells is permitted by the expression of germline-encoded receptors, called Pattern Recognition Receptors (PRRs), each responsible for recognizing a specific class of molecules, known as Pathogen Associated Molecular Patterns (PAMPs) or Damage Associated Molecular Patterns (DAMPs) (Brubaker et al., 2015). DAMPs and PAMPs are activated by various self- and foreign-derived stimuli, respectively. For example, Lipopolysaccharide (LPS), a prominent component of the outer membrane of Gram-negative bacteria, and the microbial toxin, nigericin, are common activators for PAMPs, whereas ATP and silica are common DAMP activators (Brubaker et al., 2015; Swanson et al., 2019; Ubanako et al., 2019). PRRs are essentially expressed in all cells, with the most abundant families being Toll-like receptors (TLRs) and NOD-like receptors (NLRs), which can be distinguished based on their specificity or their subcellular location (Guillemin et al., 2021).

TLRs are a family of type I transmembrane receptors, which plays a critical role in mediating the inflammatory response against pathogens (Rehani et al., 2009). Currently, there are ten identified TLRs in humans, each being activated by specific microbial components (Wicherska-Pawłowska et al., 2021). TLR4 is among the most studied TLRs due to its central role in inflammatory conditions and several immune disorders (Vaure & Liu, 2014). TLR4 specifically recognizes bacterial LPS, and its signaling pathways and contribution to the immune response have been thoroughly described (Kawagoe et al., 2008; Kawai & Akira, 2010).

The NLR family consists of several proteins, which assemble into multi-protein complexes, known as inflammasomes, in response to microbial or danger signals, leading to pyroptosis and the sequential production of mature IL-18 and IL-1β (Huang et al., 2021). Among the NLRs, NLRP3 is the best-characterized inflammasome, and it has been implicated in inflammation and the pathogenesis of a wide range of inflammatory disorders, including neurodegenerative diseases and inflammatory conditions, making it an attractive therapeutic target for understanding and, ultimately, treating these diseases (Swanson et al., 2019; Wang et al., 2020). The activation of the NLRP3 inflammasome is initiated by binding of inflammatory stimuli to TLRs or cytokine receptors, such as tumor necrosis factor receptor (TNFR), leading to transcriptional upregulation of NLRP3, pro-caspase-1, and pro-IL-1β. This step is followed by an activation signal, which facilitates inflammasome assembly and the cleavage of procaspase-1 to active caspase-1, and the promotion of activation and release of IL-1β and IL-18 (Carty et al., 2019).

Upon microbial ligand interactions, each PRR family recruits specific adaptor proteins, such as Myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) for TLRs, and apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) for NLRs (Guillemin et al., 2021). This stimulates a signaling cascade comprising the NF-κB (Nuclear factor-κB) and p38 MAPK (p 38 mitogen-activated protein kinase) pathways, leading to the expression of type I and II IFNs and the production of

inflammatory mediators, such as cytokines and chemokines, which orchestrate the inflammatory response and regulate the interactions and communication between cells (Guillemin et al., 2021; Zhang & An, 2007). Depending on the nature, timing, or strength of the activating stimuli, innate cells can be programmed to produce pro-inflammatory cytokines, such as IL-1β, TNF-α, and IL-6, or anti-inflammatory cytokines, such as IL-10 or IL-13 (Lin & Leonard, 2019).

Activation of macrophages involves a cascade of events that not only initiate rapid inflammation but also ensure that the inflammatory response is limited and switched off in due time to maintain immune homeostasis and prevent any hazardous effects (Guillemin et al., 2021; Schott et al., 2014). Therefore, the production of inflammatory cytokines is tightly regulated on the transcriptional, post-transcriptional, and translational levels (Schott et al., 2014). Glycogen synthase kinase-3 (GSK3) is a ubiquitously expressed serine/threonine kinase initially known to regulate glycogen metabolism but is now also found to be a powerful regulator of the inflammatory response (Beurel et al., 2010). In mammals, GSK3 occurs in two structurally similar but functionally distinct isoforms, GSK3α and GSK3β, with GSK3α being essentially expressed in the brain, lung, and bone marrow, whereas GSK3β is prominently expressed in the brain tissues (Giambelluca et al., 2014). Increasing evidence has demonstrated that GSK-3 is an essential factor for the LPS-induced production of pro-inflammatory cytokines, such as IL-6, IL-1β, and TNF-α, by macrophages and neutrophils (Noori et al., 2020; Wang et al., 2010). In contrast, GSK-3 is reported to diminish anti-inflammatory cytokine production, such as of IL-10. Thus, GSK3 inhibitors could have beneficial anti-inflammatory effects by acting on both arms of the inflammatory response, reducing the secretion of pro-inflammatory cytokines, and promoting the production of anti-inflammatory cytokines (Jope et al., 2017).

Among the various inflammatory cytokines regulated by GSK-3, Tumor necrosis factor (TNF-α) is a multi-functional cytokine and a critical regulator of the inflammatory response. In addition to its well-characterized anti-tumor activity, TNF-α plays a pivotal role in regulating cell proliferation and adhesion and stimulation of inflammation. Aberrant TNF-α production is associated with the pathogenesis of numerous diseases, including sepsis, rheumatoid arthritis, and cancer (Sabio & Davis, 2014). Therefore, a better understanding of the mechanisms regulating TNF-α production would open vast opportunities to cure inflammatory diseases associated with abnormal TNF-α activities.

