Degree project

UNIVERSITY OF SKÖVDE

The effect of GSK-3 inhibitor SB216763 on the expression and secretion of IL-8 in THP-1 ASC GFP macrophages cells

Bachelor Degree Project in Bioscience

First Cycle 30 credits

Spring term 2022

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Abstract

Inflammation is a part of the innate immune system. It protects the body against foreign invaders such as bacteria and viruses. Inflammation helps to restore the body by removing harmful stimuli and starting the healing process. Inflammation is produced in response to damage-associated molecular patterns (DAMPS) or pathogen-associated molecular patterns (PAMPS). Glycogen synthase kinase 3 (GSK-3) is a key regulator of a variety of pathways, making it a promising therapeutic target. Therefore, this experiment aims to see how inhibiting GSK-3 affects the generation of IL-8 in THP-1 ASC GFP macrophage cells. For this study qPCR was used to measure IL-8 expression, while ELISA was used for protein secretion. An ANOVA test was utilized for the statistical analysis. Obtained results from this study showed that there is a significant difference between stimulated cells with LPS and nigericin against unstimulated samples both in protein and mRNA levels. When it comes to the stimulated cells against inhibited cells, the ANOVA test showed there is no significant difference between the samples both in protein and mRNA levels. This might suggest that GSK-3 does not influence the development of inflammasomes in THP-1 macrophage cells. Another possible reason is that other pathways such as the MAPK and JAK-STAT may mask potential inhibitory effects on the NLRP3 inflammasome pathway by producing even more IL-8, which interfered with qPCR and ELISA results. In conclusion, additional research is needed to confirm the involvement of GSK-3 in NLRP3 inflammation.

Contents

List of abbreviations

AIM2	Absent-in-melanoma
ASC	Apoptosis-associated speck-like protein containing a caspase
CARD	Caspase recruitment domain
cDNA	Complementary DNA
CLRs	C-type lectin receptors
Cq	Quantification cycle
DAMPS	Damage-associated molecular pattern
ELISA	Enzyme-linked immunosorbent assay
GSK-3	Glycogen synthase kinase
IL-8	Interleukin 8
IL-18	Interleukin 18
IL-1β	Interleukin 1- βeta
LPS	lipopolysaccharide
NACHT	central nucleotide domain termed
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRs	NOD-like receptors.
NOD	nucleotide-binding oligomerization domain
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern-recognition receptors.
PYD	pyrin domain
qPCR	Quantitative polymerase chain reaction
SD	Standard deviation
TLR	Toll-like receptors

Introduction

Inflammation is a component of the immune system and is part of the body's defence mechanism against outside threats such as bacteria and viruses. Inflammation protects the body by boosting blood flow to injured areas or infected by pathogens. This can result in swelling, heat, redness, and tissue healing. Inflammation is a part of the innate immune system that is found in the body from birth (Kanneganti, 2015). Innate immunity is a basic form of immunity and is an evolutionarily ancient immunity that is found in many animals and plants. It acts as the first line of protection against infections that invade the body (Kanneganti, 2015).

The mammalian innate immunity consists of almost 50 pattern-recognition receptors (PRRs). They are divided into two classes the membrane-bound C-type lectin receptors (CLRs) and TLRs and the cytosolic PRRs. Examples of this are absent in melanoma 2 (AIM2)-like receptors (ALRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), RLRs as well as other nucleic acid-sensing receptors (Pandey et al., 2015; Brubaker et al., 2015). As reviewed by Lee et al. (2007) by binding to various adaptors, PAMPs like lipopolysaccharide (LPS) activate the PRRs and several downstream kinases, which in turn activate transcription factors. Consequently, cytokines are created as a result of innate immune reactions. (Lee et al., 2007). Adaptive immunity is also part of the immune system. Adaptive immunity takes a longer time to react to pathogens than the innate immune system. Therefore, adaptive immunity is considered the second line of defence after innate immunity (Bonilla et al., 2010). These two immune systems work in tandem and are essential to the body's capacity to defend itself.

Inflammasomes are cytoplasmic complexes that consist of multiple proteins. It is produced in response to damage-associated molecular patterns (DAMPS) or pathogen-associated molecular patterns (PAMPs) and acts as a molecular platform for activation of the cysteine protease caspase-1 (Martinon et al., 2002). An example of some of the inflammasomes that have been discovered and classified as an NLR family are NLRP1, NLRP3, NLRP6, and NLRC4. The NLR inflammasome's most well-studied and characterized member is NLRP3. The NLRP3 protein is a component of the inflammasome, and it is mostly expressed in macrophages. NLRP3 consists of three domains: the C-terminal leucine-rich repeats (LRRs), the central nucleotide-binding and oligomerization domain (NACHT) domain, and the N-terminal effector pyrin domain (PYD). The Apoptosis-associated speck-like protein containing a caspase (ASC) is made up of the PYD, through which it associates with NLRP3, and a C-terminal caspase recruitment domain (CARD). Caspase-1 contains (p10 and p20) and the CARD domain through which it associates with ASC (Yang et al., 2020).

For the inflammasome to produce IL-1β and IL-18 it must go through two different signals. The first is the priming signal where PAMPs will attach to the toll-like receptor (TLR). Then the nuclear factor-kappa B (NF-κB) pathway will be activated and finally produce pro-IL-1β and pro-IL-18 and NLRP3. The second signal is the activation signal. This step is necessary for the inflammasome formation (assembly). In this step, caspase-1 will cleave the pro-IL-18 and pro-IL-1β into active IL-18 and IL-1β form which will be secreted (Lebreton et al., 2018) (Figure 1).

