Degree project

The GSK-3 inhibitor has no effect on production of IL-1β in LPS- and Nigericin-stimulated THP-1 macrophages

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Abstract

Inflammation is the body's natural defense reaction and is known since ancient times. The inflammation is divided into two main phases, acute and chronic inflammation dependent on the process and cellular mechanisms of the inflammation. Inflammation has become to be an important field in research by biomedical research where it is included in many cellular processes thus being phagocytosis, chemotaxis, mitosis, and cell differentiation. Inflammasomes are pro-inflammatory intracellular multimeric protein complexes that introduce the activation of pro-inflammatory cytokines, such as interleukin-1β and interleukin-18, upon trigger by PAMPs and DAMPs signals. The most studied inflammasome is the NLRP3 inflammasome that is activated by various trigger signals, like DAMPs, ATP, uric acid crystals and amyloid-β fibrils. GSK-3β is a kinase that controls various cellular processes, such as inflammation by regulating the activity of abundant transcription factors that are valuable for cytokine production. The aim of this thesis project was to investigate if GSK-3 Inhibitor IV, SB-216763, in a concentration-dependent manner had an effect on production of IL-1β in LPS- and Nigericin-stimulated THP-1 ASC-GFP-macrophages. In addition to the gene expression analysis of IL-1β, the amount of secreted IL-1β, and the possible correlation between treated THP-1 cells with and without GSK-3 inhibitor evaluated. The gene expression analysis was performed by using qPCR and the amount of secreted IL-1β was done using sandwich enzyme-linked immunosorbent assay. The results from this study showed no significant difference in gene expression and amount secreted of IL-1β in THP-1 cells when treated with the GSK-3 Inhibitor IV, SB-216763.

List of abbreviations

ASC	Apoptosis-associated Speck-like protein Containing a CARD
ATP	Adenosine Triphosphate
cAMP	Cyclic Adenosine Monophosphate
CARD	Caspase Activation and Recruitment Domain
DAMPs	Damage-Associated Molecular Pattern
FBS	Fetal Bovine Serum
GSK	Glycogen Synthase Kinase
IL-1β	Interleukin-1β
LPS	Lipopolysaccharides
LRR	Leucine-Rich Repeat
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear Factor of Activated T cells
NLRP3	NLR family Pyrin domain containing 3
NOD	Nucleotide-binding and Oligomerization Domain
PAMPs	Pathogen-Associated Molecular Patterns
PMA	Phorbol Myristate Acetate
PRRs	Pattern Recognition Receptors
PYD	Pyrin domain
ROS	Reactive Oxygen Species

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Introduction

The innate immune system

Pathogens are disease-causing microorganisms, and they are categorized into four large groups: viruses, bacteria, fungi and parasites. Living organisms have adopted a protection against these pathogens at various levels of defense and the first line of defense is the anatomic and chemical barriers. If these barriers are breached, the immune system assume control to recover homeostasis and when cells are confronted with danger signals or pathogens, the innate immune system is the first to be activated (Murphy et al., 2017). The anatomic and chemical barriers are also part of the innate immunity, together with physiologic, phagocytic and endocytic, and inflammatory barries (Warrington et al., 2011). The efficiency of the response is highly vital as bacteria and viruses have a rapid doubling time and the innate immune system is beneficial as it is a rapid although relatively non-specific response to infection and tissue damages (Hato & Dagher, 2015). The innate immune system coordinates with the adaptive immune system, which is activated by phagocytic cells, for instance dendritic cells, and by cytokines such as IL-1β (Clark & Kupper, 2005). The primary effectors of innate immunity are specific sensor cells such as macrophages, neutrophils and dendritic cells that recognizes inflammatory inducers. These are molecular components special to pathogens, such as bacterial lipopolysaccharides (LPS), that usually is not encountered in the extracellular space (Murphy et al., 2017). The sensor cells express a variety of innate pathogen recognition receptors (PRRs) that recognizes molecules called pathogen-associated molecular patterns (PAMPs), or molecules called damage-associated molecular patterns (DAMPs) such as intracellular proteins, ATP and uric acid discharged by damaged cells. There are two valuable types of PRRs. Firstly, the transmembrane proteins called Toll-like receptors (TLRs) that recognizes PAMPs obtained from extracellular bacteria or bacteria brought to vesicular pathways by phagocytosis. Secondly, the cytoplasmic proteins called NOD-like receptors (NLRs) that detect components of bacterial peptidoglycan (McDonald & Levy, 2019; Murphy et al., 2017).

Sensor cells also produce inflammatory mediators and aid with amplification of immune responses. The two crucial groups of inflammatory mediators, cytokines and chemokines, are acting on additional immune cells, for example the innate natural killer (NK) cells and the innate lymphoid cells (ILCs). Cytokines and chemokines are secreted proteins that behave in a similar way as hormones regarding the transfer of essential signals to additional immune cells (Murphy et al., 2017). The difference between these inflammatory mediators is that cytokines are an large and varied group of pro- or anti-inflammatory factors that are classified into families depending on their structural homology or based on their receptors. Chemokines, on the other hand, are a group of small secreted proteins within the family of cytokine which also includes interleukins, interferons, the transforming growth factors (TNF), and tumor necrosis factor (TGF). The generic function of the cytokine family is to activate the migration of cells (L. Ferreira et al., 2019; Ramesh et al., 2013). The process which inflammatory mediators respond to tissue damage is called inflammation (V. Stankov, 2012). The gathering of white blood cells, leukocytes, at the place of injury is one of the most significant characteristic of inflammation (Murphy et al., 2017). The sensor cells

induce inflammatory response with aim to reduce or eliminate infection by the pathogen and to repair damaged tissues (Murphy et al., 2017; Kelley, Jeltema, Duan & He, 2019). According to Chen et al. (2017), despite the fact that inflammatory response relies upon the specific nature of the primary stimulus and its position of activity in the body, these responses share a common mechanism: 1) cell surface receptors recognize pathogens; 2) inflammatory pathways are activated; 3) inflammatory proteins are delivered; and 4) inflammatory cells are gathered.

The Inflammasome

Inflammasomes are intracellular multimeric protein complexes that activate pro-inflammatory cytokines, such as interleukin-1β (IL- 1β) and interleukin-18 (IL-18), triggered by PAMPs and DAMPs signals (Murphy et al., 2017). The inflammasome is in most cases composed of a receptor molecule, the adaptor molecule apoptosis-associated speck-like protein including a caspase recruitment domain (ASC) and an effector molecule such as caspase-1 (Wilson & Cassel, 2010). Nucleotide oligomerization domain (NOD)-like receptors (NLRs) are intracellular proteins found within the cytoplasm as monomers, where they are in an inactive state. NLRs are the largest family of PRRs that protects the cytosolic environment (Feerick & McKernan, 2016). All NLR group members have a NACHT domain or nucleotide-binding and oligomerization domain (NOD) in common that mediates self-oligomerization and nearly all have a C-terminal leucine-rich repeat (LRR) domain to control activator sensing and ligand binding, and a variable N-terminal domain primarily for protein interactions (Sharma & Kanneganti, 2016; Di Virgilio, 2013). The NLR family can be subdivided into five groups based on the N terminus; NLRA containing an acidic transactivation domain, NLRP containing a pyrin domain (PYD), NLRC a caspase activation and recruitment domain (CARD), NLRB containing a baculoviral inhibitory repeat (BIR)-like domain and NLRX with a non-homologous amino-terminal (Wilson & Cassel, 2010; Sharma & Kanneganti, 2016). Thus far, approximately 22 NLRs have been discovered in the human genome and out of these only a few genes have been completely established as qualified to form inflammasomes, being NLRP1, NLRP3 and NLRC4. Others, like NLRP6 and NLRP12, are assumed to be inflammasome sensors and possibly form inflammasome complexes (Di Virgilio, 2013; Sharma & Kanneganti, 2016).

The NLR pyrin domain containing 3 (NLRP3) inflammasome is the most commonly studied and characterized inflammasomes so far, partially because of its association with various human diseases and it is the main target in this study (Kawashima et al., 2017). First defined by Martinon (2002), the NLRP3 is a protein composed of three domains: an amino-terminal pyrin domain (PYD), a carboxyl-terminal leucine-rich repeat (LRR) and a nucleotide-binding and oligomerization domain (NOD). The NOD domain has ATPase activity which is crucial for the self-association and oligomerization of the NLRP3 inflammasome (Biasizzo & Kopitar-Jerala, 2020). In NLRP3 inflammasome, the enrollment of pro-caspase-1 is by the adaptor associated speck- like protein (ASC) that mediates interaction between NLRP3 and the effector caspase-1. Further, pro-caspase-1 self-cleave into active caspase-1 and later cleaves immature pro-inflammatory cytokines, pro-IL-1β and pro-IL-18, to the biological effective and mature forms that are secreted (Kelley et al., 2019; Owen et al., 2013).

Interleukin-1β is a pro-inflammatory cytokine produced and secreted by different cell types, mainly by monocytes and macrophages as well as by endothelial and epithelial cells

upon stimulation (La Pine & Hill, 2011; Lopez-Castejon & Brough, 2011). It is crucial mediator of the inflammatory response and autoimmune disorders and serves a valuable role in controlling immune responses and inflammation (Lopez-Castejon & Brough, 2011; Ren & Torres, 2009). Besides the key homeostatic functions of IL-1β in organism in normal condition, like regulation of ingestion, sleep, and temperature, it can be harmful with overproduction of IL-1β. The overproduction is a common cause for various disease conditions, such as Alzheimer's disease, multiple sclerosis, rheumatoid arthritis, neuropathic pain, inflammatory bowel disease, osteoarthritis, and vascular disease (Braddock & Quinn, 2004; Dinarello, 1996; Dinarello, 2004). Lipopolysaccharide is an important pro-inflammatory agent that stimulates monocytes and macrophages through TLR4, successive activates signaling pathways that produce IL-1β and other inflammatory cytokines (Fang et al., 2004; Ren & Torres, 2009).

Priming and activation of the NLRP3 Inflammasome

For an active NLRP3 inflammasome complex to be formed in macrophages there are two separate signals required, the first being the priming signal while the second is the activation signal to complete the formation of NLRP3 inflammasome (Figure 1) (Kelley et al., 2019). Priming occurs when ligands for toll-like receptors such as LPS, binds to TLRs and it is a fundamental step in the inflammasome establishment. Other priming signals are microbial components or endogenous cytokines (Kelley et al., 2019). A major effect of the priming signal is the upregulation of gene expression of NLRP3, pro-interleukin-1β (pro-IL-1β), pro-IL-18 and procaspase-1. This upregulation is normally initiated by the recognition of PAMPs, such as lipopolysaccharides and peptidoglycans, by PRRs and further activation of the nuclear factor-κB (NF-κB), a transcriptional activator of NLRP3 and pro-IL-1β genes which both serves as essential mediators of inflammatory responses (Juliana et al., 2012; Lio et al., 2017; Bauernfeind et al., 2009).