Consequently, this study aimed to gain a better understanding of the role of GSK-3 in modulating the inflammatory response, specifically its contribution to TNF-α biosynthesis in macrophages. The main objectives were to examine potential dose-dependent activities of GSK-3 inhibition on TNF-α protein and mRNA levels in LPS- and nigericin-stimulated THP-1 macrophages.

Materials and methods

Cell culture

THP1-ASC-GFP cells (Invivogen) were cultured in RPMI-1640 medium supplemented with L-glutamine (0.3 g/L) and sodium bicarbonate (2.0 g/L) (Merck) containing 10% heat-inactivated, Ultra-low endotoxin Fetal Bovine Serum (FBS) (Biowest), 10 mM HEPES (Merck), 1x penicillin-streptomycin (Merck), 0.45% glucose, and 1mM sodium pyruvate, and incubated in a humidified incubator at 37 °C and 5% CO_2. The viability of the cells was determined using the trypan blue staining method, and cultures were maintained at a density between 5×10^5 and 1.5×10^6 cells/mL by changing the culture medium every 2-3 days. Zeocin (100 µg/mL, Merck) was added to the growth medium every other passage to maintain selection pressure.

Differentiation and stimulation of THP-1 cells

THP-1-ASC-GFP reporter cells were seeded at a density of 3×10^5 cells/mL on 6-wells plates or T-25 cell culture flasks and differentiated into macrophages with Phorbol 12-myristate 13-acetate (PMA) (100 ng/mL, Invivogen). A 24 h incubation period with PMA was followed by washing with 1X PBS (Sigma-Aldrich) and a 48h resting period in fresh growth media without PMA. Subsequently, the growth media was changed, and THP-1 macrophages were incubated for 1 h with growth media only or with 0.1, 1.0, or 10 µM of the GSK-3 inhibitor SB-216763 (Merck) or <0.1% DMSO (vehicle control). The cells were then left unstimulated or stimulated with Ultrapure lipopolysaccharide (LPS) from *E. coli* K12 (500 ng/mL, Invivogen) for 4 h, followed by 45 min incubation with nigericin (10 µM, Invivogen). All incubation steps were performed in a humidified incubator at 37 °C and 5% CO_2. The differentiation of THP-1 cells into macrophages was confirmed by observing the morphological changes and adherence of THP-1 cells under an optical microscope (VWR).

Enzyme-linked immunosorbent assay (ELISA): quantification of TNF-α cytokine

Cell culture supernatant of stimulated THP-1 macrophages was aspirated and stored at −20°C until further use. The amount of TNF-α cytokine secreted into the medium was measured using the Human TNF-α ELISA Max™ Deluxe Set (Biolegend) following the manufacturer's instructions. Appropriate dilution factors were determined in preliminary experiments. The absorbance was read using The Multiskan FC microplate photometer (ThermoFisher) at 570 nm and 450 nm, and the absorbance at 570 nm was subtracted from the absorbance at 450 nm for wavelength correction. A standard graph was plotted from absorbance values of the standards using the Skanlt Software (ThermoFisher). All samples were measured in triplicates, and ELISA analysis was repeated for three experimental replicates.

RNA extraction and Reverse transcription

THP-1 macrophages were harvested by washing the cells with 1X PBS followed by scraping to collect the cells. Cells were centrifuged for 5 min at 300 x *g*, and the pellets were stored at -80 °C until further use. Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol. The quantity and purity of the extracted RNA were measured at 260 and 280 nm using the DS11 spectrophotometer (DeNovix). Samples with an A_{260}/A_{280} ratio of ≥1.8

were considered pure. Reverse transcription of the RNA was performed using 10 µl of RNA (70 – 167 ng) in a 20 µl reaction with the High-capacity RNA-to-cDNA Kit (Applied Biosystems) following the manufacturer's instructions without RNase inhibitor. Synthesized complementary DNA (cDNA) was stored at -20°C.

Primer design and Reference gene validation

A suitable reference gene was selected by evaluating the expression stability of eight putative reference genes (*ACTB, GAPDH, GUSB, TBP, HPRT1, YWHAZ, UBC,* and *PPIA*) from the Human Endogenous Control Gene Panel (TAATA Biocenter). *B2M* and *RRN18s* were excluded from the analysis as they do not span an exon-exon junction and may amplify genomic DNA. *TUBB* and *RPLP* were also excluded as they were shown to have low amplification efficiency by a previous experiment. The mRNA expression of each candidate reference gene was measured by qPCR under all the experimental conditions, and raw Cq data were exported to RefFinder (Xie et al., 2012) to evaluate the stability of the genes using four commonly used algorithms (GeNorm, NormFinder, BestKeeper, and the delta Cq method). For this project, *YWHAZ* was selected as the most stably expressed gene.