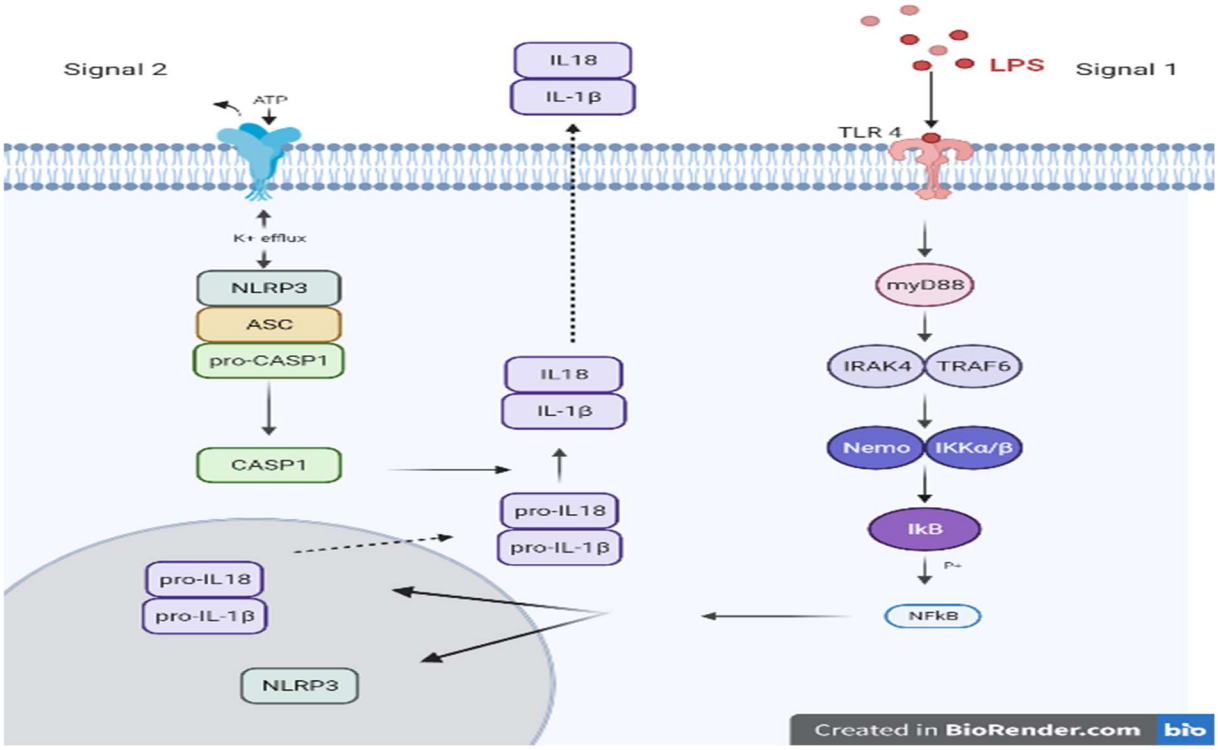

Figure 1. The two signals that induce inflammation. The first signal is the priming signal which acts through the NF-κB pathway and ends by producing pro-IL-18 or pro-IL-1β. In the second signal which is the inflammasome pathway the pro-interleukins cleave into their active forms.

The non-canonical NLRP3 inflammasome is a different type of inflammation. This type of activation requires caspase 4 (also known as caspase-11 in mice) and caspase 5 (Kayagaki et al.,2011). The caspase in the non-canonical inflammasome can sense the gram-negative bacteria LPS without a receptor. This enables immune cells to recognize infections that have devised ways to avoid detection by receptors. Caspase-1 together with caspase 4/5 cleaves the pore to form gasdermin-D (GSDMD). GSDMD causes the cell membrane to permeabilize, which leads to pyroptosis, an inflammatory programmed cell death (Kayagaki et al., 2015).

GSK-3 was initially identified as an enzyme that phosphorylates glycogen synthase. Martin et al. (2005) first discovered the role of Glycogen synthase kinase 3 (GSK-3) in the regulation of inflammation. Recent research has demonstrated the ability of GSK-3 to phosphorylate more than 100 substrates, making it a confluence of many signalling pathways and the control of many cellular functions (Martin et al., 2005). GSK-3 has been connected to several disorders, including Alzheimer's disease, type 2 diabetes, and inflammation (Jope et al., 2004). GSK-3 is a serine/threonine protein kinase that phosphorylates Ser21 in GSK-3α Isozyme or Ser9 in GSK-3β Isozyme. Activation of GSK-3 is essential to produce several pro-inflammatory cytokines (Zhao et al., 2020).

A study that used LPS-stimulated human monocytes showed that the production of pro-and anti-inflammatory cytokines is differently regulated by GSK-3 (Wang et al., 2018). GSK-3 inhibition significantly decreased the production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, and IFN-γ whereas the production of anti-inflammatory cytokines like (IL-10) resulted in a significant increase (Beurel, 2011; Wang et al., 2018).

The chemokine IL-8 (also known as CXCL8) is largely produced by macrophages. IL-8 is an inflammatory protein that helps neutrophils and other immune cells find their way to the infection

site. A range of biological activities have been linked to this chemokine, including cell proliferation, tissue remodelling, and angiogenesis (El Ayadi et al., 2018).

Aim and objectives

As mentioned, GSK-3 phosphorylates molecules in several distinct pathways. It is involved in a range of disorders, making it an important drug development target. However, no study has shown how the inhibition of GSK-3 affects the expression and secretion of IL-8 in THP-1 macrophages. Therefore, this study aimed to see how inhibiting GSK-3 affects the expression and secretion of IL-8 and the creation of ASC-specks in THP-1 ASC GFP macrophage cells. This was accomplished by treating THP-1 cells with 0.1 μM, 1 μM, and 10 μM of the GSK-3 inhibitor SB216763 and stimulating these cells with LPS and nigericin. IL-8 secretion was measured using Sandwich Enzyme-Linked Immunosorbent Assays (ELISA). The IL-8 expression was measured using the quantitative polymerase chain reaction (qPCR).