The second signal, the activation signal, is necessary to complete the formation of an active NLRP3 inflammasome and it can be activated by DAMPs like adenosine triphosphate and hyaluronan. Besides these molecular patterns there are others triggers that can activate the NLRP3 inflammasome. Examples of these are bacterial, viral and fungal PAMPs, toxins, and DAMPs such as crystals and aggregates like β-amyloid (Lamkanfi & Dixit, 2012). In order to detect these triggers the inflammasome observes host-derived factors that are changed by these activators. According to Lamkanfi and Dixit (2014), the activation can occur by five different cellular effects; potassium efflux, translocation of NLRP3 to mitochondria, reactive oxygen species (ROS) produced by mitochondria, presence of mtDNA and lysosome in cytosol, and lysosomal cathepsins. Other studies suggest that not all of these effects appear with all NLRP3-activating agents, or they are linked with several inflammasomes (Bauernfeind et al., 2011; Muñoz-Planillo et al., 2013; Pétrilli et al., 2007; Zhou et al., 2011).
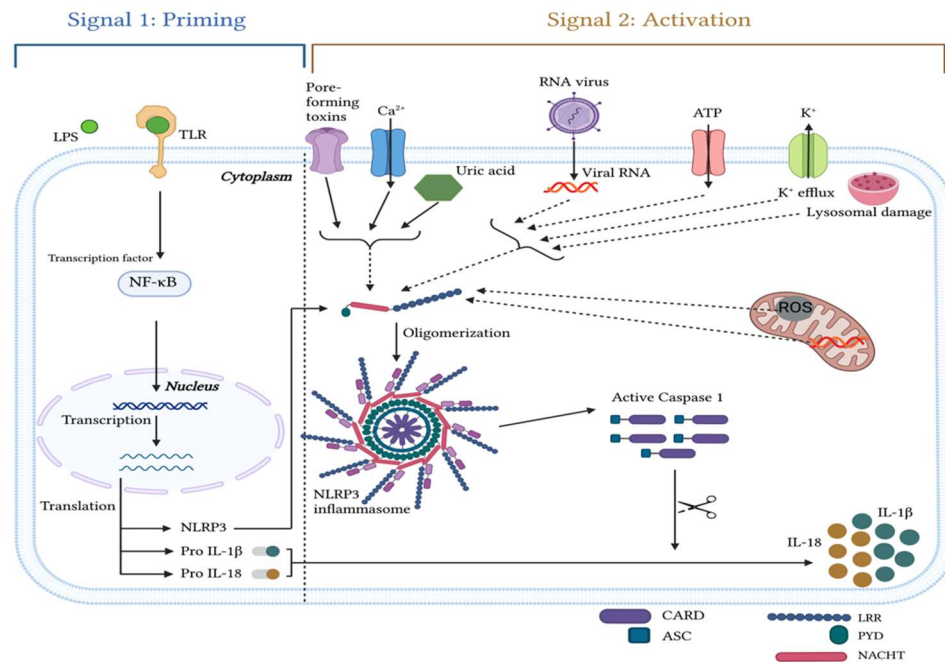
Figure 1. Priming and activation of NLRP3 inflammasome. Signal 1 (priming) is initiated by activation from binding of PAMPs to TLR and leads to the transcription of NLRP3 inflammasome components. Signal 2 (activation) is initiated by many different PAMPs or DAMPS, like crystals and ATP. These activates various upstream signaling processes, such as K+ efflux, Ca+ flux, lysosomal damage, the production of mitochondrial reactive oxygen species (mtROS) and the release of oxidized mitochondrial DNA (Swanson et al., 2019). The oligomerization and formation of NLRP3 triggers active caspase 1 and that cleaves pro IL-1β and pro IL-18 (Swanson et al., 2019).

Glycogen Synthase Kinase

Glycogen synthase kinase (GSK)-3β is a serine/threonine kinase that controls various cellular processes, for example cell cycle control, differentiation, motility, apoptosis and inflammation by regulating the activity of abundant transcription factors, such as NFAT, NF-κB, AP-1, T-bet, and cyclic adenosine monophosphate (cAMP)-response element-binding protein (Tsai, Tsai, Tseng, Lin & Chen, 2020; Wang, Brown & Martin, 2011). Nearly all of those transcription factors are valuable for T lymphocyte activation and cytokine production (Li, Spolski, Liao & Leonardo, 2014). Abnormal activity of GSK3 has been associated with different human diseases, such as diabetes, inflammation, and neurodegenerative and psychiatric disorders. The hypothesis of inhibition of GSK3 will lead to therapeutic benefit, is supported by the link between abnormal activity of GSK3 and human diseases (Eldar-Finkelman & Martinez, 2011). *In vivo* studies investigating the therapeutic ability of GSK3 have established that the inactivation of GSK3 have a possibility to defend the host from immune-mediated pathology and death (Wang, Brown & Martin, 2011).

Research question and aim

The inhibition of GSK3 has been shown in studies to change the repertoire of cytokines produced by peripheral and central cells. The results of these studies have shown that the

pro-inflammatory cytokines have been reduced and anti-inflammatory cytokines increased, proving that GSK3 inhibitors can be advantageous in condition involving inflammation and can provide new therapeutic actions to reduce neuroinflammation and various human diseases (Beurel, 2011). The effect of GSK3 inhibitor on the gene expression and secretion levels of IL-1β in LPS- and Nigericin-stimulated THP-1 macrophages has not yet been investigated. This thesis project therefore aims to investigate the effect of GSK3 Inhibitor on IL-1β production in THP-1 macrophages stimulated with LPS and Nigericin.

To complete this aim the gene expression levels of IL-1β will be analyzed using qPCR and secretion will be quantified by Enzyme Linked Immunosorbent Assays (ELISA). This was done in a concentration-dependent manner with three different concentrations of GSK3 inhibitor: 0.1 μM, 1 μM and 10 μM.

Materials and Methods

Cell line and culture

THP-1 ASC-GFP cells (Invivogen) were cultured in RPMI-1640 medium (Sigma Aldrich) containing L-glutamine, 10% heat inactivated Premium Grade Fetal Bovine Serum (FBS; Biowest), 0.45% Glucose solution (Sigma Aldrich), 10 mM HEPES (Sigma Aldrich), 1X Penicillin-streptomycin (Sigma Aldrich) and 1 mM Sodium pyruvate (Sigma Aldrich) at 37°C, 5% CO_2 in a humified incubator. Density was maintained between 5×10^5 cells/mL and 1.6×10^6 cells/mL. To care for selective pressure, 0.01 μg/mL Zeocin (Invivogen) was added every other passage as per the manufacturer's instructions.

Differentiation and Activation of THP-1 cells

THP-1 cells (3×10^5 cells/mL) were seeded into 6-well plate (1.5×10^5 cells per well) and incubated with 100ng/ml Phorbol Myristate Acetate (PMA; Invivogen) for 24 h at 37°C, 5% CO_2 in a humified incubator. Cells were subsequently washed two time with fresh media and incubated for 48 h at 37°C, 5% CO_2 in a humified incubator. To inhibit GSK3, 0.1, 1 and 10 μM of SB-216763 inhibitor (Merck) was added in fresh media and to respective wells. 0.1% of Dimethelsulfooxide (DMSO) was used as a vehicle control, incubated for 1 h. GSK3 inhibited, or uninhibited cells were stimulated with lipopolysaccharide (LPS; 500 ng/mL) for 4 h followed by 10 μM Nigericin for 45 min to activate the NLRP3 inflammasome. Supernatant of each sample was collected and used for quantification of IL-1β by ELISA and total RNA was extracted for reverse transcription to use cDNA for gene expression analysis by qPCR. The experiment included a total of six samples in three experimental replicates; LPS + Nigericin + 0.1 μM SB216763, LPS + Nigericin + 1 μM SB216763, LPS + Nigericin + 10 μM SB216763, LPS + Nigericin, DMSO and untreated. The different concentrations of the stimulants are as mentioned above.

Isolation and Purification of total RNA

Purification of total RNA from THP-1 macrophages was done using RNeasy® Plus Mini kit (Qiagen) following the manufacturer's protocol using $< 5 \times 10^6$ cells. To homogenize the

lysate, the RNase-free syringe method was chosen. The quantity and purity of the extracted RNA were measured by using a DS11 spectrophotometer (DeNovix).

Reverse Transcription

Complementary DNA (cDNA) was reverse-transcribed from total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's protocol for reverse transcription without RNase Inhibitor. No Reverse Transcription controls were performed. The reverse transcription (25°C for 10 min, 37 °C for 120 min, followed by 85 °C for 5 min and with final step on hold at 4 °C) was performed using a MJ Research PTC-400 Thermal cycler (Marshall Scientific). To provide the same concentration in all samples, 75 ng in a 2 μL reaction was used.

Quantitative PCR

Quantitative PCR (qPCR) was performed using the SYBR® Select Master Mix (Applied Biosystems) according to the manufacturer's instructions and carried out on the AriaMx Real-time PCR System (Agilent). Five different primers for the target gene IL-1β were designed using Primer-BLAST/NCBI, see complete sequences for these primers in Table 1. Primers for seven candidate reference genes (*GAPDH*, *ACTB*, *GUSB*, *TBP*, *YWHAZ*, *HRPT1* and *PPIA*) were obtained from the Human Endogenous Control Gene Panel (TATAA Biocenter) and divided between colleagues. Furthermore, the primers were tested to analyze the efficiency and linearity by qPCR and was done by performing a seven-step, 10-fold serial dilution with DNA amounts ranging from 37.5 ng to 0.75×10^{-5}, all run in triplicates. The selected primer sequence used for gene expression analysis was as follows: IL-1β foward (5'- TTCGAGGCACAAGGCACAA -3') and reverse (5'- TGGCTGCTTCAGACACTTGAG -3') and *YWHAZ* forward (5' – CGAAGCTGAAGCAGGAGAAG – 3') and reverse (5' – TTTGTGGGACAGCATGGATG – 3'). The qPCR final volume per reaction was set to 10 μL with 1 ng of input cDNA and 200nM of each primer. When analyzing the gene expression by qPCR, triplicates was used for all samples in the three experimental replicates together with no-template controls, also performed in triplicates. The thermal cycling conditions used were 50 °C for 2 min followed by 95 °C for 2 min, 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. The change in gene expression was calculated by $2^{-\Delta\Delta C}$.

Table 1. Primer sequences for forward and reverse primer for IL-1β.

Primer pair	Forward primer (5'->3')	Reverse primer (5'->3')
1	AGCCATGGCAGAAGTACCTG	CCTGGAAGGAGCACTTCATCT
2	TTCGAGGCACAAGGCACAA	TGGCTGCTTCAGACACTTGAG
3	AGCTTGGTGATGTCTGGTCC	TGGAGAACACCACTTGTTGC
4	GACACATGGGATAACGAGGCT	AGGACATGGAGAACACCACTTG
5	ATCTGTACCTGTCCTGCGTG	TTTTTGGGATCTACACTCTCCAGC

Enzyme-linked immunosorbent assays

To detect IL-1β and measure the cytokine secretion in the THP-1 supernatants, sandwich ELISA was used. The kit used in this study was Human IL-1β – ELISA MAX™ Deluxe Set, obtained from BioLegend®. All washing steps were performed with Thermo Scientific Wellwash and the plate reader used was Thermo Scientific Multiskan FC. The ELISA was carried out with four experimental replicates and all samples were run in technical triplicates. All samples were diluted 1:100 prior to ELISA except the vehicle and untreated controls. SkanIt™ Software (Thermo Scientific) was used to analyze the results and generate the standard curve. Absorbance was measured at 450 nm and 571 nm.

Statistical analysis

Statistical analysis of all data was performed using IBM® SPSS® Statistics 28.0.1.0 software (IBM Corp.) GenEx version 6.0 was used for data pre-processing and efficiency correction was made for the gene of interest and reference gene. The ΔΔCt method of comparative quantification was calculated in Microsoft Excel software to determine IL-1β gene expression levels by normalizing the target gene with the reference gene and the two calibrator samples (LPS + Nigericin and vehicle control). To investigate if any statistical significance was shown between the groups studied, for both gene and protein expression, a Kruskal-Wallis test was performed with pairwise comparisons of treatment groups. Significant difference was set to be when p-value< 0.05 (*), p< 0.01 (**) and p< 0.001 (***). The data in the box plots for gene expression of IL-1β (Figure 4 and 5), is shown with the 1.5 interquartile range (IQR) with the upper quartile Q3, median and the lower quartile Q1. The max and min Log2 fold change values are shown with the upper and lower whiskers. Considering the bar chart for protein expression of IL-1β (Figures 6 and 7), the bars represent mean values ± standard deviation.

Ethical consideration

Cell lines are animal cells that can be cultured frequently and endlessly. The THP-1 cell line are immortalized monocyte-like cells, stemming from a patient with peripheral blood acute monocyte leukemia, and the great principle of this cell line is to mimic the role of monocytes in an artificial environment. THP-1 cells also show different advantages of use over peripheral blood mononuclear cell-derived monocytes or macrophages, such as higher growth rate of THP-1 cells compared to that of PBMC-derived monocytes. Additionally, THP-1 cells can be stored for a longer time without any impact on monocyte-macrophage features or cell viability (Chanput et al., 2014). In this study, no direct or indirect ethical considerations could be found. Since the human THP-1 cells were used *in vitro,* no consent or permission from authorities was needed. Furthermore, using THP-1 cell lines allows this research to be performed in accordance with the 3R principle, as it supports alternative method to not use animals for research purposes. The principle of the Three Rs were developed by William Russell and Rex Burch, which was formulated to improve the welfare of animals used in research (Hubrecht & Carter, 2019). Russell and Burch defined the Three Rs as: Replacement, Reduction, and Refinements of animal use (Russell & Burch, 1959).

Results

Concentration and purity of extracted RNA

After isolation and purification of total RNA, concentration and purity were measured for every sample in each experimental replicate. The 260/280 values ranged from 1.60 to 2.15. The concentration and purity readings for extracted RNA can be seen in Table 1 in Appendix 1.