Gene-specific primers for *TNF-α* and selected reference gene, *YWHAZ*, were collected from scientific literature (Giambelluca et al., 2014; Jeon et al., 2019; Morris et al., 2014; Vandesompele et al., 2002), or designed using the Primer-Blast tool (NCBI) (See Table 1, Appendix 1). Primers selected for qPCR analysis are found in Table 1. Primers were designed to span an exon-exon junction to avoid the risk of amplifying potential contaminating genomic DNA. The presence of hairpins, self-and hetero-dimers, and potential secondary structure formation was checked using the OligoAnalyzer tool (IDT).

The amplification efficiency of the designed primer pairs was determined by performing seven-step 10-fold serial dilutions of each primer pair using a cDNA sample from LPS-stimulated THP-1 monocytes, and the PCR efficiency was automatically calculated from the standard curve by the AriaMx Software (Agilent Technologies). Primers resulting in PCR efficiency of 90-110% were considered suitable.

Table 1. qPCR primers used in this study.

Gene	Primer	Sequence (5' → 3')	qPCR Efficiency
TNF-α	Forward	AGCCCATGTTGTAGCAAACC	103.7 %
	Reverse	TGAGGTACAGGCCCTCTGAT	
YWHAZ	Forward	CGAAGCTGAAGCAGGAGAAG	97.5 %
	Reverse	TTTGTGGGACAGCATGGATG	

Real-time PCR (qPCR): Measuring TNF-α gene expression

The qPCR reaction was performed using the AriaMX Real-time PCR system (Agilent Technologies) in a 10 uL reaction volume containing 1X of the SYBR Select Master Mix (Applied Biosystems), 200 nM of each gene-specific primer, cDNA equivalent to 5 ng of the reverse-transcribed RNA, and Molecular Grade water. The following thermal cycling conditions were used: UDG activation for 2 min at 50°C, DNA Polymerase activation for 2 min at 95°C, followed by 40 cycles of denaturation

for 15 s at 95°C and annealing for 1 min at 60°C. A melt curve analysis was included after the amplification cycles following the AriaMX standard protocol to assess the amplification specificity. qPCR analysis was performed for three experimental replicates, and each sample (condition) was measured in triplicates. A no-template control (NTC) and No-reverse-transcriptase control (NO RT) were included in each qPCR experiment. Changes in TNF-α gene expression were normalized to the reference gene, *YWHAZ*, and calculated relative to the unstimulated THP-1 macrophages (calibrator) using the ΔΔCq method. Relative expression levels were log2 transformed for further data analysis.

Statistical analysis

The data presented are the mean ± Standard deviation (SD) of three experimental replicates. Data were analyzed using SPSS Statistics software, version 28 (IBM). Statistical significance was determined using independent or paired-sample t-test for comparing differences between two groups and One-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple groups comparison. Homogeneity of variances was confirmed by Levene's Test. p-value < 0.05 was considered significant.

Results

Evaluation of reference gene stability and qPCR amplification efficiency

Initially, raw qPCR data of the candidate reference genes were evaluated. *PPIA* and *UBC* were excluded from the analysis due to large variation (SD ≥ 2.0) within the Cq values of their technical replicates, suggesting that the Cq data are unreliable for the analysis (Nolan et al., 2006). Analysis of the qPCR data of *ACTB* and *GAPDH* showed a significant change in the Cq values of these genes between the unstimulated and LPS-stimulated samples, p=0.016 and p=0.033 for *ACTB* and *GAPDH*, respectively (Figure 1). The remaining genes, *YWHAZ*, and *HPRT1* did not show significant differences in their Cq values between the unstimulated and LPS-stimulated samples.

GeNorm analysis identified *ACTB* and *GAPDH* as the most suitable reference gene combination, with a stability value (M-value) of 0.244, while the least suitable genes being *HPRT1* and *TPB*, with an M-value of 0.800 and 0.839, respectively. *ACTB* was also ranked as the most suitable gene according to NormFinder and the delta Cq method. However, BestKeeper ranked *YWHAZ* as the most stably expressed gene, followed by *HPRT1*, and *TPB*, whereas *ACTB*, *GAPDH* were the least stably expressed genes according to BestKeeper.