Materials and methods

Cell culturing and stimulation

THP-1 ASC-GFP cells (Invivogen) were cultured at 37°C, 5% CO2 in RPMI 1640 with L-glutamine (Merck), 10% heat-inactivated Fetal Bovine Serum (FBS) (Biowest), 1 mM sodium pyruvate (Sigma Aldrich), 1 X penicillin-streptomycin (Merck), 10 mM HEPES (Merck) and 0.45% glucose (Sigma Aldrich). The cells went through up to 12 passages with 0.01 µg/mL zeocin being added to every other passage before being differentiated. Cells were then subsequently differentiated using 100 ng/mL PMA on a 6-well plate in a humidified incubator for 24 hours at 37°C, 5% CO2. Followed by two washes with culture medium and a 48-hour incubation in fresh media at 37°C and 5% CO2 in a humidified incubator.

Differentiated cells were then treated with 0.1 µM, 1 µM, and 10 µM of SB216763 inhibitor (Merck) and 0.1 % DMSO. Except for the control, cells were stimulated with LPS; 500 ng/mL for 4 h. For the activation step 10 µM nigericin was added for 45 min.

Primer design and validation

Primers were designed using (NCBI primer-blast) (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), (Primer3Plus) (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). When primer pair IL-8-4 Table 1. was taken from a previous study (Chanput et al., 2010). Primer pairs were analysed using the IDT OligoAnalyzer (https://eu.idtdna.com/calc/analyzer.

All primer pairs were validated using complementary DNA (cDNA) from THP-1 cells. The cDNA was then diluted with a 1:10 dilution factor and with 7 points of dilution starting at 100 ng. A qPCR reaction was then performed using SYBR® Select Master Mix and with 200 nM of each primer. Efficiency was then determined using AriaMx Software. The primer pair with the best efficiency level (between 90 and 110%) was used for further reactions.

RNA extraction

RNA extraction was performed using the RNeasy Plus Mini Kit (Qiagen). All cells were extracted in the same way. Cells were pelleted by centrifugation for 5 min. at 300 x g. The number of pelleted cells was < $5x10^6$. After lysing pelleted cells with RLT plus, for homogenization cells were passed through a 20-gauge needle fitted to an RNase-free syringe 10 times. Other steps of the extraction were according to the RNeasy Plus Mini handbook (Qiagen). After the extraction cells concentration and purity were measured using a DS11 spectrophotometer (DeNovix) Table 1 (Appendix).

Reverse transcription and qPCR

After extracting the RNA, the High-Capacity RNA-to-cDNA™ Kit and protocol (Applied Biosystems) were used to generate complementary DNA to the extracted RNA. All the processes were done according to the protocol. A PTC-200 Thermal Cycler (BIO-RAD) was used in this process.

The SYBR® Select Master Mix protocol (Applied Biosystems) was utilized for the qPCR reactions. IL-8 primers (FP 5'-CACTGCGCCAACACAGAAAT-3') (RP 5'-TTCTCAGCCCTCTTCAAAAACTTC-3')

with a concentration of 200 nM, and cDNA with a 0.5 ng was used in all reactions. No reverse transcriptase control (NRT) and non-template control (NTC) were run in triplicate.

ELISA

A sandwich ELISA was used to measure IL-8 secretion in the supernatant. ELISA MAX™ Deluxe Set Human kit IL-8 and protocol were used. ELISA was performed in triplicate. The required dilution factors were determined experimentally. The acceptable limit for CV% was below 15%.

Statistical analysis

The ΔΔCq values were used to see if there were any significant differences between samples. The independent sample T-test and one-way ANOVA with Tukey's post hoc analysis were used. IBM® SPSS ® Statistics 28.0.1.0 software (IBM Corp) was used for all statistical analysis. Raw Cq values were efficiency corrected using GenEx version 6.0. Samples were considered statistically significant if the P-value was < 0.05.

Ethical considerations, future aspects, and the impact on society

The cells that were used in this experiment were commercial THP-1 ASC-GFP macrophages. So, they can be used in inflammasome research, but no human or animal organisms were involved in this particular experiment. As a result, there were no ethical considerations taken in the laboratory setup. KLARA software was used to assess the risks of this experiment to ensure that the chemicals used in this experiment had no negative impact on the environment and to guarantee that these chemicals are appropriately managed and properly disposed of so that they do not damage the environment.

This is important immunological research that helps people understand the role of GSK-3 in regulating IL-8 production in THP-1 ASC-GFP macrophages. The results from this paper could play an important role for future researchers to understand the role of GSK-3 in macrophage cells. This research uses commercial THP-1 instead of using actual animals in the experiment. This goes with the 3R principle that was launched in 1960 by Russell & Burch (1959). The 3R principle is referred to as Reduction, Refinement, and Replacement. In the case of this laboratory, animal use is replaced with cell culture which will have a positive impact on society.

Results

Primer validation

A total of four primer pairs were tested for the IL-8 gene. Two primer pairs were inside the acceptable efficiency range of (90 % – 110%). IL-8-2 got an efficiency of 99.8% and 108% for IL-8-3. The rest of the primer pairs are found in Table 1. This study used the primer pair IL-8-2 since it was the most efficient. For the reference gene YWHAZ, the efficiency was 107.3%. The standard curves for the IL-8-2 primers and the YWHAZ reference gene are shown in Figures 2 and 3 (Appendix).

Table 1. Primer efficiency for IL-8 and YWHAZ primers.