Expression levels of reference genes and primer validation

The raw Cq values of the seven selected candidate reference genes can be seen in Table 2. With the obtained result the difference in Cq values for each candidate reference gene could be calculated by subtraction of the lowest and highest Cq value. According to these results, it can be seen that *YWHAZ* has the smallest difference among the Cq values (difference of 0.37) and *GUSB* the largest difference (difference of 3.31). *HPRT1* has a sligthly larger variation in the difference in Cq values than *YWHAZ* (difference of 0.47). Based on the small difference among Cq values and similar Cq values between unstimulated and LPS- and Nigericin-stimulated cells, *HPRT1* could also be a potential reference gene. However, *YWHAZ* was the most stable candidate reference with no irregular shifting in Cq values among the different treatments.

Table 2. Raw Cq values of seven selected candidate reference genes *ACTB*, *GAPDH*, *TPB*, *GUSB*, *YWHAZ*, *HPRT1* and *PPIA* and the different treatments used in this experiment.

	ACTB	GAPDH	TPB	GUSB	YWHAZ	HPRT1	PPIA
10µM SB216763 + LPS and Nigericin	17.12	21.74	25.23	22.87	26.10	21.29	20.79
1µM SB216763 + LPS and Nigericin	17.08	22.18	25.69	23.47	26.01	20.93	20.89
0.1µM SB216763 + LPS and Nigericin	17.65	22.60	26.42	24.47	26.19	21.16	21.75
LPS+Nigericin	17.16	21.93	25.84	23.60	26.10	21.14	21.00
Vehicle Control	15.51	20.04	25.32	21.16	26.38	21.40	19.49
Unstimulated	15.55	20.65	23.55	22.41	26.38	21.33	19.94
Difference in Cq	2.14	2.56	2.87	3.31	0.37	0.47	2.26

The results from qPCR for the candidate reference genes were also examined using RefFinder to investigate whether they could be used for normalization. An appropriate reference gene is defined as one that is expressed at rather high but stable levels (Huggett et al., 2005). Later the expression level of the most stable reference gene was used to normalize the gene expression level of the gene of interest. Normalization calculations were done by the ΔΔCt method of comparative quantification.

According to the comprehensive ranking by RefFinder, the most stable gene was *PPIA* while the least stable gene showed to be *HPRT1* (Figure 2). The overall final ranking was calculated using the geometric mean of each gene weights, individually, and by using four different methods; delta CT, BestKeeper, Normfinder and Genorm. In the comprehensive gene stability tool in RefFinder, *YWHAZ* had a general average value for stability of 3.8 (Figure 2). These numbers are general values determined by the algorithms of all four different methods mentioned before adopted by RefFinder and they are mainly developed from online datasets and references. Another disadvantage of RefFinder is that the data from the algorithms are not evaluated in accordance with the unavailability of its cut-offs and appropriate weights (Wu et al., 2014; Wu et al., 2022). By these reasons the value of stability for *YWHAZ* from the comprehensive ranking was not taken in account in this current study. When comparing the stability of the seven selected candidate reference genes it varied in regard to the chosen analysis method although partial consistency was shown in results from delta Ct, Normfinder and Genorm, and the results of BestKeeper were distinct from those of delta Ct, Normfinder and Genorm.
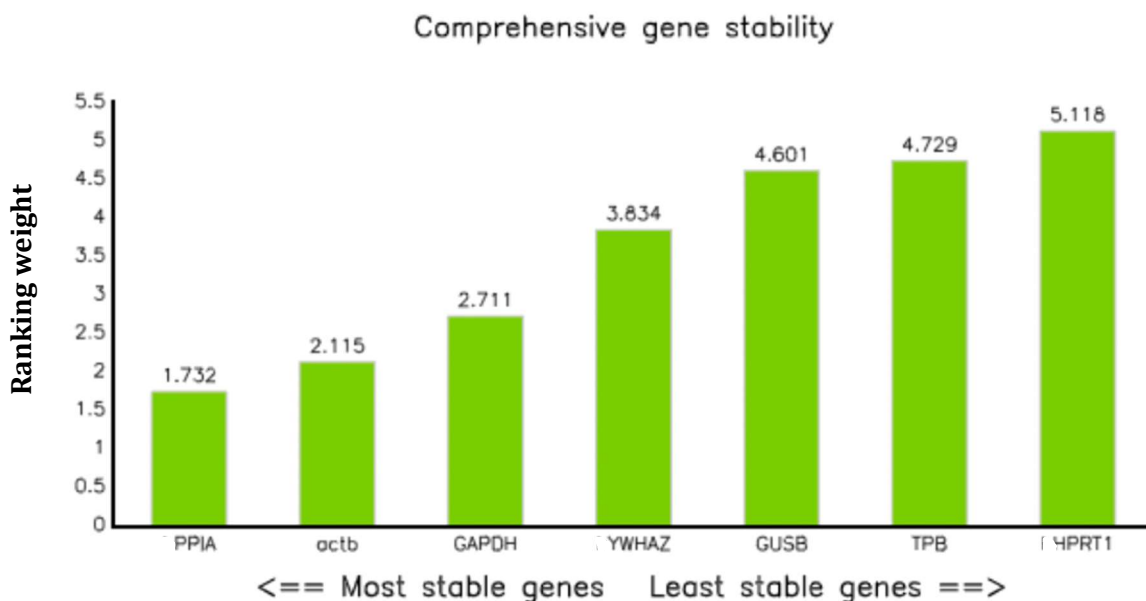

Figure 2. Bar chart obtained from RefFinder. Ranking weight calculated by the geometric mean of the individual genes. Genes are sorted in descending order of stability from left to right, with PPIA being most stable and HPRT1 least stable.

In Figure 3, the gene stability of the seven selected candidate reference genes is shown and presents the Standard Deviation of means determined by BestKeeper. According to these results, the most stable genes were *YWHAZ* and *HPRT1* with SD [+/- CP] of 0.124 and 0.132,

respectively. *GUSB* and *GAPDH* showed to be the two least stable genes with SD [+/- CP] of 0.85 and 0.786, respectively (Figure 3). BestKeeper confirmed *YWHAZ* to be the most stable gene with least SD among the technical triplicates, and based on the results from Figure 3 and Table 2 *YWHAZ* was chosen as reference gene to use in this current study.
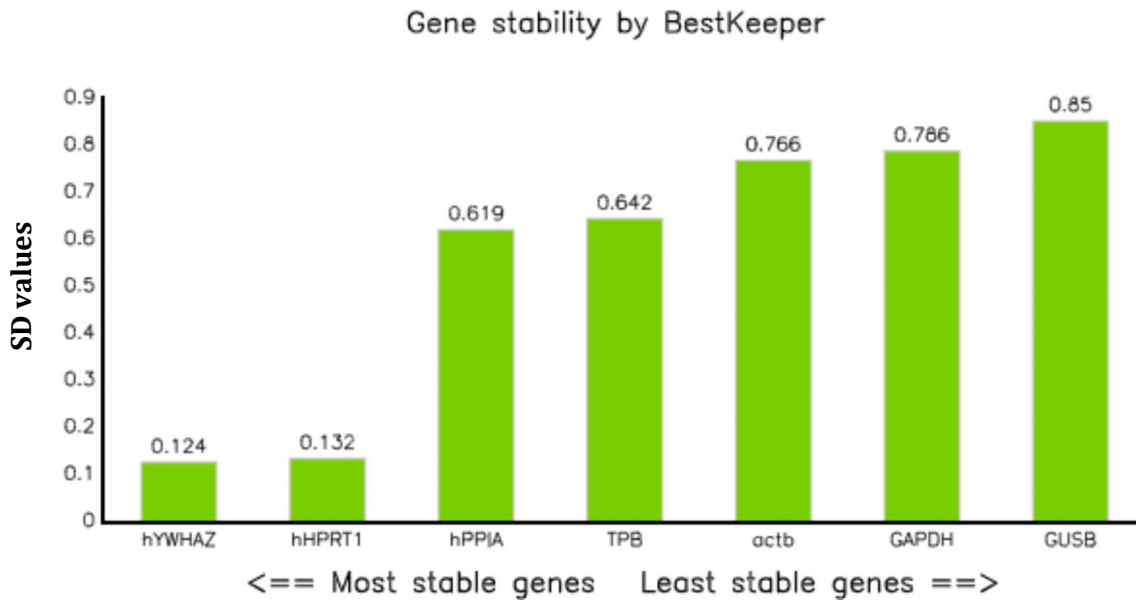
Gene stability by BestKeeper

Figure 3. Bar chart obtained from RefFinder and showing results using BestKeeper. Genes are sorted in descending order of stability from left to right, with YWHAZ being most stable and GUSB least stable.

The amplification efficiencies of designed primer pairs for *IL-1β* and *YWHAZ* were calculated from the amplification curve generated for each primer pair. From the standard curve the slope was used to measure the efficiency of the qPCR reaction. In an optimally efficient (100%) reaction, a PCR product is doubling every cycle and is represented by a slope of -3.32. Primers with amplification efficiencies between 90% and 110% can be treated as adequate (Hellemans & Vandesompele, 2011). The chosen primer pairs for IL-1β and *YWHAZ* were considered to have the best efficiencies where primer pair for IL-1β had a slope of -3.26, corresponding to a 102.6% efficiency, and primer pair for *YWHAZ* had a slope of -3.42, corresponding to a 95.8% efficiency (Table 2 in Appendix 1). Additionally, only single peaks were observed in the melting curves for each primer pair, which indicates the absence of non-specific amplification products (Hellemans & Vandesompele, 2011).

Gene expression of IL-1β

Gene expression of IL-1β was analyzed by qPCR. Using the ΔΔCt method, normalization of IL-1β with the reference gene *YWHAZ* and the two calibrator samples (unstimulated and stimulated) was done. A boxplot with log2 FC values was generated in SPSS, with unstimulated cells as calibrator sample to investigate if any possible difference would be shown between the unstimulated cells and cells stimulated with LPS and Nigericin (Figure 4). Using Kruskal-Wallis test with a significance level of 0.05, a pairwise comparison of the treatment groups was performed (Table 3 in Appendix 2). As shown in Figure 4, there is a significant difference between unstimulated cells and cells stimulated with LPS and

Nigericin (p=0.047). As illustrated in Figure 4 and in Table 1 in Appendix 2, the expression levels of IL-1β increased significantly after stimulation of THP-1 macrophages with LPS and Nigericin with an approximately 28-fold higher expression of IL-1β in stimulated compared to unstimulated cells (mean Log2=5.29, p=0.047).
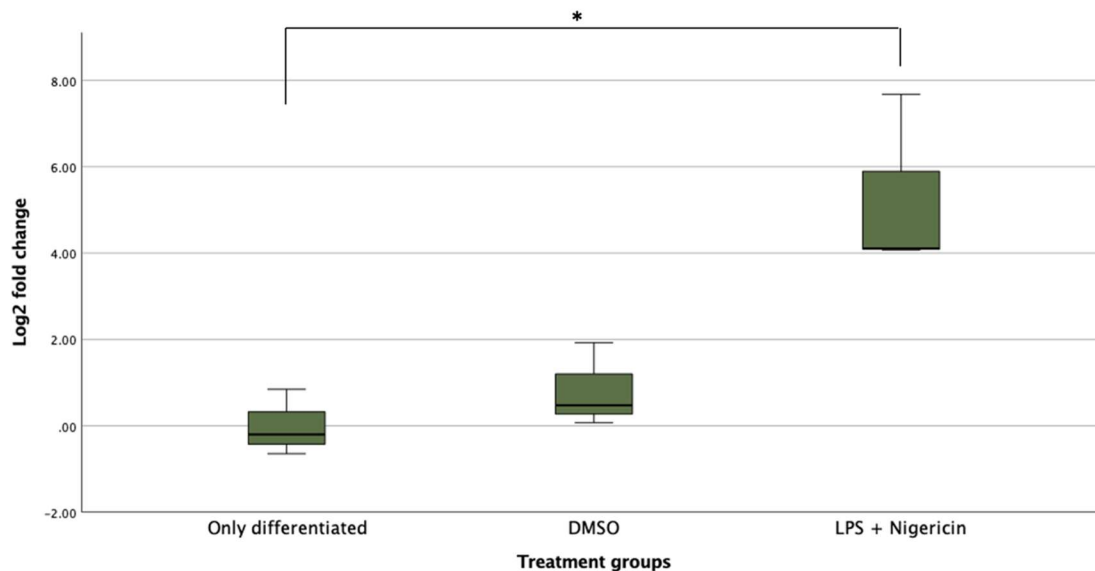

Figure 4. Boxplot showing relative Log2 fold change of IL-1β gene expression for the different treatment groups (n=3 in each group) when unstimulated cells were used as calibrator sample. Statistical significance was determined by Kruskal-Wallis test with pairwise comparisons of the treatment groups. Asterisks show significant difference: p< 0.001 = ***, p< 0.01 = ** and p< 0.05 = *.