The amplification efficiency of the designed primer pairs for TNF-α and *YWHAZ* was calculated from the slope of the standard curve generated for each primer pair, and primers with efficiency between 90-110% were considered suitable (Bustin et al., 2009). Selected primer pairs for *TNF-α* and *YWHAZ* had an efficiency of 103.7% and 97.5%, respectively (Table 1). Evaluation of the melting curves revealed single peaks for each primer pair, which further confirmed the accuracy of the amplifications.
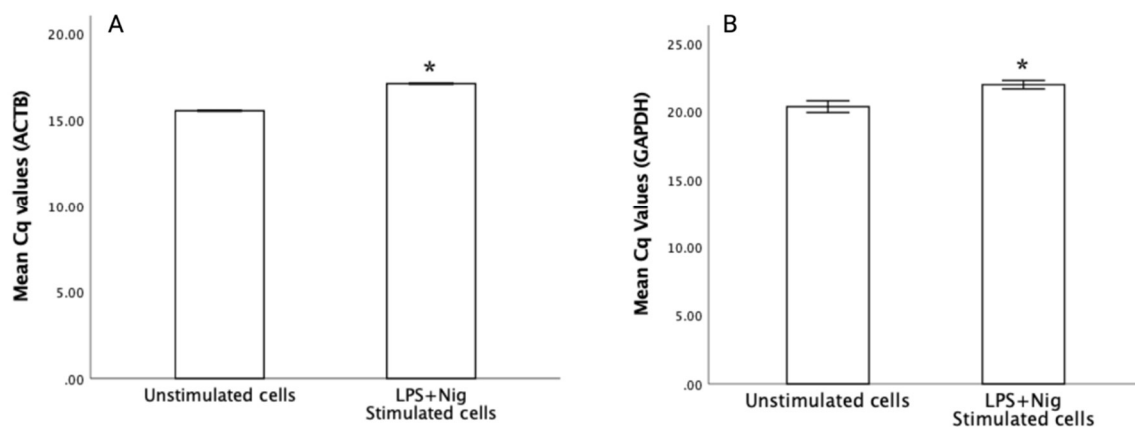

Figure 1. Significant changes in the Cq values of **(A)** *ACTB* and **(B)** *GAPDH* between the LPS-stimulated and unstimulated samples measured by qPCR. Statistical significance was determined by paired samples t-test. Bars represent the mean Cq values ± 1 SD. Asterisks represent significant differences compared to the unstimulated cells (* p<0.05).

GSK-3 inhibition dose-dependently attenuates LPS-induced TNF-α secretion

To characterize the potential anti-inflammatory activities of GSK-3, the effect of GSK-3 inhibition on the secretion of TNF-α in LPS- and nigericin-stimulated THP-1 macrophages was assessed. THP-1 cells were stimulated as previously described, and the amount of TNF-α protein released into the cell culture media was analyzed by ELISA. As shown in Figure 2A, stimulation with LPS for 4 h followed by nigericin for 45 min significantly enhanced the secretion of the TNF-α cytokine compared to the vehicle control (<0.1% DMSO) and unstimulated cells ($p<0.001$). Vehicle control was included as SB-216763 and Nigericin were dissolved in DMSO. As seen in Figure 2, the addition of DMSO (<0.1%) in the presence or absence of LPS and nigericin did not have any significant inhibitory or stimulatory influence on TNF-α secretion.

Furthermore, ELISA analysis revealed that SB-216763 treatment markedly reduced the LPS- and nigericin-induced TNF-α cytokine production in a dose-dependent manner (Figure 2B), with an approximate 50% down-regulation at 10 µM inhibitor concentration ($p=0.002$). Treatment with SB-216763 at lower concentrations did not exhibit any statistically significant effect on TNF-α protein levels, $p=0.273$ at 0.1 µM and $p=0.988$ at 1.0 µM concentration.
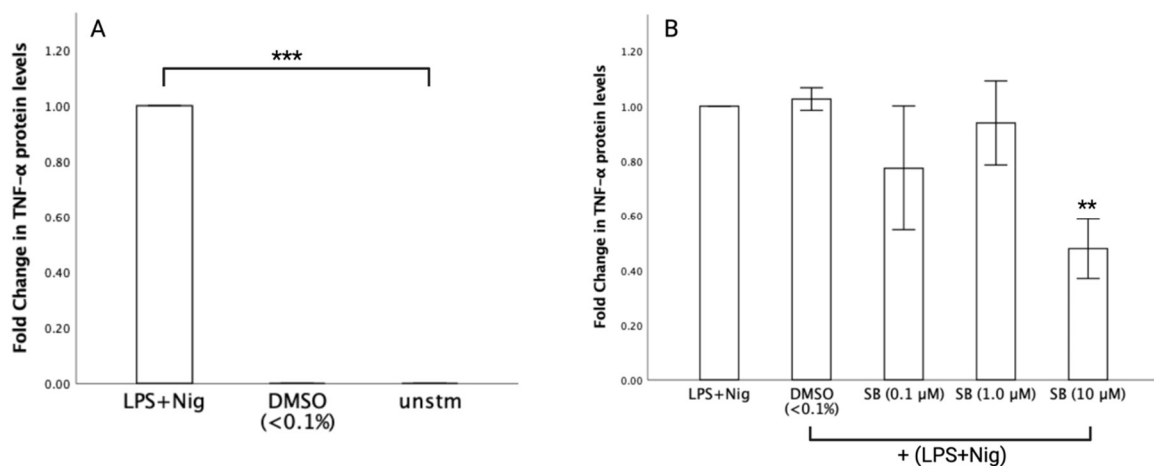

Figure 2. Effect of GSK-3 inhibition on LPS- and nigericin-induced TNF-α protein levels in THP-1 macrophages. Cells culture supernatant was collected from stimulated THP-1 macrophages, and TNF-α cytokine production was assessed by ELISA. **(A)** Stimulation with LPS and nigericin significantly enhanced TNF-α protein levels compared to the vehicle control (<0.1% DMSO) and unstimulated cells. **(B)** GSK-3 inhibition significantly reduced TNF-α protein levels in a dose-dependent manner. Statistical significance was determined by one-way ANOVA, followed by Tukey's post-hoc test (n=3 in each group). Bars represent the fold change relative to the response to LPS and nigericin ± 1 SD. Asterisks represent significant differences compared with the LPS and nigericin-stimulated cells (*** $p<0.001$ and ** $p<0.01$).