Primer pair	Forward primer (5'->3')	Reverse primer (5'->3')	Efficiency
Il8-1	AGTTTTTGAAGAGGGCTGAGA	TGCTTGAAGTTTCACTGGCATC	117.4%
Il8-2	CACTGCGCCAACACAGAAAT	TTCTCAGCCCTCTTCAAAAACTTC	99.8%
Il8-3	CAGGAATTGAATGGGTTTGC	AAACCAAGGCACAGTGGAAC	108%
Il8-4	CTGATTTCTGCAGCTCTGTG	GGGTGGAAAGGTTTGGAGTATG	116%
YWHAZ	CGAGCCAGCAGAACATCCA	ACCTACGGGCTCCTACAACA	107.3%

Validation of the housekeeping gene

Of the seven reference genes investigated YWHAZ and HPRT1 were the two most stable genes. According to BestKeeper YWHAZ and HPRT1 got the least standard deviation with 0.124 and 0.132 respectively. The two most unstable genes were GAPDH and GUSB with standard deviations of 0.786 and 0.85 respectively as shown in Figure 2. BestKeeper compares the genes based on the standard deviation of the cq values. In Figure 2, the y-axis shows the standard deviation values. The x-axis shows the most stable genes on the left and the least stable genes on the right.

Based on the comprehensive tool in RefFinder algorithm the most stable genes are PPIA and ACTB while the least stable genes are HPRT1 and TPB Figure 1 (Appendix) RefFinder comprehensive tool finds the geometric mean of the weights of each gene calculated by each program, including geNorm, NormFinder, Bestkeeper, and the comparative delta Cq method. The YWHAZ gene was chosen for this investigation because it showed the least change when compared to the other reference genes analysed. Furthermore, it was unaffected by experimental conditions and demonstrated consistent Cq values across all samples.

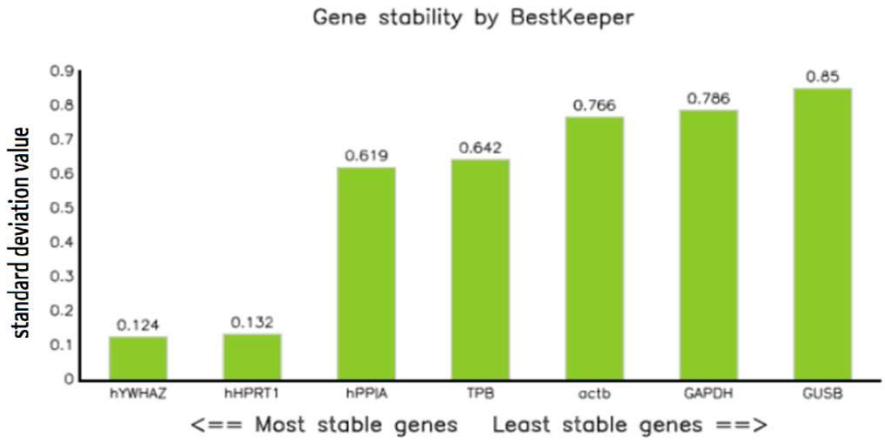

Figure 2. Bar chart showing the most stable genes on the left side and the least stable genes on the right, as determined using BestKeeper. Standard deviation values are shown on the y-axis.

IL-8 expression

The stimulated cells with LPS and nigericin revealed a considerable upregulation by 64.76 folds when compared to the IL-8 mRNA levels of PMA differentiated THP-1 cells without stimuli. In the chart below Figure 3, the x-axis shows the difference between the unstimulated samples and the stimulated samples with LPS and nigericin. The y-axis shows the log2 fold change of the three experimental replicates. An independent sample T-test was done to see if this upregulation was significant. A P-value of ≤ 0.006. Indicating that there is a significant difference between the two samples (Figure 3). In this test n=3 in each group and the unstimulated samples act as the control. Using the ΔΔCq method, the expression of the IL-8 gene was normalized to that of the YWHAZ gene.

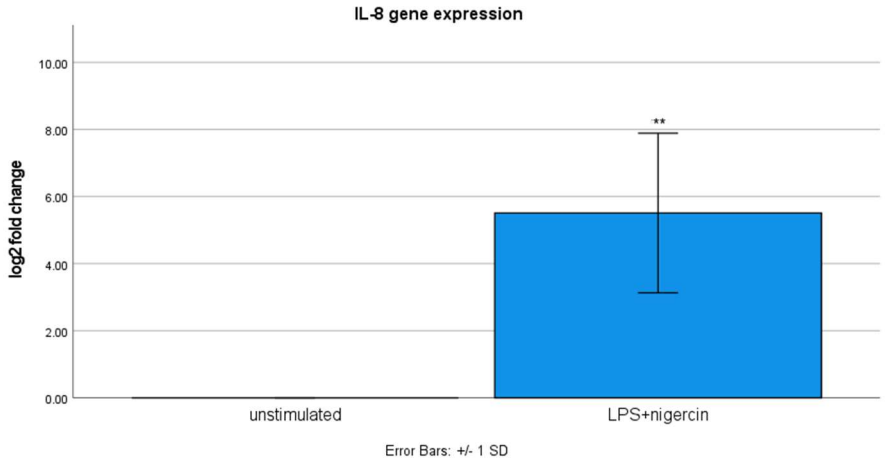

Figure 3. Bar chart showing the log 2-fold change ± 1 SD for IL-8 expression in THP-1 macrophages. The calibrator in this test was an unstimulated sample. The statistical test used was the independent sample t-test (P =≤ 0.001) (n=3 in each group). Asterisks denote significant differences from the control (**P <0.01).

When it comes to the stimulated sample with LPS + nigericin versus the inhibited samples with 0.1 µM, 1 µM, and 10 µM GSK-3 inhibitor. The inhibited samples are compared to the stimulated sample with LPS + nigericin here which means that the stimulated sample with LPS + nigericin acts as the calibrator. The difference between inhibited samples and their calibrator (stimulated sample) is shown on the x-axis. The log2 fold change is displayed on the y axis and it represents the three experimental replicates. The one-way ANOVA test showed a P-value of 0.64 which means that the samples are not significantly different (Figure 4). Therefore, no further investigation was done on these samples. Data were normalized using the ΔΔCq method.