Furthermore, analysis was done in order to compare the expression levels of IL-1β in cells stimulated with LPS and Nigericin to the one with stimulated cells combined with varying concentrations of the GSK3 inhibitor SB216763. The Kruskal-Wallis test resulted in p-values >0.05 (Table 3 in Appendix 2). This demonstrate no significant difference in gene expression of IL-1β between LPS- and Nigericin-stimulated samples treated with 0.1µM, 1µM and 10µM of SB216763 and the calibrator sample, in this case cells stimulated with LPS and Nigericin but no inhibitor. As illustrated in Figure 5, the mean Log2 fold change of the samples treated with varying concentration of inhibitor are similar to the calibrator sample. The calculated Log2 fold changes of IL-1β with stimulated cells as calibrator sample can be seen in Table 2 in Appendix 2. This indicates that different concentration of inhibitor does not affect IL-1β mRNA levels in this experimental setup.
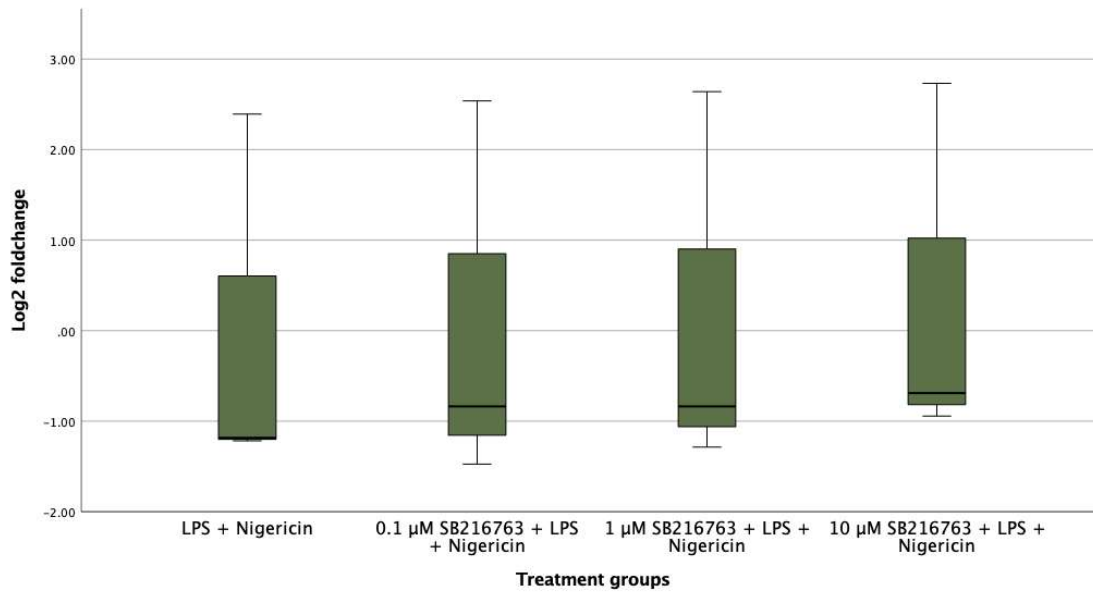
Figure 5. Boxplot showing relative Log2 fold change of IL-1β gene expression for the different treatment groups (n=3 in each group) when cells stimulated with LPS and Nigericin was used as calibrator sample. Statistical significance was determined by Kruskal-Wallis test with pairwise comparisons of the treatment groups.

To conclude, the GSK3 inhibitor SB216763 was unsuccessful in changing the IL-1β mRNA levels in THP-1 macrophages stimulated with LPS and Nigericin, although treated with different concentrations of inhibitor, 0.1μM, 1μM and 10μM (p=0.939, p=0.760, and p=491, respectively).

Quantification of secreted IL-1β

To obtain the concentrations of secreted IL-1β, ELISA was performed. As with gene expression, protein expression levels were analyzed to be able to do a comparison between stimulated cells and unstimulated cells to investigate if LPS and Nigericin alter the secretion of IL-1β from THP-1 macrophages (Figure 6).
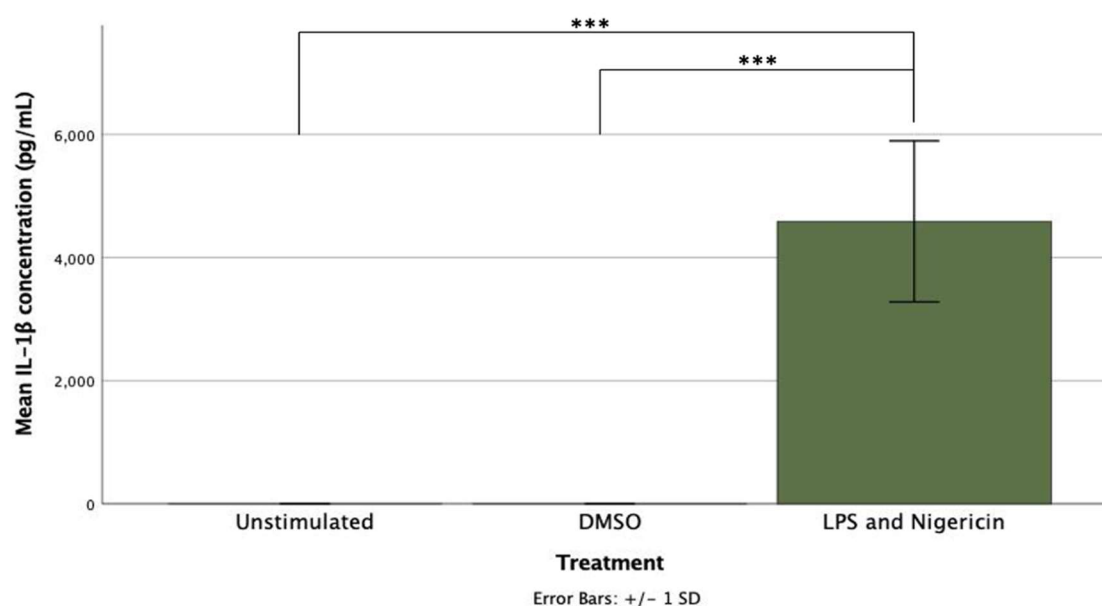
Figure 6. Bar chart representing the significant difference in mean concentrations of IL-1β between cells stimulated with LPS and Nigericin and unstimulated cells (n=4 in each group). Bars represent mean value ± SD. Statistical significance was determined by Kruskal-Wallis test with pairwise comparisons of the treatment groups. Asterisks to show significant difference according: $p < 0.001$ = ***, $p < 0.01$ = ** and $p < 0.05$ = *.

As illustrated in Figure 6, mean concentrations of IL-1β between cells stimulated with LPS and Nigericin and unstimulated cells are compared. The results show significant (p-value <0.001) higher concentration of secreted IL-1β in stimulated cells than in unstimulated cells. This indicates that following stimulation of THP-1 macrophages with LPS and Nigericin, the amount of IL-1β protein released is notably increased. The Kruskal-Wallis test with a significance level of 0.05, supports the findings with the p-value <0.001 (Table 2 in Appendix 3).

Furthermore, a comparison between LPS- and Nigericin-stimulated cells and stimulation combined with varying concentrations of SB216763 was done to investigate if GSK3 inhibitor affects the production of pro-inflammatory cytokine IL-1β in LPS- and Nigericin-stimulated THP-1 macrophages (Figure 7). To investigate if there is any difference in mean concentration of IL-1β between the treatment groups, the concentration of samples within each experimental replocate (n=4) was plotted in SPSS, see concentrations of secreted IL-1β in Table 1 in Appendix 3.
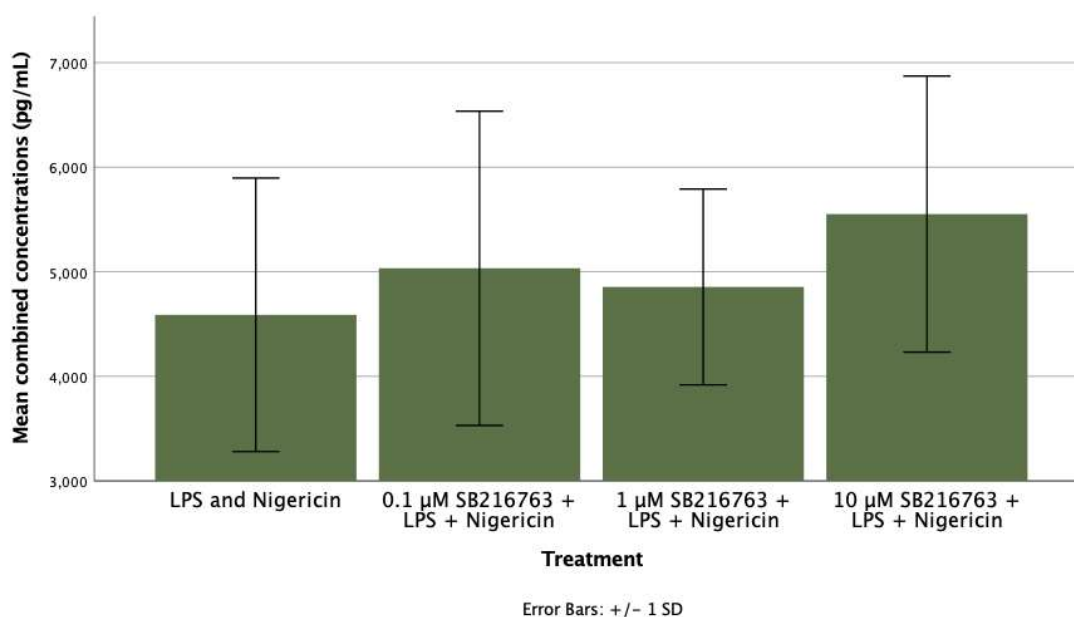
Figure 7. Bar chart representing mean concentration of IL-1β between cells stimulated with LPS and Nigericin and the samples containing varying concentrations of SB216763 (n=4 in each group). Bars represent mean value ± SD.

In Figure 7, the comparison of mean concentration of IL-1β between LPS- and Nigercin-stimulated THP-1 macrophages and the stimulated cells combined with varying concentrations of SB216763 inhibitor can be seen. Stimulated cells treated with 0.1μM, 1μM and 10μM of inhibitor showed nearly the same amounts of secreted IL-1β protein. This suggest that the GSK3 inhibitor, SB216763, did not have any significant inhibitory effect on the production of IL-1β in LPS- and Nigericin-stimulated THP-1 macrophages. The mean concentrations of secreted IL-1β from macrophages treated with varying concentration of inhibitor did not show significant alteration in form of increase or decrease of the amount of secreted IL-1β in comparison to the mean concentration of secreted IL-1β generated from the LPS- and Nigericin-stimulated cells. No significant difference in amount of secreted IL-1β when stimulation is combined with GSK3 inhibitor at various concentration can be supported with p-values, p=0.706 at 0.1μM, p=0.691 at 1μM and p=0.237 at 10μM.

To conclude, there is a stable increase in concentration of IL-1β within the experimental replicates after LPS- and Nigericin-stimulation, although no significant change was observed when stimulation is combined with SB216763 at various concentrations.

Discussion

The secretion of cytokines and their regulation, followed by the role of GSK3 in inflammation and the effect of inhibiting GSK3, continue to be of great interest in the field of medical research. To gain better and deeper understanding of these complexities, this thesis project investigate the effect of an GSK3 inhibitor on gene expression and secretion levels of IL-1β in LPS- and Nigericin-stimulated THP-1 macrophages.