GSK-3 inhibition does not affect TNF-α mRNA levels

TNF-α mRNA levels in stimulated THP-1 macrophages were measured by qPCR to evaluate whether the observed downregulation in TNF-α secretion in response to GSK-3 inhibition was exerted at the transcriptional level. As illustrated in Figure 3A, stimulation of THP-1 macrophages with LPS and nigericin resulted in a significant increase by approximately 19 folds in *TNF-α* expression compared to the unstimulated cells (mean Log2=4.3, p<0.001). However, GSK-3 inhibition with SB-216763 failed to alter *TNF-α* mRNA levels in LPS- and nigericin-stimulated THP-1 macrophages, even at the highest inhibitor concentration, p=1.00, p=0.994, and p=0.998, for 0.1, 1.0, and 10 μM, respectively. Additionally, as shown in Figure 3B, <0.1% DMSO treatment (vehicle control) did not show any significant effects on TNF-α mRNA expression compared to the LPS and nigericin-treated cells (p=0.162).
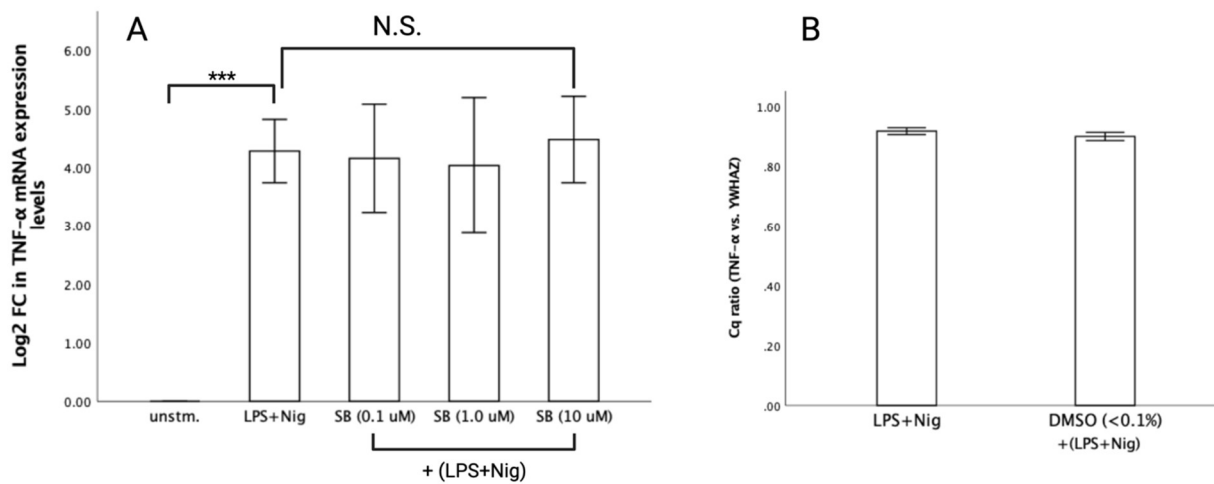

Figure 3. GSK-3 inhibition does not affect LPS- and nigericin induced TNF-α mRNA expression by THP-1 macrophages. Total RNA was harvested from stimulated THP-1 macrophages. Real-time PCR was performed to determine the expression levels of *TNF-α*. **(A)** Log2 fold change (ΔΔCt values) in *TNF-α* mRNA expression normalized to *YWHAZ* and calculated relative to unstimulated cells ± 1 SD. Statistical significance was determined by one-way ANOVA, followed by Tukey's post-hoc test (*n*=3 in each group). Homogeneity of variance was confirmed by Levene's Test (p=0.188). **(B)** Effect of DMSO (<0.1%) on LPS+Nig-induced *TNF-α* expression. Relative gene expression was obtained by calculating the ratios of Cq values between *TNF-α* and *YWHAZ*. Statistical significance was determined by independent sample t-test (*n*=3 in each group). Bars represent the mean Cq ratios between the vehicle control (<0.1% DMSO) and LPS and nigericin-stimulated cells ± 1 SD. Asterisks represent significant difference compared to the unstimulated cells (*** p<0.001). N.S. No significant difference.

Discussion

Reference gene selection

The success and reliability of the qPCR analysis are strongly dependent on the selection of the reference gene(s) used for normalization (Yang et al., 2012). Therefore, an appropriate reference gene (RG) must be carefully selected and validated for each experiment to avoid incorrect interpretations of the RT-qPCR results.