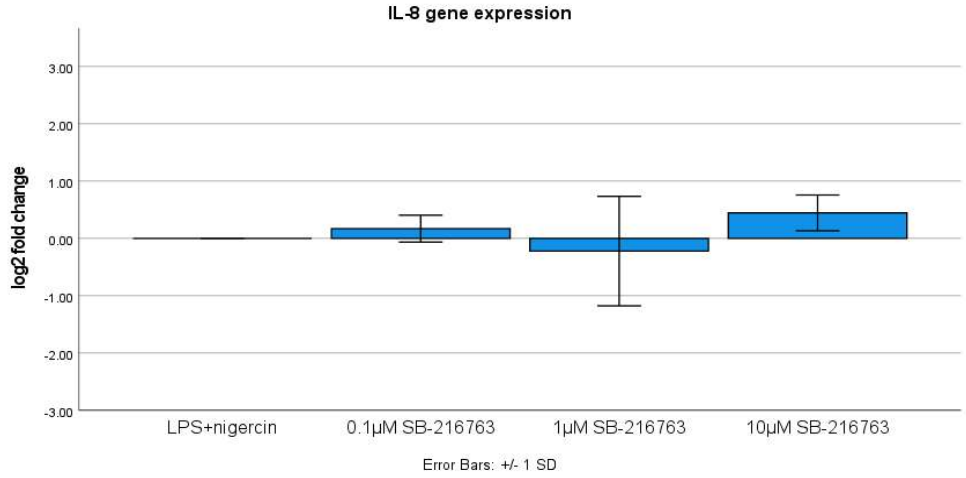

Figure 4. Bar chart showing the log 2-fold change ± 1 SD for IL-8 expression in stimulated THP-1 macrophages with and without inhibitor. The calibrator in this test was LPS + nigericin (stimulated sample). The statistical test used was one-way ANOVA (n = 3 in each group). P = 0.64 which means there is no significant difference between the groups.

IL-8 secretion

In Figure 5 unstimulated sample (PMA differentiated THP-1) was compared with the stimulated cells with LPS + nigericin. In Figure 5 the unstimulated sample shows a mean concentration of ≈2000 pg/ml. when the stimulated sample shows a mean concentration of ≈ 8500 pg/ml. to see if these concentrations were significantly different an independent sample t-test was used. An independent sample t-test was done, and it showed a P-value of < 0.001 which is less than the significant level of 0.05 (Figure 5). This means there is a significant difference between the unstimulated and the stimulated sample (LPS + nigericin) at the protein level. In this test n = 3 and error bars with ± 1 SD were used.

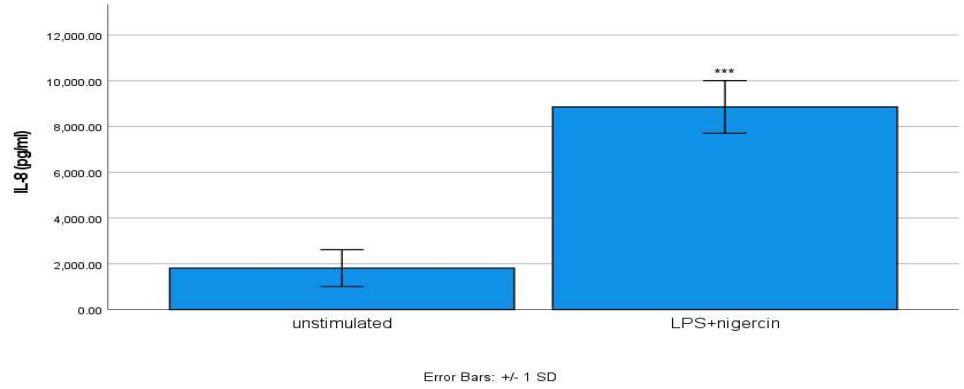

Figure 5. Bar chart showing the mean concentration (pg/ml) ± 1 SD for IL-8 secretion in THP-1 macrophage cells. The statistical test used was an independent sample t-test (P =< 0.001) (n = 3 in each group). Asterisks denote significant differences from the control (***p <0.001).

All samples are normally distributed with a P-value of 0.088 for 0.1 µM of SB-216763, 0.123 for 1 µM of SB-216763, and 0.454 for 10 µM of SB-216763. When the stimulated (LPS + nigericin) and the unstimulated got a P-value of 0.094 and 0.253 respectively, the sample size for this test was (n = 3). To check if the samples are significantly different first test of homogeneity of variances was used to check if the samples have equal variance, the P-value for this test was =0.077 which is more than 0.05 which means that samples have equal variance and thus Tukey's post-hoc test can be used. For further analysis, one-way ANOVA was used to test for any significance and the P-value = <0.001 which means one or more samples are significantly different from the other. To check the different samples Tukey's post-hoc test was used, and it showed that there is no significant difference between the stimulated samples and the 0.1 µM, 1 µM and 10 µM of SB-216763 inhibitor concentrations with P-values = 0.997, 0.884, 0.967 respectively (Figure 6).