According to literature, active GSK3 is essential for the production of pro-inflammatory cytokine subsequent of TLRs stimulation (Beurel et al., 2010). In TLR-stimulated monocytes, there has been studies done showing that GSK3 inhibitor reduces the production by 67-90% of pro-inflammatory cytokines, such as IL-6, IL-1β, IL-12p40, interferon-γ (IFN-γ) and tumor necrosis factor (TNF) (Beurel et al., 2010). In a study by Martin (2005), mice were given a lethal (LD_{100}) dose of LPS and GSK3 inhibitor, SB216763 was administrated *in vivo* to produce protection against endotoxin shock. This protection was enough to let the most mice survive. Feeding mice with GSK3 inhibitor reduced proinflammatory cytokines and improved survival rate of 60% when treated with LPS, compared to mice on normal diet that had survival rates of 0%. The ability of GSK3 to regulate inflammatory response after TLR activation makes GSK3 a potential therapeutic target for sepsis and other inflammatory diseases (Martin et al., 2005). The study by Martin et al (2005) was one of the first to prove the ability of GSK3 inhibitors to change the stability of the inflammatory response from pro-inflammatory to anti-inflammatory, and to disclose the therapeutic possibilities in inflammatory states.

Concentration and purity of RNA

The quality of RNA and thereby the cDNA can have impact on the qPCR results. A study by Krsek and Wellington (1999), stated that the A260/280 affected the outcome of PCR amplification to a greater extent than by the A260/230 ratio. There have also been studies done where researchers did not find any considerable relationship between low A260/230 value and efficiency of qPCR amplification (Cicinnati et al., 2008; Kuang et al., 2018). The cDNA samples used in this study was obtained from the various RNA samples, see Table 1 in Appendix 1 for the concentration and purity of total RNA extractions. The purity (Absorbance 260/280) for all samples are within the accepted range (optimal ratio of ~2.0) for RNA except for six samples with 260/280 values ranging from 1.60 to 19.98. The samples can be considered pure since the 260/280 values were very close to the optimal ratio that is accepted as ''pure'' for RNA (Desjardins & Conklin, 2010). The reason why some samples had a lower A260/280 value could be change in sample acidity (Wilfinger et al., 1997). As seen in Table 1 in Appendix 1, the A260/230 values ranged from 0.04 to 1.66, with all samles being lower than the suggested range of 1.8 – 2.2 for ''pure'' RNA (Desjardins & Conklin, 2010). The reason for this could be that samples were contaminated with residue of reagents used in purification of total RNA like guanidine, glycogen and carbohydrate carryover, that are absorbed at 230 nm (Matlock, 2015). These contaminations can impact on the qPCR results and efficiency of qPCR amplification (Carvalhais et al., 2013; Unger et al., 2019).

Selection of reference gene

When performing quantitative polymerase chain reaction (qPCR) it is required to normalize to a reference gene. To obtain correct results and data, the stability of candidate reference genes needs to be evaluated (Li et al., 2014). In this study, the expression stability of seven candidate reference genes was evaluated. Using geNorm, NormFinder and BestKeeper algorithms, *YWHAZ* and *PPIA* are suggested as the most favorable reference genes for normalizing the qPCR data in this experiment (Figure 2 and 3). When using RefFinder and analyzing the comprehensive gene stability where ranking weight was calculated by the geometric mean of the individual genes, the results showed *PPIA* as the most stable gene and *HPRT1* as the least stable gene (Figure 2) whereas results of gene stability by BestKeeper showed *HPRT1* to be the second most stable gene (Figure 3). The difference in stability seen in the geNorm, NormFinder and BestKeeper results, could be due to different algorithms used in these three programs (Chang et al., 2012). The *YWHAZ* gene had the lowest crossing point, SD value of 0.12, when using BestKeeper algorithm (Figure 3) which indicated minimum variation. For this reason *YWHAZ* was chosen as reference gene in this study. Another reason was that *YWHAZ* also showed stable and acceptable SD C_q values in all samples, threshold for acceptable difference in C_q value between the PCR replicates being 0.5 cycles (corresponds to SD of 0.35) (Hellemans & Vandesompele, 2011). These criterias are the most important when selecting a reference gene (Chervoneva et al., 2010).

IL-1β gene expression

To measure the gene expression level of IL-1β, comparative quantification was used which allows to quantify the relative changes in gene expression of a gene of interest between the target in different treatments groups to that of another sample such as a calibrator sample (Livak & Schmittgen, 2001). The qPCR raw data was analyzed using the ΔΔCt method of comparative quantification, data was pre-processed before with effiency correlation due to the sensitivity of the ΔΔCt method (Ramakers et al., 2003). In this study, the calibrator chosen for confirmation of stimulation in LPS- and Nigericin-stimulated THP-1 cells was the unstimulated cells. The study by Livak and Schmittgen (2001), stated that the decision of calibrator for the ΔΔCt method should depend on the aim of the gene expression experiment and that the generally chosen one is the untreated control.

The results of Log2 fold change of IL-1β showed that the gene was up-regulated 28 folds after stimulation with LPS and Nigericin when compared to unstimulated cells (Table 1 in Appendix 2). To present the relative changes in IL-1β gene expression level for the different treatment groups, Log2 Fold change (FC) was used. The FC is the proportion in gene expression normalized to an internally originated reference gene and relative to the chosen control (Livak & Schmittgen, 2001). The FC in gene expression relative to the control is explained to be equal to one since the ΔΔCt of control sample equals zero and 2^0 equals one (Livak & Schmittgen, 2001). There has been one problem adressed with using the FC ratios to display the change in gene expression on a linear scale , since they treat the up- and down-regulated genes differently the results shows that down-regulated genes are limited to be between one and zero whereas up-regulated can be between one and positive infinity. To solve this problem of asymmetric scale, the fold change values where log transformed (Log₂ base 2) to obtain a a symmetric scale and for treating the up and down-regulated genes equally (Causton et al., 2009). It should be noted that for the data analysis, the ΔΔCt values

were used since expression ratios elimate information about absolute gene expression levels (Causton et al., 2009). It was also recommended by Yuan et al. (2006) to perform all statistics on the ΔΔCt values and not on the FC.

The aim of this study was to investigate whether the GSK3 inhibitor has an effect on the gene expression levels of IL-1β in LPS- and Nigericin-stimulated THP-1 cells. For that reason a second calibrator was chosen and it was the LPS- and Nigericin-simulated sample. When comparing the stimulated cells with stimulation combined with SB216763 at various concentrations, a slight but not significant difference in the log2 fold change was observed (Table 2 in Appendix 2). To conclude, IL-1β is up-regulated significantly in the stimulated cells but there was not any change in gene expression between the cells with inhibitor and those without inhibitor added.

LPS- and nigericin-stimulation increases IL-1β mRNA levels

NF-κB is an essential transcriptional factor family containing five different groups in eukaryotic cells, containing NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB and c-Rel (Yu et al., 2009). The release and translocation of nuclear factor-κB p65 and p50 to the nucleus is a result of proteolysis of IκBα, the main NF-κB inhibitor protein in almost all cells. When translocated to the nucleus, NF-κB p65 and p50 activates the transcription of genes encoding pro-inflammatory cytokines (Akira & Takeda, 2004; Durand & Baldwin, 2017; Muri et al., 2020). A study by Henkel et al (1993) indicates that NF-κB activation is dependent on the proteolysis of IκBα. One of the important function of NF-κB is that activated NF-κB translocates to the nucleus and triggers the transcription of proinflammatory cytokines such as IL-1β (Zielinski & Krueger, 2012). Sakai et al, (2017) investigated the NF-κB activation which plays a essential role in LPS-induced inflammatory cytokine production. In a study by Bauernfeind et al, (2009) it has been stated that macrophages requires a priming stimuli being ligands such as LPS since the existence of only activators such as Nigericin does not activate the inflammasome. Results from previous studies shows that stimulation with LPS enhance the binding action of NF-κB and further induce release of IL-1β and significantly enhance the IL-1β gene expression in THP-1 cells (Bauernfeind et al., 2009; Yang et al., 2019). These results confirms those from this current study, considering the significant increase in the production of IL-1β in LPS- and Nigericin-stimulated THP-1 macrophages (Figure 4). The mean log2 fold change (5.29) of IL-1β gene expression and corresponds to approximately 28-folds of up-regulation of IL-1β gene expression in THP-1 cells that were stimulated with LPS and Nigericin (Figure 4).

LPS- and nigericin-stimulation increases IL-1β protein levels

Sandwich ELISA is a very quick and precise tool for protein expression analysis with high-affinity antibodies, making it a powerful method to use for measuring the amount of secreted IL-1β in this study (Chiswick et al., 2011). The assay is useful in measuring secreted cytokine levels in an immune response for the reason that the non-purified antigens can be tested and the method itself has high sensitivity and specificity ("Types of ELISA | Bio-Rad", 2022). In a study by Zhao et al (2019), THP-1 macrophages were treated with 1μg/mL LPS for 3 h to measure secretion levels of IL-1β before and after stimulation. The results showed that the secretion of IL-1β significantly increased in LPS induced THP-1 macrophages (Zhao et al., 2019). In this work Figure 6 illustrates the amount of secreted IL-1β from LPS- and

Nigericin-stimulated and unstimulated THP-1 macrophages. The results showed that cells that were stimulated with 500 ng/mL LPS significantly increased secretion of IL-1β, compared to the control (unstimulated cells) (Figure 6). These findings supports the results from study by Zhao et al. (2019). Previous studies have further shown that LPS stimulates the NF-κB pathway in macrophages, where activation of this particular pathway is the initial signal for transcriptional upregulation of IL-1β (Boaru et al., 2015; Guha & Mackman, 2001; Oeckinghaus et al., 2011). These results demonstrates that when THP-1 macrophages were induced with LPS, NF-κB and its biochemical cascade was activated.

GSK3 inhibitor has no effect on IL-1β mRNA and protein levels in LPS- and nigericin-stimulated THP-1 macrophages

GSK3 is a well active kinase involved in LPS induced cytokine production and it is activated by phosphorylation at Tyr216 and inactivated by phosphorylation at Ser9 (Cortés-Vieyra et al., 2021; Noori et al., 2020). Previous studies have shown that inhibition of GSK3 attenuates the expression of pro-inflammatory cytokines in response to LPS (Morris et al., 2014). The study done by Noori et al (2020), brings evidence for compounds that suppress GSK3 to be promising therapeutics for inflammatory diseases. In this current study, LPS- and Nigericin stimulated THP-1 macrophages were treated with 0.1μM, 1μM, and 10μM of GSK3 inhibitor, SB216763, nevertheless no significant difference was observed in the IL-1β mRNA nor protein levels (Figure 5 and 7). Regarding mRNA levels, the transcription levels of IL-1β were not altered by the GSK3 inhibitor in cells stimulated with LPS- and Nigericin consequently the IL-1β gene expression was not affected (Figure 5). Same is true about IL-1β protein levels, no significant difference was seen in mean concentration of IL-1β in THP-1 macrophages when LPS- and Nigericin stimulation was combined with 0.1μM, 1μM, and 10μM of SB216763 (Figure 7).

Previous studies stated that GSK3 regulates the production of proinflammatory cytokines, including IL-1β, by the TLR4-MyD88-dependent pathway. Activated GSK3 inhibits AP-1 and binding of CREB to CBP, and is involved in the activation of NF-κB (Jope et al., 2017; Ko & Lee, 2016). NF-κB is activated through the Toll/IL-1 receptor (TIR)-domain-containing adaptors, myeloid differentiation primary response gene 88 (MyD88) or TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway, that regulate the expression of inflammatory cytokine genes (Cortés-Vieyra et al., 2021). Furthermore, studies in human monocytes and human peripheral blood mononuclear cells (PBMCs) stimulated with TLR4 agonist showed that GSK3 inhibitor SB216763 reduced the production of proinflammatory cytokines, including IL-1β, with 50-90% while production of the anti-inflammatory cytokine IL-10 was promoted (Martin et al., 2005). Another study done by Green & Nolan (2012), demonstrate that GSK3 mediates the release of IL-1-1β, TNF-α and IL-10 from cortical glia. The results show that GSK3 inhibitor SB216763 reduced the LPS-stimulated levels of pro-inflammatory cytokines (Green & Nolan, 2012).

A study by Noori et al (2020) used LPS induced THP-1 macrophages to investigate the potential of GSK3, COB-187, to reduce the protein and gene expression of different cytokines, such as IL-1β, IL-6, TNF-α, IFN-β and CXCL10. The results showed that COB-187 significantly attenuated the production of cytokines, both on protein and mRNA levels. According to the research, the explanation for these results could be that GSK3 inhibitor used reduced the NF-κB (p65/p50) DNA binding activity (Chen et al., 2008; Noori et al.,

2020). However, there is some difference between Noori et al, study and this current study. The first being use of a different GSK3 inhibitor and second they used 10 ng/mL of LPS while this study used 500 ng/mL, which may have an impact.