The relative gene expression in LPS-stimulated monocytes and macrophages has been in most studies normalized to the expression of *ACTB* and/or *GAPDH* as RGs (Maess et al., 2010; Morris et al., 2014; Noori et al., 2020; Piehler et al., 2010; Ubanako et al., 2019). To identify a suitable RG for the present study and to investigate whether traditional RGs are suitable for studying the gene expression in LPS- and nigericin-stimulated THP-1 macrophages in the presence or absence of GSK-3 inhibitor, the expression stability of eight candidate internal control genes, including *ACTB* and *GAPDH*, have been evaluated. A candidate RG was selected based on: (1) how the expression of the candidate RG varies among all the different experimental conditions, and (2) whether the expression of the gene changes significantly in the presence of inflammatory stimuli compared to the unstimulated samples (Cao et al., 2012). Additionally, the RefFinder tool was used to assist in the selection of appropriate RG, as it comprises four commonly used RG evaluation statistical algorithms (geNorm, NormFinder, BestKeeper, the delta Cq method).

ACTB and *GAPDH* were ranked as the most suitable RGs based on all used statistical algorithms, except for BestKeeper, which ranked *YWHAZ* as the most suitable RG. However, when analyzing the raw Cq values, *ACTB* and *GAPDH* had significantly lower Cq values for unstimulated cells compared to the LPS- and nigericin-stimulated cells (Figure 1), indicating that they are unsuitable candidates for this study (Taylor et al., 2010).

These observations suggest inadequate assessment of the intra- and inter-group variability by the used algorithms (St-Pierre et al., 2017). For example, GeNorm ranks the most suitable RG pair based on the similarities in their expression ratio and ignores the variation across the different sample groups or treatments (Andersen et al., 2004). This could explain the reason why *ACTB* and *GAPDH* were top ranked by GeNorm, as both genes had similar expression profiles; both had lower Cq values for the unstimulated samples compared to stimulated samples. A more reliable GeNorm analysis can be achieved by selecting RGs involved in distinct biological processes, because genes from the same biological processes tend to have similar expression profiles (Mehdi Khanlou & Van Bockstaele, 2012).

Consequently, with the support of the BestKeeper analysis, which uses more critical parameters to identify suitable RGs, such as standard deviation (SD), coefficient of variation (CV), and pairwise correlation, *YWHAZ* was selected as a reference gene for normalizing the gene expression data during this study as it had the lowest SD (0.13) and stable expression among all experimental conditions (Pfaffl et al., 2004).

In conclusion, the current study highlights the drawbacks of using commonly used reference genes without carefully evaluating their stability. For instance, the traditionally used reference genes, *ACTB* and *GAPDH*, showed a significantly different expression between stimulated and unstimulated THP-1 macrophages. The study further emphasizes the significance of preliminary verification and assessment of the variations in the gene expression among all tested conditions using the raw qPCR data rather than merely relying on the results of the used algorithm(s).

GSK-3 inhibition reduces TNF-α protein but not mRNA levels

TNF-α is a central regulator of the immune and inflammatory response, produced by several cell types, including macrophages, dendritic cells, natural killers (NK) cells, and T- and B- lymphocytes in response to various stress or inflammatory stimuli (Grivennikov et al., 2006). TNF-α biosynthesis is tightly regulated in a cell type- and stimulus-specific manner to maintain cellular homeostasis and prevent the development of diseases caused by aberrant TNF-α production, such as diabetes, rheumatoid arthritis, septic shock, and cancer (Falvo et al., 2010). Hence, it is of utmost importance to enhance the current understanding of the pathways involved in TNF-α biosynthesis. Earlier studies reported that GSK-3 regulates the production of inflammatory cytokines, including TNF-α, through the TLR4-MyD88-dependent pathway by promoting the activation of NF-κB while inhibiting the activity of AP-1 and CREB (Beurel et al., 2010; Götschel et al., 2008; Martin et al., 2005; Wang et al., 2010). Additionally, studies in human monocytes and mouse peripheral blood mononuclear cells (PBMC) showed that GSK-3 inhibition negatively regulates TLR4-induced pro-inflammatory cytokine production while concurrently promoting the production of the anti-inflammatory cytokine IL-10 (Cortés-Vieyra et al., 2021; Jope et al., 2007; Noori et al., 2020; Zailan et al., 2020). Collectively, these data emphasize the importance of GSK-3 in the TLR4-mediated inflammatory response.

In the present study, stimulation of THP-1 macrophages with LPS and nigericin resulted in a significant increase in TNF-α mRNA and protein levels. This observation is consistent with previous studies that demonstrated that LPS stimulation induces an inflammatory response by activating the MyD88-dependent pathway of TLR4 (Kleveta et al., 2012; Ubanako et al., 2019). Signal transduction via MyD88 initiates the sequential events of recruitment of IRAK family (interleukin-1 receptor-associated kinase), activation of TRAF6 (TNF receptor-associated factor 6), ubiquitination of TAK1 (Transforming growth factor-β), phosphorylation of MAPKs (mitogen-activated protein kinases) and IKK (I kappa B kinase), activation of NF-κB and AP-1 and translocation to the nucleus, and subsequent transcription of pro-and anti-inflammatory cytokines, respectively (Cui et al., 2014; Kawagoe et al., 2008; Liu et al., 2018).