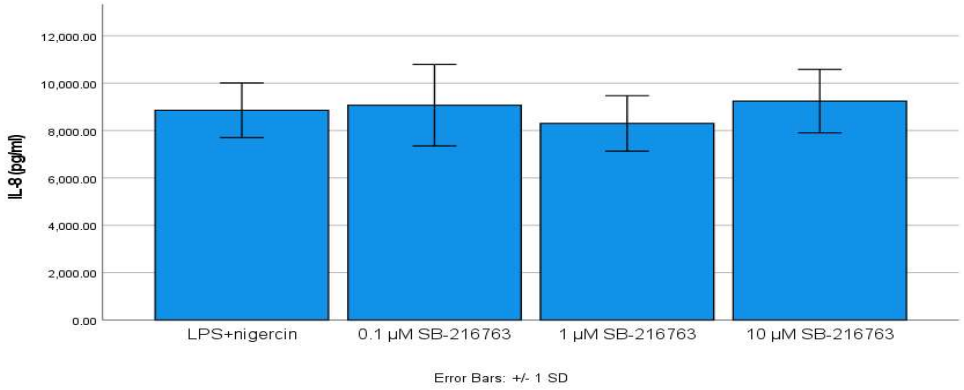

Figure 6. Bar chart showing the mean concentration (pg/ml) ± 1 SD for IL-8 secretion in stimulated THP-1 macrophages with and without inhibitor. The statistical test used was one-way ANOVA followed by Tuckey's post-hoc test (P =0.997, 0.884, 0.967) respectively (n = 3 in each group). which means no significant difference between the groups.

Discussion

GSK-3 phosphorylates molecules in several distinct pathways. It is involved in a range of disorders making it an important drug development target (Jope et al., 2004). GSK-3 is linked to a variety of diseases like inflammation, and various studies have revealed how GSK-3 regulates the inflammasome and influences the release of many cytokines in a variety of cell types, including monocytes, microglia, and macrophages (Jope et al., 2004). However, no study has shown how the inhibition of GSK-3 affects the expression and secretion of IL-8 in THP-1 macrophages. Therefore, this study aimed to see how inhibiting GSK-3 would affect IL-8 production at the mRNA and protein levels using the qPCR and ELISA methods, respectively.

Selection of reference genes

Finding a stable reference gene is crucial to have reliable and normalized qPCR results. Reference genes are important to adjust the changes in the target gene expression level caused by technical errors or cDNA synthesis (Freitas et al., 2019). Using a common reference gene like GADPH or B-actin is not possible for every experiment. Even though these genes are supposed to remain stable, transcription variability may prevent them from doing so in certain experimental situations. Therefore, a new set of reference genes should be tested for every experiment since there are no such things as stable reference genes in every condition (Tanaka et al., 2017). The RefFinder website was used to evaluate a total of seven reference genes for this experiment (Figure 3). RefFinder is a robust tool for assessing and screening reference genes, as well as providing a weight to each gene based on rankings from four distinct databases, including geNorm, Normfinder, BestKeeper, and the comparative ΔCq technique. To calculate the geometric mean and generate a full comprehensive rating for the four databases (Xie et al., 2012). In this study, the most appropriate reference gene found was YWHAZ from BestKeeper (Figure 4). BestKeeper simply uses the coefficient of variance (CV) and the standard deviation (SD) value to rank the genes. The gene with the smallest standard deviation is considered the most stable reference gene (Pfaffl et al., 2004). In the RefFinder comprehensive tool, PPIA was shown to be the most stable reference gene, while the YWHAZ gene was ranked in the fourth place Figure 1 (Appendix). In this study, the YWHAZ gene was chosen because it showed the smallest fluctuations compared to other tested reference genes. In addition, it was not affected by experimental factors and showed stable Cq values in all samples and these trades are the most important when selecting a reference gene (Chervoneva et al., 2010).

A previous study by Cao et al. (2012) evaluated a set of reference genes to use in normalizing gene expression in THP-1 and K562 cells cultured in vitro. PPIA, ACTB, GAPDH, TBP, and HPRT1 were among the genes evaluated in the reference gene set. The LPS-stimulated or un-stimulated cells were used and the raw Cq values were used to analyse the results using BestKeeper, geNorm, and NormFinder (Cao et al., 2012). The findings from this study indicate that the often-used reference genes ACTB, GAPDH, and TBP are inappropriate to use, and they may potentially lead to misinterpretation. According to NormFinder and Genorm PPIA has also been shown as unstable. In a similar study using THP-1 monocyte cells, the results showed PPIA as a not suitable gene for THP-1 cells using Normfinder, Bestkeeper, and GeNorm (Diehl et al., 2022).

IL-8 production increases in LPS-induced THP-1 macrophage cells.

Figures 3 and 5 show stimulated (LPS and nigericin) vs. unstimulated samples. The results showed a significant difference between the two samples, both in mRNA and protein levels. Bacterial lipopolysaccharide (LPS) was used for the priming step in this experiment when nigericin was

used as a second signal. This could be due to the activation of TLR4, which leads to the phosphorylation of the NF-κB pathway. The NF-κB pathway is one of the important pathways that gets induced after LPS priming. Therefore, IL-8 production was increased for both the protein and the mRNA levels after phosphorylated NF-κB translocated to the nucleus (Lund et al., 2016).

Consistent with the results in Figure 5. A previous study used 0.5 μg/mL of LPS for 3 h, followed by ATP (5 mM) for 1 h in THP-1 macrophage cells treated with LPS. An ELISA test revealed significantly increased levels of several pro-inflammatory cytokines in immune and non-immune cells, including IL-6, IL-8, and TNF- α (Suryavanshi et al., 2022). Another study investigated the role of the NF-κB, STAT3, or AP-1 pathway activation in LPS-mediated cytokine expression in THP-1 cells. This study treated THP-1 cells for four hours with or without PDTC (NF-κB inhibitor) or static (a STAT3 inhibitor). Then, cells were exposed for 48 hours to 1 g/ml LPS. And then the expression of IL-8 was measured. The results showed a significant increase in the cells treated with LPS alone which corresponds to the results in Figure 3. On the other hand, compared to cells treated with LPS alone, PDTC or static treatment significantly reduced the LPS-induced expression of IL-8 and TNF- α in THP-1 cells (Liu et al., 2018) The results from these two studies indicate that both NF-κB and STAT3 pathways play an important role in producing IL-8 after LPS treatment.