Another possible reason for the results of this study to differ from those mentioned above that used the same GSK3 inhibitor SB216763, could be the choice of nigericin-stimulation as the second signal. Since the second signal is mediated by numerous PAMPs or DAMPs stimulation and is required for the activation of NLRP3 inflammasome following the priming step, several other stimuli could be used (Jo et al, 2015). Nigericin has been shown to directly cause the efflux of potassium by creating pores on the plasma membrane. It has further been reported that potassium efflux is fundamental for calcium influx (Muñoz-Planillo et al., 2013; Xu et al., 2020; Yaron et al., 2015). A study by Yaron et al (2015), demonstrates the crucial role for calcium influx upstream of mitochondrial reactive oxygen generation, inflammasome assembly and pro-inflammatory cytokine release. A study by Ainscough et al (2015), investigated the role of calcium in IL-1β up-regulation and release. The findings demonstrated an important role for calcium in IL-1β secretion as following calcium influx within the cell, pro-IL-1β interrelate with calmodulin and this interplay is vital for the processing and release of IL-1β (Ainscough et al., 2015). These results are supported by earlier study by Brough et al (2003), which present that the release of intracellular calcium stores is necessary for the secretion of IL-1β. However, further research should be done to get a deeper insight of the role of GSK3 inhibitor on TLR4-mediated IL-1β production, and one example would be to investigate the IL-1β mRNA and protein levels in LPS-stimulated THP-1 macrophages without adding nigericin to examine if nigericin triggered other pathways that effected and still produced IL-1β.

Another possible reason for the lack of results of GSK3 inhibitor on IL-1β production may be due to the inhibition of GSK3 and at the same time the cells found different pathways to continue to produce IL-1β. GSK3 is involved in the regulation of NF-κB which regulates several cellular processes and there are various signaling pathways that result to the activation of NF-κB (Martin et al., 2005; Oeckinghaus & Ghosh, 2009). A study by Martin et al (2005), investigated what step(s) of the NF-κB pathway that GSK3 inhibition can alter. The findings showed that IκBα degradation was noticeable at 30 min after LPS stimulation and that GSK3 inhibitor SB216763 was unsuccessful in altering the amount of degradation or resynthesis of IκBα (Martin et al., 2005). In other word, the extent of degradation of IκBα was the same in human monocytes stimulated with LPS combined with GSK3 inhibitor compared to the cells stimulated with LPS alone. This demonstrates that IκBα pathway is activated even with the presence of GSK3 inhibitor hence IL-1β is even so produced (Martin et al., 2005).

Furthermore, Martin et al (2005) study the PI(3)K/Akt pathway that regulates NF-κB and also activates the IL-1β transcription. Akt, a key mediator of the PI(3)K pathway, phosphorylates different downstream targets of PI(3)K pathway when being activated. One downstream target is the GSK3-β and when phosporylated GSK3 is inhibited (Martin et al., 2005). Martin et al (2005) further explained the ability of the PI(3)K/Akt pathway to separately control the synthesis of cytokines by inhibiting GSK3-β after LPS-stimulation combined with SB216763. The observations of the present study could be supported by the findings by Martin et al (2005) and indicates that this study possibly managed to inhibit GSK3 but in the PI(3)K/Akt pathway. Regarding the other observation by Martin et al (2005) of the NF-κB pathway mediated by IκBα, GSK3 inhibitor SB216763 did not have an

effect on cytokine production since IL-1β was still produced.

Conclusion

GSK3 is an valuable target for drug development. The collected data in this study was assumes to show if GSK3 inhibitor had an effect on gene expression and in the production of IL-1β in LPS- and Nigericin-stimulated macrophages. The results from relative gene expression and protein expression analysis of IL-1β showed that it was significantly up-regulated after LPS- and Nigericin-stimulation, on the other hand no significant difference in mRNA levels was observed upon treatment with different concentrations of GSK3 inhibitor.

For the quantification of secreted IL-1β, the results did not show the amount of secreted IL-1β to be reduced nor induced upon treatment with different concentrations of GSK3 inhibitor. In other words, the LPS- and Nigericin-stimulated THP-1 macrophages treated with GSK3 inhibitor did not effect the IL-1β mRNA and protein level. As, further research should be done of GSK3 inhibitors or activators. For further studies, this study should be repeated with some alterations in methods, such as concentration of LPS and investigate if the results would change. Also to determine the IL-1β mRNA and protein levels in macrophages stimulated with LPS only and to use another NLRP3 activator than Nigericin to study whether other activator than Nigericin has a link to GSK and if that would alter the results of the study.

Acknowledgments

References

Ainscough, J., Gerberick, G., Kimber, I., & Dearman, R. (2015). Interleukin-1β Processing Is Dependent on a Calcium-mediated Interaction with Calmodulin. *Journal Of Biological Chemistry, 290*(52), 31151-31161. https://doi.org/10.1074/jbc.m115.680694

Akira, S., & Takeda, K. (2004). Toll-like receptor signalling. *Nature Reviews Immunology, 4*(7), 499-511. https://doi.org/10.1038/nri1391

Bauernfeind, F., Bartok, E., Rieger, A., Franchi, L., Núñez, G., & Hornung, V. (2011). Cutting Edge: Reactive Oxygen Species Inhibitors Block Priming, but Not Activation, of the NLRP3 Inflammasome. *The Journal Of Immunology, 187*(2), 613-617. https://doi.org/10.4049/jimmunol.1100613

Bauernfeind, F., Horvath, G., Stutz, A., Alnemri, E., MacDonald, K., & Speert, D. et al. (2009). Cutting Edge: NF-κB Activating Pattern Recognition and Cytokine Receptors License NLRP3 Inflammasome Activation by Regulating NLRP3 Expression. *The Journal Of Immunology, 183*(2), 787-791. https://doi.org/10.4049/jimmunol.0901363

Beurel, E. (2011). Regulation by glycogen synthase kinase-3 of inflammation and T cells in CNS diseases. *Frontiers In Molecular Neuroscience, 4.* https://doi.org/10.3389/fnmol.2011.00018

Beurel, E., Michalek, S., & Jope, R. (2010). Innate and adaptive immune responses regulated by glycogen synthase kinase-3 (GSK3). *Trends In Immunology, 31*(1), 24-31. https://doi.org/10.1016/j.it.2009.09.007

Biasizzo, M., & Kopitar-Jerala, N. (2020). Interplay Between NLRP3 Inflammasome and Autophagy. *Frontiers In Immunology, 11*. doi: 10.3389/fimmu.2020.591803

Boaru, S., Borkham-Kamphorst, E., Van de Leur, E., Lehnen, E., Liedtke, C., & Weiskirchen, R. (2015). NLRP3 inflammasome expression is driven by NF-κB in cultured hepatocytes. *Biochemical And Biophysical Research Communications, 458*(3), 700-706. https://doi.org/10.1016/j.bbrc.2015.02.029

Braddock, M., & Quinn, A. (2004). Targeting IL-1 in inflammatory disease: new opportunities for therapeutic intervention. *Nature Reviews Drug Discovery, 3*(4), 330-340. https://doi.org/10.1038/nrd1342

Brough, D., Le Feuvre, R., Wheeler, R., Solovyova, N., Hilfiker, S., Rothwell, N., & Verkhratsky, A. (2003). Ca2+Stores and Ca2+Entry Differentially Contribute to the Release of IL-1β and IL-1α from Murine Macrophages. *The Journal Of Immunology, 170*(6), 3029-3036. https://doi.org/10.4049/jimmunol.170.6.3029

Bustin, S., Benes, V., Garson, J., Hellemans, J., Huggett, J., & Kubista, M. et al. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry, 55*(4), 611-622. https://doi.org/10.1373/clinchem.2008.112797

Carvalhais, V., Delgado-Rastrollo, M., Melo, L., & Cerca, N. (2013). Controlled RNA contamination and degradation and its impact on qPCR gene expression in S. epidermidis biofilms. *Journal Of Microbiological Methods, 95*(2), 195-200. https://doi.org/10.1016/j.mimet.2013.08.010

Carvalho, R., Silva, A., Santos, L., Andrade, W., Sá, K., & Zamboni, D. (2019). Macrophage priming is dispensable for NLRP3 inflammasome activation and restriction of Leishmania amazonensis replication. *Journal Of Leukocyte Biology, 106*(3), 631-640. https://doi.org/10.1002/jlb.ma1118-471r

Causton, H., Brazma, A., & Quackenbush, J. (2009). *Microarray Gene Expression Data Analysis* (pp. 49-51). Blackwell Science Ltd.

Chang, E., Shi, S., Liu, J., Cheng, T., Xue, L., & Yang, X. et al. (2012). Selection of Reference Genes for Quantitative Gene Expression Studies in Platycladus orientalis (Cupressaceae) Using Real-Time PCR. *Plos ONE, 7*(3), e33278. https://doi.org/10.1371/journal.pone.0033278

Chanput, W., Mes, J., & Wichers, H. (2014). THP-1 cell line: An in vitro cell model for immune modulation approach. *International Immunopharmacology, 23*(1), 37-45. https://doi.org/10.1016/j.intimp.2014.08.002

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., & Deng, J. et al. (2017). Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget, 9*(6), 7204-7218. doi: 10.18632/oncotarget.23208

Chen, W., Srinath, H., Lam, S., Schiffer, C., Royer, W., & Lin, K. (2008). Contribution of Ser386 and Ser396 to Activation of Interferon Regulatory Factor 3. *Journal Of Molecular Biology, 379*(2), 251-260. https://doi.org/10.1016/j.jmb.2008.03.050

Chervoneva, I., Li, Y., Schulz, S., Croker, S., Wilson, C., Waldman, S., & Hyslop, T. (2010). Selection of optimal reference genes for normalization in quantitative RT-PCR. *BMC Bioinformatics, 11*(1). https://doi.org/10.1186/1471-2105-11-253

Chiswick, E., Duffy, E., Japp, B., & Remick, D. (2011). Detection and Quantification of Cytokines and Other Biomarkers. *Methods In Molecular Biology*, 15-30. https://doi.org/10.1007/978-1-61779-527-5_2

Cicinnati, V., Shen, Q., Sotiropoulos, G., Radtke, A., Gerken, G., & Beckebaum, S. (2008). Validation of putative reference genes for gene expression studies in human hepatocellular carcinoma using real-time quantitative RT-PCR. *BMC Cancer, 8*(1). https://doi.org/10.1186/1471-2407-8-350

Clark, R., & Kupper, T. (2005). Old Meets New: The Interaction Between Innate and Adaptive Immunity. *Journal Of Investigative Dermatology, 125*(4), 629-637. doi: 10.1111/j.0022-202x.2005.23856.x

Cortés-Vieyra, R., Silva-García, O., Gómez-García, A., Gutiérrez-Castellanos, S., Álvarez-Aguilar, C., & Baizabal-Aguirre, V. (2021). Glycogen Synthase Kinase 3β Modulates the Inflammatory Response Activated by Bacteria, Viruses, and Parasites. *Frontiers In Immunology*, 12. https://doi.org/10.3389/fimmu.2021.675751

Desjardins, P., & Conklin, D. (2010). NanoDrop Microvolume Quantitation of Nucleic Acids. *Journal Of Visualized Experiments*, (-1). https://doi.org/10.3791/2565

Di Virgilio, F. (2013). The Therapeutic Potential of Modifying Inflammasomes and NOD-Like Receptors. *Pharmacological Reviews, 65*(3), 872-905. doi: 10.1124/pr.112.006171

Dinarello, C. (1996). Biologic basis for interleukin-1 in disease. *Blood, 87*(6), 2095-2147. https://doi.org/10.1182/blood.v87.6.2095.bloodjournal8762095

Dinarello, C. (2004). Therapeutic strategies to reduce IL-1 activity in treating local and systemic inflammation. *Current Opinion In Pharmacology, 4*(4), 378-385. https://doi.org/10.1016/j.coph.2004.03.010

Durand, J., & Baldwin, A. (2017). Targeting IKK and NF-κB for Therapy. *Chromatin Proteins And Transcription Factors As Therapeutic Targets,* 77-115. https://doi.org/10.1016/bs.apcsb.2016.11.006

Eldar-Finkelman, H., & Martinez, A. (2011). GSK-3 Inhibitors: Preclinical and Clinical Focus on CNS. *Frontiers In Molecular Neuroscience, 4*. https://doi.org/10.3389/fnmol.2011.00032

Fang, H., Pengal, R., Cao, X., Ganesan, L., Wewers, M., Marsh, C., & Tridandapani, S. (2004). Lipopolysaccharide-Induced Macrophage Inflammatory Response Is Regulated by SHIP. *The Journal Of Immunology, 173(*1), 360-366. https://doi.org/10.4049/jimmunol.173.1.360

Feerick, C., & McKernan, D. (2016). Understanding the regulation of pattern recognition receptors in inflammatory diseases - a 'Nod' in the right direction. *Immunology, 150*(3), 237-247. https://doi.org/10.1111/imm.12677

Green, H., & Nolan, Y. (2012). GSK-3 mediates the release of IL-1β, TNF-α and IL-10 from cortical glia. *Neurochemistry International, 61*(5), 666-671. https://doi.org/10.1016/j.neuint.2012.07.003

Guha, M., & Mackman, N. (2001). LPS induction of gene expression in human monocytes. *Cellular Signalling, 13*(2), 85-94. https://doi.org/10.1016/s0898-6568(00)00149-2

Hato, T., & Dagher, P. (2014). How the Innate Immune System Senses Trouble and Causes Trouble. *Clinical Journal Of The American Society Of Nephrology, 10(*8), 1459-1469. doi: 10.2215/cjn.04680514

Hayden, M., & Ghosh, S. (2004). Signaling to NF-κB. *Genes &Amp; Development, 18*(18), 2195-2224. https://doi.org/10.1101/gad.1228704

Hellemans, J., & Vandesompele, J. (2011). *qPCR data analysis – unlocking the secret to successful results.* Qbaseplus.com. Retrieved 28 May 2022, from https://www.qbaseplus.com/sites/default/files/publication/hellemans_vandesompele_2011.pdf.