However, the observations of the present study differ significantly from those of some recent reports. While TNF-α protein production was significantly impaired by GSK-3 inhibition in LPS- and nigericin-stimulated THP-1 macrophages, TNF-α mRNA levels remained unaffected. This lack of effect of GSK-3 inhibition on TNF-α transcriptional level may partially be due to the concurrent treatment of LPS-stimulated THP-1 macrophages with nigericin, which among many other stimuli, serves as the second signal required for inducing NLRP3 inflammasome activation following a priming step with LPS (Swanson et al., 2019). Nigericin triggers potassium efflux, which was shown to induce calcium influx to regulate the assembly and activation of the NLRP3 inflammasome (Yaron et al., 2015). On the other hand, previous studies have revealed that calcium influx promotes TNF-α gene transcription by binding to specific binding motifs on the TNF promoter, without altering the *de novo* protein synthesis (Brown et al., 2004; Falvo et al., 2000). Nevertheless, a better understanding of the regulatory role of GSK-3 in the TLR4-mediated TNF-α production can be achieved by measuring TNF-α mRNA levels in LPS-stimulated macrophages without the addition of nigericin to eliminate the possibility of triggering other pathways that might contribute to TNF-α production.

Emerging studies have also revealed that GSK-3 contributes to the transcriptional activation of NF-κB in a promoter-specific manner, suggesting that GSK-3 selectively promotes the expression of a subset of NF-κB target genes, and GSK-3 inhibition does not influence all NF-κB activities (Jope et al., 2007). For example, Steinbrecher et al. (2005) reported that GSK-3 was required for the NF-κB-mediated IL-6 and monocyte chemoattractant protein-1 (MCP-1) expression, while it was dispensable for the expression of IκBα and macrophage inflammatory protein-2 (MIP-2) (Steinbrecher et al., 2005). These selective, gene-specific activities of GSK-3 could also explain the lack of influence of GSK-3 inhibition on TNF-α mRNA expression observed in this study. However, the knowledge of the mechanisms by which GSK-3 selects its target molecules is inadequate, but it may be due to a specific sequence within the mRNA of the target molecule. Further studies clarifying the selective involvement of GSK-3 in regulating the inflammatory response in human microphages would be insightful.

Furthermore, the observed effects of GSK-3 inhibition on LPS-induced TNF-α protein levels but not mRNA levels may also reflect potential post-transcriptional regulation of TNF-α biosynthesis by GSK-3. TNF-α is early and rapidly secreted cytokine in response to TLR4 stimulation (Falvo et al., 2010). In resting cells, TNF-α shows relatively high mRNA levels compared to most of the other cytokines, but its translation is strongly suppressed. Upon stimulation with LPS, TNF-α mRNA expression oscillates, and the translational suppression is relieved, leading to immediate induction of the protein production (Schott et al., 2014). The suppression of TNF-α translation is thought to be caused by specific proteins that bind to the Adenylate-uridylate (AU)-rich elements (ARE) found within the 3′-untranslated region of the TNF-α mRNA (Gueydan et al., 1999). The activity of ARE-binding-proteins is regulated by the p38 MAPK pathway and its target kinase, MAP kinase-activated protein kinase 2 (MK2). For example, previous studies have indicated that the activation of the p38 MAPK pathway by LPS phosphorylates ZFP36, an ARE-binding protein, which suppresses TNF-α mRNA translation in resting cells to protect against its overexpression (Schott et al., 2014). Phosphorylation of ZFP36 leads to the stabilization of TNF-α mRNA and initiation of its translation (Tiedje et al., 2012).

Interestingly, the TRIF-dependent pathway of TLR4 has been shown to modulate TNF-α translation through direct regulation of the p38 MAPK pathway. For example, Gais et al. (2010) indicated that p38 and MK2 activation was severely reduced in *Trif*-deficient dendritic cells, while their activation was unaltered in *Myd88*-deficient bone marrow-derived dendritic cells (BMDC) and macrophages (BMM). The study further reported that MK2 inhibition or *Trif*-gene inactivation markedly impaired LPS-induced TNF-α protein levels in BMM and BMDC, without affecting the mRNA levels (Gais et al., 2010). Collectively, these data suggest that the TLR4-TRIF-dependent signaling pathway is strongly involved in TNF-α translational regulation through direct activation of the p38 protein kinase, MK2. In the current study, GSK-3 inhibition showed a similar phenomenon; reduced TNF-α production and unaffected mRNA levels. Therefore, it is tempting to speculate that GSK-3 might contribute to TNF-α translation by regulating the activity of specific ARE-binding-proteins through the TRIF-dependent activation of the p38-MK2 signaling cascade.

Taken together, this study demonstrated that TNF-α production in stimulated THP-1 macrophages can be regulated by GSK-3 at the post-transcriptional level. Future studies elucidating the selective ability of GSK3 to control the repertoire of produced inflammatory cytokines, especially its contribution to TNF-α biosynthesis, through the TLR4-MyD88-dependent and MyD88-independent pathways could reveal novel therapeutic targets that facilitate the treatment of inflammatory conditions caused by uncontrolled TNF-α production.