The GSK3 inhibitor did not affect the production of IL-8 in LPS-stimulated THP-1 ASC GFP macrophage cells

As for the qPCR and ELISA results (Figures 4 and 6), In the stimulated samples with LPS + nigericin versus different concentrations of SB-216763 inhibitor. As indicated in the results section there are no significant differences between these samples. Both in mRNA and protein levels. ANOVA followed by Tuckey's post-hoc test was used to show the significant difference in qPCR and ELISA results (Figures 4 and 6). Even though it is challenging to determine whether small data sets are normally distributed and may show non-homogenous variance (Figure 4). But small data sets require the use of the parametric test, while nonparametric methods lack statistical power with small samples (Grech & Calleja, 2018). Therefore, it was found that the optimum option to show the significant difference is to use one-way ANOVA followed by Tuckey's post-hoc test.

A previous study that used adipose tissue and skeletal muscle from women with gestational diabetes was stimulated with LPS for 20 h and inhibited with SB216763 (20 μM) for 60 min. TNF-α, IL-1, IL-6, and IL-8 gene expression and secretion were significantly decreased after GSK-3 inhibitor SB216763 treatment (Cortés-Vieyra et al., 2021). Another study was done by Wang et al. (2006) and treated the cells with SB216763 (10 μM) GSK-3 inhibitor for 4 h. It determined that inhibition of GSK-3 induces IL-8 expression in HT29 cells unlike the findings in the current study that found no significant difference in IL-8 expression level (Figure 4) or IL-8 secretion (Figure 6). Along with Wang et al. (2006) findings, other studies that used other cell types also found that GSK-3 inhibition induces IL-8 expression and secretion (Tang et al., 2001; Rao et al., 2004). The results from these previous studies identified that GSK-3 regulates IL-8 production in many different cell types. This may be because these previous studies used higher doses of the GSK-3 inhibitor for a shorter time or lower doses of the GSK-3 inhibitor for a longer time, which resulted in the appearance of an effect. In this report, the doses of GSK-3 inhibitor were 0.1, 1, and 10 μM, and cells were inhibited for only 30 min. Therefore, inhibiting these cells for a longer period may have some effects when using such low doses of a GSK-3 inhibitor.

Having no significant difference in Figures 4 and 6 could also be due to several reasons. First, GSK-3 simply does not regulate the cytokine production in THP-1 macrophages, so maybe even if this study was repeated in different circumstances the results would be the same. Another reason is that IL-8 is also produced as a response to several pathways other than through NF-κB and NLRP3.

For example, it is also produced by the MAPK signalling pathway that is also activated by LPS through the ERK, JNK, and p39 pathways (Yin et al., 2019). IL-8 is also produced by the JAK-STAT signalling pathway, which can be activated by binding to ligands such as growth factors, interferons, or interleukins (Bousoik et al., 2018). So, the JAK-STAT pathway could be activated by the interleukins produced by the other pathways such as the NF-κB and MAPK pathways. All these pathways can also be found in macrophage cells (Li et al., 2021). These mentioned pathways can easily interfere with the inhibition of cytokine production in the inflammation pathway by producing even more cytokines, which will make it impossible to see any of the inhibition effects. Therefore, potential inhibitory effects on the NLRP3 inflammasome pathway may be hidden by other pathways. A possible solution to this could be to inhibit any other pathways that could produce IL-8. For example, the MAPK pathway can be blocked by utilizing the U0126 inhibitor, which prevents ERK phosphorylation induction (Wang et al., 2006).

Conclusion

Although several studies have shown that GSK-3 regulates the generation of cytokines in various cell types, this was not the case in this study. According to the qPCR and ELISA results, the effect of GSK-3 inhibition on IL-8 production was not observed in stimulated THP-1 macrophages. This could simply mean that GSK-3 does not regulate the inflammasome production in THP-1 macrophage cells and therefore GSK-3 is not the ideal therapeutic target to regulate the NLRP3 inflammasome. Or that using the GSK-3 inhibitor for a longer time instead of 30 min. could have shown some effect. Another possible reason is that the inhibition of the inflammasome occurred but could not be observed due to the activation of other pathways such as the MAPK and JAK-STAT signalling pathways that produced IL-8 that interfered with qPCR and ELISA results. A way to solve this in the future is by trying to inhibit other possible pathways. For example, the MAPK pathway could be inhibited by using a U0126 inhibitor which would cause blockage of the induction of ERK phosphorylation. In conclusion, more studies are required to be certain of the GSK-3 role in inflammation.