Henkel, T., Machleidt, T., Alkalay, I., Krönke, M., Ben-Neriah, Y., & Baeuerle, P. (1993). Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. *Nature, 365*(6442), 182-185. https://doi.org/10.1038/365182a0

Hubrecht, R., & Carter, E. (2019). The 3Rs and Humane Experimental Technique: Implementing Change. *Animals, 9*(10), 754. https://doi.org/10.3390/ani9100754

Huggett, J., Dheda, K., Bustin, S., & Zumla, A. (2005). Real-time RT-PCR normalisation; strategies and considerations. *Genes &Amp; Immunity, 6*(4), 279-284. https://doi.org/10.1038/sj.gene.6364190

Jo, E., Kim, J., Shin, D., & Sasakawa, C. (2015). Molecular mechanisms regulating NLRP3 inflammasome activation. Cellular &Amp; *Molecular Immunology, 13*(2), 148-159. https://doi.org/10.1038/cmi.2015.95

Jope, R., Cheng, Y., Lowell, J., Worthen, R., Sitbon, Y., & Beurel, E. (2017). Stressed and Inflamed, Can GSK3 Be Blamed?. *Trends In Biochemical Sciences, 42*(3), 180-192. https://doi.org/10.1016/j.tibs.2016.10.009

Juliana, C., Fernandes-Alnemri, T., Kang, S., Farias, A., Qin, F., & Alnemri, E. (2012). Non-transcriptional Priming and Deubiquitination Regulate NLRP3 Inflammasome Activation. *Journal Of Biological Chemistry, 287(*43), 36617-36622. https://doi.org/10.1074/jbc.m112.407130

Kawashima, A., Karasawa, T., Tago, K., Kimura, H., Kamata, R., & Usui-Kawanishi, F. et al. (2017). ARIH2 Ubiquitinates NLRP3 and Negatively Regulates NLRP3 Inflammasome Activation in Macrophages. *The Journal Of Immunology, 199*(10), 3614-3622. doi: 10.4049/jimmunol.1700184

Kelley, N., Jeltema, D., Duan, Y., & He, Y. (2019). The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. *International Journal Of Molecular Sciences, 20*(13), 3328. doi: 10.3390/ijms20133328

Ko, R., & Lee, S. (2016). Glycogen synthase kinase 3β in Toll-like receptor signaling. *BMB Reports, 49*(6), 305-310. https://doi.org/10.5483/bmbrep.2016.49.6.059

Krsek, M., & Wellington, E. (1999). Comparison of different methods for the isolation and purification of total community DNA from soil. *Journal Of Microbiological Methods, 39*(1), 1-16. https://doi.org/10.1016/s0167-7012(99)00093-7

Kuang, J., Yan, X., Genders, A., Granata, C., & Bishop, D. (2018). An overview of technical considerations when using quantitative real-time PCR analysis of gene expression in human exercise research. *PLOS ONE, 13*(5), e0196438. https://doi.org/10.1371/journal.pone.0196438

L. Ferreira, V., H.L. Borba, H., de F. Bonetti, A., P. Leonart, L., & Pontarolo, R. (2019). Cytokines and Interferons: Types and Functions. *Autoantibodies And Cytokines*. https://doi.org/10.5772/intechopen.74550

La Pine, T., & Hill, H. (2011). Host Defense Mechanisms Against Bacteria. *Fetal And Neonatal Physiology*, 1553-1566. https://doi.org/10.1016/b978-1-4160-3479-7.10148-x

Lamkanfi, M., & Dixit, V. (2012). Inflammasomes and Their Roles in Health and Disease. Annual Review Of Cell And Developmental Biology, 28(1), 137-161. https://doi.org/10.1146/annurev-cellbio-101011-155745

Lamkanfi, M., & Dixit, V. (2014). Mechanisms and Functions of Inflammasomes. Cell, 157(5), 1013-1022. https://doi.org/10.1016/j.cell.2014.04.007

Li, P., Spolski, R., Liao, W., & Leonard, W. (2014). Complex interactions of transcription factors in mediating cytokine biology in T cells. Immunological Reviews, 261(1), 141-156. doi: 10.1111/imr.12199

Li, X., Yang, Q., Bai, J., Xuan, Y., & Wang, Y. (2014). Identification of appropriate reference genes for human mesenchymal stem cell analysis by quantitative real-time PCR. *Biotechnology Letters, 37*(1), 67-73. https://doi.org/10.1007/s10529-014-1652-9

Liu, T., Zhang, L., Joo, D., & Sun, S. (2017). NF-κB signaling in inflammation. *Signal Transduction And Targeted Therapy, 2*(1). https://doi.org/10.1038/sigtrans.2017.23

Livak, K., & Schmittgen, T. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods, 25*(4), 402-408. https://doi.org/10.1006/meth.2001.1262

Lopez-Castejon, G., & Brough, D. (2011). Understanding the mechanism of IL-1β secretion. *Cytokine &Amp; Growth Factor Reviews, 22*(4), 189-195. https://doi.org/10.1016/j.cytogfr.2011.10.001

Martin, M., Rehani, K., Jope, R., & Michalek, S. (2005). Toll-like receptor–mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nature Immunology, 6*(8), 777-784. https://doi.org/10.1038/ni1221

Martinon, F., Burns, K., & Tschopp, J. (2002). The Inflammasome. *Molecular Cell, 10*(2), 417-426. doi: 10.1016/s1097-2765(02)00599-3

Matlock, B. (2015). *Assessment of Nucleic Acid Purity.* Assets.thermofisher.com. Retrieved 28 May 2022, from https://assets.thermofisher.com/TFS-Assets/CAD/Product-Bulletins/TN52646-E-0215M-NucleicAcid.pdf.

McDonald, D., & Levy, O. (2019). Innate Immunity. *Clinical Immunology*, 39-53.e1. https://doi.org/10.1016/b978-0-7020-6896-6.00003-x

Morris, M., Gilliam, E., Button, J., & Li, L. (2014). Dynamic Modulation of Innate Immune Response by Varying Dosages of Lipopolysaccharide (LPS) in Human Monocytic Cells. *Journal Of Biological Chemistry, 289*(31), 21584-21590. https://doi.org/10.1074/jbc.m114.583518

Muñoz-Planillo, R., Kuffa, P., Martínez-Colón, G., Smith, B., Rajendiran, T., & Núñez, G. (2013). K+ Efflux Is the Common Trigger of NLRP3 Inflammasome Activation by Bacterial Toxins and Particulate Matter. *Immunity, 38*(6), 1142-1153. https://doi.org/10.1016/j.immuni.2013.05.016

Muri, J., Thut, H., Feng, Q., & Kopf, M. (2020). Thioredoxin-1 distinctly promotes NF-κB target DNA binding and NLRP3 inflammasome activation independently of Txnip. *Elife, 9*. https://doi.org/10.7554/elife.53627

Murphy, K., Weaver, C., Mowat, A., Berg, L., Chaplin, D., & Janeway, C. et al. (2017). *Janeway's immunobiology* (9th ed., pp. 1-77). New York: Garland Science.

Noori, M., Courreges, M., Bergmeier, S., McCall, K., & Goetz, D. (2020). Modulation of LPS-induced inflammatory cytokine production by a novel glycogen synthase kinase-3 inhibitor. *European Journal Of Pharmacology, 883*, 173340. https://doi.org/10.1016/j.ejphar.2020.173340

Oeckinghaus, A., & Ghosh, S. (2009). The NF- B Family of Transcription Factors and Its Regulation. *Cold Spring Harbor Perspectives In Biology, 1*(4), a000034-a000034. https://doi.org/10.1101/cshperspect.a000034

Oeckinghaus, A., Hayden, M., & Ghosh, S. (2011). Crosstalk in NF-κB signaling pathways. *Nature Immunology, 12*(8), 695-708. https://doi.org/10.1038/ni.2065

Owen, J., Kuby, J., Punt, J., & Stranford, S. (2013). *Immunology.* Macmillan Higher Education.

Pétrilli, V., Papin, S., Dostert, C., Mayor, A., Martinon, F., & Tschopp, J. (2007). Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death &Amp; Differentiation, 14*(9), 1583-1589. https://doi.org/10.1038/sj.cdd.4402195

Ramakers, C., Ruijter, J., Deprez, R., & Moorman, A. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience Letters, 339*(1), 62-66. https://doi.org/10.1016/s0304-3940(02)01423-4

Ramesh, G., MacLean, A., & Philipp, M. (2013). Cytokines and Chemokines at the Crossroads of Neuroinflammation, Neurodegeneration, and Neuropathic Pain. *Mediators Of Inflammation*, 2013, 1-20. https://doi.org/10.1155/2013/480739

Ren, K., & Torres, R. (2009). Role of interleukin-1β during pain and inflammation. *Brain Research Reviews, 60*(1), 57-64. https://doi.org/10.1016/j.brainresrev.2008.12.020

Sakai, J., Cammarota, E., Wright, J., Cicuta, P., Gottschalk, R., & Li, N. et al. (2017). Lipopolysaccharide-induced NF-κB nuclear translocation is primarily dependent on MyD88, but TNFα expression requires TRIF and MyD88. *Scientific Reports, 7*(1). https://doi.org/10.1038/s41598-017-01600-y

Sharma, D., & Kanneganti, T. (2016). The cell biology of inflammasomes: Mechanisms of inflammasome activation and regulation. *Journal Of Cell Biology, 213*(6), 617-629. doi: 10.1083/jcb.201602089

Swanson, K., Deng, M., & Ting, J. (2019). The NLRP3 inflammasome: molecular activation and regulation to therapeutics. *Nature Reviews Immunology, 19*(8), 477-489. https://doi.org/10.1038/s41577-019-0165-0

Tsai, C., Tsai, C., Tseng, P., Lin, C., & Chen, C. (2020). Glycogen Synthase Kinase-3β Facilitates Cytokine Production in 12-O-Tetradecanoylphorbol-13-Acetate/Ionomycin-Activated Human CD4+ T Lymphocytes. *Cells, 9*(6), 1424. doi: 10.3390/cells9061424

Types of ELISA | Bio-Rad. Bio-Rad. (2022). Retrieved 6 August 2022, from https://www.bio-rad-antibodies.com/elisa-types-direct-indirect-sandwich-competition-elisa-formats.html?JSESSIONID_STERLING=64188C3DA5B923E1FA1F1DFA77206097.ecommerce2&EU_COOKIE_PREFS=111&&evCntryLang=SE-enthirdPartyCookieEnabled=true#Sandwich.