Conclusion

In conclusion, this study showed that GSK-3 inhibition significantly reduced TNF-α production by LPS-and nigericin-stimulated THP-1 macrophages without altering TNF-α mRNA levels, reflecting a potential post-transcriptional regulation of TNF-α biosynthesis by GSK-3. TNF-α is a potent mediator of the inflammatory response. Aberrant TNF-α production is implicated in the pathogenesis of numerous inflammatory disorders, including sepsis and cancer. Therefore, therapeutic strategies targeting TNF-α are being evaluated. GSK-3 inhibitors hold promise as potential therapeutic agents against several inflammatory conditions due to their ability to attenuate the production of pro-inflammatory cytokines while promoting the production of anti-inflammatory cytokines. Therefore, future studies elucidating the overall role of GSK-3 in the TLR signaling pathways and its precise contribution to the TNF-α biosynthesis would not only help understand the regulatory mechanisms involved in TNF-α biosynthesis and overall signaling but would also promote the identification of selective therapeutic mechanisms superior to the current TNF-α targeted therapeutics. However, it is worth mentioning that TNF-α and GSK-3 are pleiotropic molecules involved in myriad signaling pathways and physiological processes other than the inflammatory response, and their inhibition must be viewed as a double-edged sword as it might lead to unforeseeable side effects.

Ethical considerations

The THP-1 cell line used in the present study is an immortalized human leukemia monocytic cell line derived from the blood of a year-old boy with acute monocytic leukemia (Tsuchiya et al., 1980). In addition to their low costs and absence of any ethical constraints regarding their use, THP-1 cells exhibit a high resemblance to primary monocytes and macrophages in both morphology and differentiation properties and can be differentiated into various types of macrophages and dendritic cells (Chanput et al., 2014). Hence, it is not surprising that the THP-1 cell line has been widely exploited in immunology research to study the functions, regulatory mechanisms, and signaling pathways of monocytes and macrophages (Bosshart & Heinzelmann, 2016). THP-1 cells also display several advantages over human primary monocytes and macrophages. For example, THP-1 cells have a homogenous genetic background, which decreases the variability in the cell phenotype and eases the reproducibility of the experiment (Cousins et al., 2003). Furthermore, compared to peripheral blood mononuclear cell (PBMC)-derived monocytes, THP-1 cells exhibit a higher growth rate and can be stored for a considerably longer time without impacting the sensitivity or activity of the cells (Chanput et al., 2014).

However, it is vital to underline the main drawbacks of using the THP-1 cell line. For example, the malignant background of THP-1 cells presents a high risk of experimental bias. Hence, culturing these cells outside their natural environment may result in biased sensitivity and different responses compared to normal somatic cells in their natural environment (Schildberger et al., 2013). Additionally, mimicking relevant interactions between the target cell and neighboring cells, as in primary tissues, is mightily challenging using the THP-1 cell line (Chanput et al., 2014). Moreover, studies comparing the functions and responses between the THP-1 monocytes and human PBMC-derived monocytes have revealed slight differences in the gene expression and cytokine production levels. For example, PBMC monocytes showed higher sensitivity to LPS-stimulation and resulted in greater inflammatory cytokine production (IL-6, IL-8, and IL-10) compared to THP-1 cells (Schildberger et al., 2013). Other studies showed that TLR3 mRNA expression was significantly weaker in THP-1 cells compared to PBMC monocytes upon LPS treatment (Hijiya et al., 2002).

Collectively, the THP-1 cell line has been shown to be a suitable and simplified *in vitro* model to study the activities of monocytes and macrophages and determine their responses to external stimuli, thereby promoting the understanding of the immune response and facilitating the identification of novel therapeutic strategies for immune disorders, such as inflammatory conditions and cancer. Additionally, the use of THP-1 cell lines enables conducting high-quality research in accordance with the 3Rs framework (Russell & Burch, 1959) as it provides an alternative method to compensate for the need to sacrifice animals for research. However, a thorough investigation demonstrating the correspondence of the *in vitro* findings to those from *ex vivo* or *in vivo* should be first executed. Carefully optimized animal and human experimentation will be required to make definite conclusions about the activity and effectiveness of potential treatment strategies in the maintenance of human health and quality of life.

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Appendices

Appendix 1: Designed primers for qPCR

Table 1. Details of dsigned primers for *TNF-α* and *YWHAZ*.

Gene	Primer	Sequence (5' → 3')	GC content (%)	Tm (°C)	Amplicon Length (bp)	qPCR Efficiency (%)
TNF-α	Forward	AGCCCATGTTGTAGCAAACC	50	59	134	103.7
	Reverse	TGAGGTACAGGCCCTCTGAT	55	59		
	Forward	TCAATCGGCCCGACTATCTC	55	58	65	116.5
	Reverse	CAGGGCAATGATCCCAAAGT	50	56		
	Forward	CATCTACTCCCAGGTCCTCTT	52	58	105	118.2
	Reverse	TTGACCTTGGTCTGGTAGGA	50	58		
YWHAZ	Forward	CGAAGCTGAAGCAGGAGAAG	55	59	110	97.5
	Reverse	TTTGTGGGACAGCATGGATG	50	59		
	Forward	TGATCCCCAATGCTTCACAAG	48	60	76	130.3
	Reverse	GCCAAGTAACGGTAGTAATCTCC	48	60		