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Appendixes

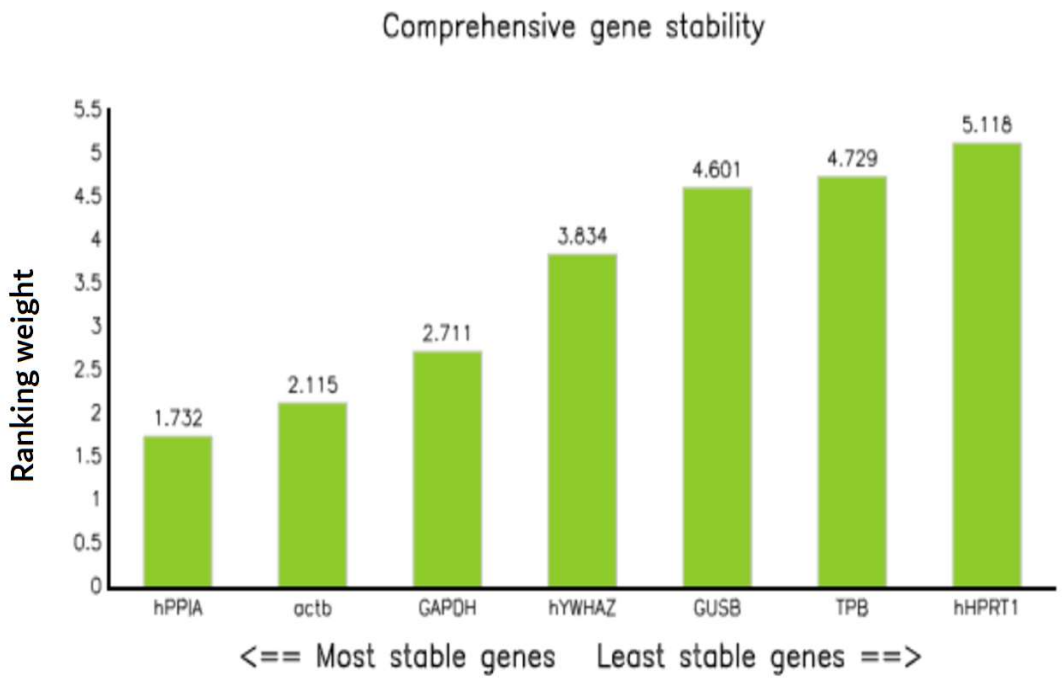

Figure 1. Bar chart from RefFinder showing the most stable genes to the least stable genes from left to right. RefFinder show their result by weighting the geometric mean from four programs geNorm, NormFinder, Bestkeeper, and the comparative delta Cq method.

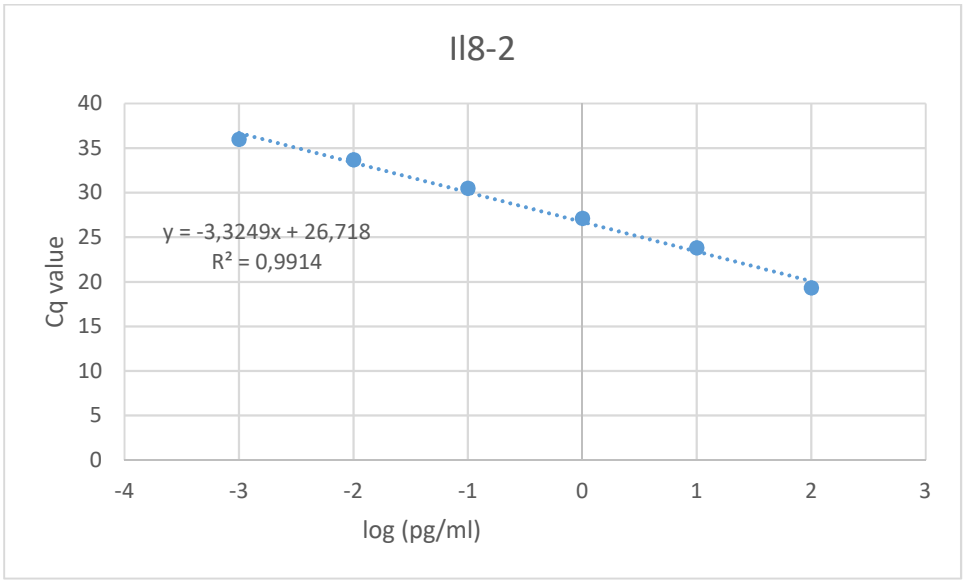

Figure 2. The standard curves for IL-8-2 primer showing a slope of -3.325 and R^2 value of 0.99.

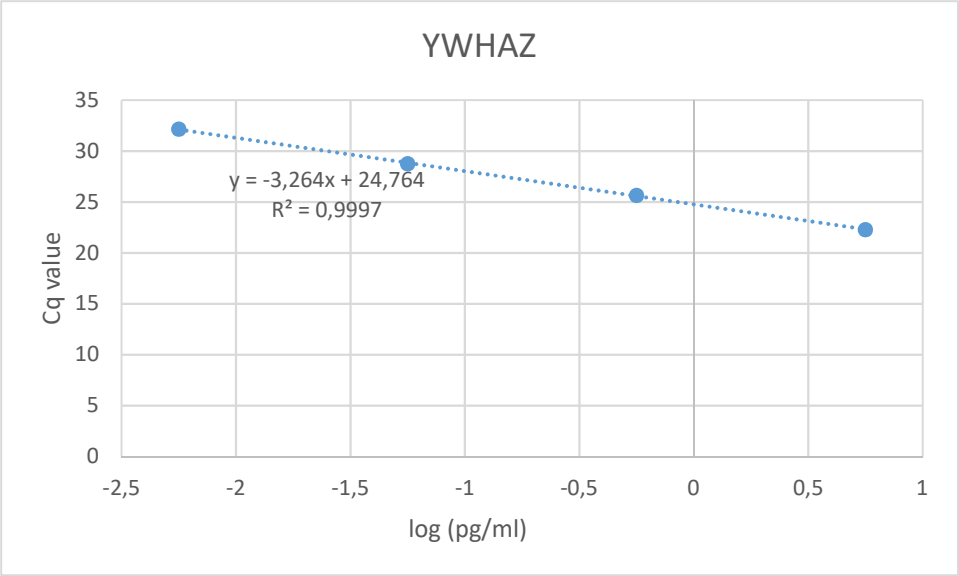

Figure 3 The standard curves for YWHAZ primer showing a slope of -3.264 and R^2 value of 0.99.

Table 1. Concentrations and purity after RNA extraction.

Samples	Replicates	Concentration ng/µl	A 260/A 230	A 260/A 280
0.1 uM	1	24.085	0.663	2.014
	2	14.64	1.511	2.086
	3	20.651	0.3	2.073
1 uM	1	25.446	1.115	1.842
	2	17.29	0.219	2.069
	3	27.893	1.17	1.985
10 uM	1	22.025	0.522	2.040
	2	17.15	0.22	2.14
	3	24.4	0.585	1.999
stimulated	1	33.367	0.406	1.980
	2	24.5	1.66	2.054
	3	16.35	1.566	1.863
THP-1 diff cells	1	31.57	1.160	2.062
	2	26.015	0.265	2.14
	3	16.37	0.136	1.602