Unger, C., Lokmer, N., Lehmann, D., & Axmann, I. (2019). Detection of phenol contamination in RNA samples and its impact on qRT-PCR results. *Analytical Biochemistry, 571*, 49-52. https://doi.org/10.1016/j.ab.2019.02.002

V. Stankov, S. (2012). Definition of Inflammation, Causes of Inflammation and Possible Anti-inflammatory Strategies. *The Open Inflammation Journal, 5*(1), 1-9. https://doi.org/10.2174/1875041901205010001

Wang, H., Brown, J., & Martin, M. (2011). Glycogen synthase kinase 3: A point of convergence for the host inflammatory response. *Cytokine, 53*(2), 130-140. doi: 10.1016/j.cyto.2010.10.009

Warrington, R., Watson, W., Kim, H., & Antonetti, F. (2011). An introduction to immunology and immunopathology. *Allergy, Asthma &Amp; Clinical Immunology, 7*(S1). https://doi.org/10.1186/1710-1492-7-s1-s1

Wilfinger, W., Mackey, K., & Chomczynski, P. (1997). Effect of pH and Ionic Strength on the Spectrophotometric Assessment of Nucleic Acid Purity. *Biotechniques, 22*(3), 474-481. https://doi.org/10.2144/97223st01

Wilson, S., & Cassel, S. (2010). Inflammasome-Mediated Autoinflammatory Disorders. *Postgraduate Medicine, 122*(5), 125-133. doi: 10.3810/pgm.2010.09.2209

Wu, H., Taki, F., Zhang, Y., Dobbins, D., & Pan, X. (2014). Evaluation and identification of reliable reference genes for toxicological study in Caenorhabditis elegans. *Molecular Biology Reports, 41*(5), 3445-3455. https://doi.org/10.1007/s11033-014-3206-6

Wu, Y., Zhou, J., Liu, Y., Gu, Y., Zhang, H., & Ahmad, F. et al. (2022). Selection and Validation of Reliable Reference Genes for qRT-PCR Normalization of Bursaphelenchus xylophilus from Different Temperature Conditions and Developmental Stages. *Applied Sciences, 12*(6), 2880. https://doi.org/10.3390/app12062880

Xu, Z., Chen, Z., Wu, X., Zhang, L., Cao, Y., & Zhou, P. (2020). Distinct Molecular Mechanisms Underlying Potassium Efflux for NLRP3 Inflammasome Activation. *Frontiers In Immunology*, 11. https://doi.org/10.3389/fimmu.2020.609441

Yang, Q., Liu, Q., Lv, H., Wang, F., Liu, R., & Zeng, N. (2019). Effect of pulegone on the NLPR3 inflammasome during inflammatory activation of THP-1 cells. *Experimental And Therapeutic Medicine*. https://doi.org/10.3892/etm.2019.8327

Yaron, J., Gangaraju, S., Rao, M., Kong, X., Zhang, L., & Su, F. et al. (2015). K+ regulates Ca2+ to drive inflammasome signaling: dynamic visualization of ion flux in live cells. *Cell Death &Amp; Disease, 6*(10), e1954-e1954. https://doi.org/10.1038/cddis.2015.277

Yu, Y., Wan, Y., & Huang, C. (2009). The Biological Functions of NF-&#954;B1 (p) and its Potential as an Anti-Cancer Target. *Current Cancer Drug Targets, 9*(4), 566-571. https://doi.org/10.2174/156800909788486759

Yuan, J., Reed, A., Chen, F., & Stewart, C. (2006). Statistical analysis of real-time PCR data. *BMC Bioinformatics, 7*(1). https://doi.org/10.1186/1471-2105-7-85

Zhao, W., Ma, L., Cai, C., & Gong, X. (2019). Caffeine Inhibits NLRP3 Inflammasome Activation by Suppressing MAPK/NF-κB and A2aR Signaling in LPS-Induced THP-1 Macrophages. *International Journal Of Biological Sciences, 15*(8), 1571-1581. https://doi.org/10.7150/ijbs.34211

Zhou, R., Yazdi, A., Menu, P., & Tschopp, J. (2010). A role for mitochondria in NLRP3 inflammasome activation. *Nature, 469*(7329), 221-225. https://doi.org/10.1038/nature09663

Zielinski, M., & Krueger, J. (2012). Inflammation and Sleep. *Therapy In Sleep Medicine*, 607-616. https://doi.org/10.1016/b978-1-4377-1703-7.10048-9

Appendices

Appendix 1: RNA concentration and purity

Table 1. Concentration and purity of extracted RNA, measured by using DeNovix DS11 spectrophotometer.

Biological Replicate	Sample	Concentration (ng/µl)	Purity $A_{260/280}$	$A_{260/230}$
1	0.1 µM SB216763 + LPS and Nigericin	24.09	2.01	0.66
	1 µM SB216763 + LPS and Nigericin	25.45	1.84	1.12
	10 µM SB216763 + LPS and Nigericin	22.03	2.04	0.52
	LPS and Nigericin	26.03	2.06	0.04
	0.1% DMSO	33.37	1.98	0.41
	Unstimulated	31.58	2.06	1.16
2	0.1 µM SB216763 + LPS and Nigericin	14.66	2.09	1.51
	1 µM SB216763 + LPS and Nigericin	17.29	2.07	0.22
	10 µM SB216763 + LPS and Nigericin	17.15	2.15	0.22
	LPS and Nigericin	24.50	2.05	1.66
	0.1% DMSO	25.79	2.03	0.43
	Unstimulated	26.02	2.14	0.27
3	0.1 µM SB216763 + LPS and Nigericin	20.65	2.07	0.29

1 µM SB216763 + LPS and Nigericin	27.89	1.99	1.17
10 µM SB216763 + LPS and Nigericin	24.42	2.00	0.59
LPS and Nigericin	16.35	1.86	1.57
DMSO	11.33	1.87	0.04
Unstimulated	16.57	1.60	0.14

Table 2. Designed primers for IL-1β and *YWHAZ*.

Gene name	Primer sequence 5'-3'	GC content (%)	Tm (C°)	Amplicon length (bp)	qPCR efficiency (%)	Reference
IL-1β	F AGCCATGGCAGAAGTACCTG	55	59.75	116	114.1	Designed with Primer-Blast (NCBI)
	R CCTGGAAGGAGCACTTCATCT	52.38	59.44			
	F TTCGAGGCACAAGGCACAA	52.63	60.15	78	102.6	Designed with Primer-Blast (NCBI)
	R TGGCTGCTTCAGACACTTGAG	52.38	60.54			
YWHAZ	F CGAAGCTGAAGCAGGAGAAG	55	58.64	110	95.8	PMID: 31240211
	R TTTGTGGGACAGCATGGATG	50	58.45			
	F ACTTTTGGTACATTGTGGCTTCAA	37.5	59.6	94	109.1	PMID: 12184808
	R CCGCCAGGACAAACCAGTAT	55	60			

Appendix 2: Gene expression of IL-1β

Table 1. Log2 fold changes of IL-1β after the different treatments of samples. Calculated with calibrator sample (only differentiated/unstimulated).

Replicates	Sample	Log2 Fold Change	Average Log2 Fold Change
1	0.1 µM SB216763 + LPS and Nigericin	7.82	5.36
2	0.1 µM SB216763 + LPS and Nigericin	4.44	
3	0.1 µM SB216763 + LPS and Nigericin	3.81	
1	1 µM SB216763 + LPS and Nigericin	7.39	5.46
2	1 µM SB216763 + LPS and Nigericin	4.45	
3	1 µM SB216763 + LPS and Nigericin	4.00	
1	10 µM SB216763 + LPS and Nigericin	8.02	5.66
2	10 µM SB216763 + LPS and Nigericin	4.35	
3	10 µM SB216763	4.59	
1	LPS and Nigericin	7.68	5.29
2	LPS and Nigericin	4.08	
3	LPS and Nigericin	4.11	
1	DMSO	1.92	0.83
2	DMSO	0.07	
3	DMSO	0.48	
1	Unstimulated	0.84	0.00
2	Unstimulated	-0.64	
3	Unstimulated	-0.20	

Table 2. Log2 fold changes of IL-1β after the different treatments of samples. Calculated with calibrator sample (Stimulated/LPS and Nigericin).

Replicates	Sample	Log2 Fold Change	Average Log2 Fold Change
1	0.1 µM SB216763 + LPS and Nigericin	2.54	0.07

2	0.1 µM SB216763 + LPS and Nigericin	-0.85	
3	0.1 µM SB216763 + LPS and Nigericin	-1.48	
1	1 µM SB216763 + LPS and Nigericin	2.64	0.17
2	1 µM SB216763 + LPS and Nigericin	-0.84	
3	1 µM SB216763 + LPS and Nigericin	-1.29	
1	10 µM SB216763 + LPS and Nigericin	2.73	0.37
2	10 µM SB216763 + LPS and Nigericin	-0.94	
3	10 µM SB216763 + LPS and Nigericin	-0.69	
1	LPS and Nigericin	2.39	0.00
2	LPS and Nigericin	-1.21	
3	LPS and Nigericin	-1.18	
1	DMSO	-3.36	-4.46
2	DMSO	-5.21	
3	DMSO	-4.81	
1	Only differentiated	-4.44	-5.29
2	Only differentiated	-5.93	
3	Only differentiated	-5.49	

Table 3. Results from Kruskal-Wallis test showing significant difference between the groups from the gene expression analysis. The significance level is 0.050.

Sample 1 – Sample 2		Std. Error	Sig.
10 µM SB216763 + LPS and Nigericin	1 µM SB216763 + LPS and Nigericin	4.359	0.702
10 µM SB216763 + LPS and Nigericin	0.1 µM SB216763 + LPS and Nigericin	4.359	0.541
10 µM SB216763 + LPS and Nigericin	LPS and Nigericin	4.359	0.491

10 µM SB216763 + LPS and Nigericin	DMSO	4.359	0.022
10 µM SB216763 + LPS and Nigericin	Unstimulated	4.359	0.007
1 µM SB216763 + LPS and Nigericin	0.1 µM SB216763 + LPS and Nigericin	4.359	0.819
1 µM SB216763 + LPS and Nigericin	LPS and Nigericin	4.359	0.760
1 µM SB216763 + LPS and Nigericin	DMSO	4.359	0.056
1 µM SB216763 + LPS and Nigericin	Unstimulated	4.359	0.022
0.1 µM SB216763 + LPS and Nigericin	LPS and Nigericin	4.359	0.939
0.1 µM SB216763 + LPS and Nigericin	DMSO	4.359	0.092
0.1 µM SB216763 + LPS and Nigericin	Unstimulated	4.359	0.039
LPS and Nigericin	DMSO	4.359	0.108
LPS and Nigericin	Unstimulated	4.359	0.047
DMSO	Unstimulated	4.359	0.702

Appendix 3: Data for IL-1β secretion

Table 1. Concentration of secreted IL-1β (combined data average)

Treatment	Concentration (pg/mL)
Experimental replicate 1	
0.1 µM SB216763 + LPS and Nigericin	6187
1 µM SB216763 + LPS and Nigericin	6113
10 µM SB216763 + LPS and Nigericin	7031
LPS and Nigericin	6566
DMSO	1.972
Unstimulated	-
Experimental replicate 2	
0.1 µM SB216763 + LPS and Nigericin	6664
1 µM SB216763 + LPS and Nigericin	5080
10 µM SB216763 + LPS and Nigericin	6354
LPS and Nigericin	4202
DMSO	0.021
Unstimulated	0.021
Experimental replicate 3	
0.1 µM SB216763 + LPS and Nigericin	3946
1 µM SB216763 + LPS and Nigericin	3768
10 µM SB216763 + LPS and Nigericin	3791
LPS and Nigericin	3188
DMSO	-0.036
Unstimulated	-0.036
Experimental replicate 4	
0.1 µM SB216763 + LPS and Nigericin	3338
1 µM SB216763 + LPS and Nigericin	4459
10 µM SB216763 + LPS and Nigericin	5031
LPS and Nigericin	4399
DMSO	-0.036
Unstimulated	-0.036

Table 2. Results from Kruskal-Wallis test showing significant difference between the groups of mean concentration of secreted IL-1β. The significance level is 0.050.

Sample 1 – Sample 2		Sig.
Unstimulated	DMSO	0.766
Unstimulated	LPS and Nigericin	< 0.001
Unstimulated	0.1 µM SB216763 + LPS and Nigericin	< 0.001
Unstimulated	1 µM SB216763 + LPS and Nigericin	< 0.001
Unstimulated	10 µM SB216763 + LPS and Nigericin	< 0.001
DMSO	LPS and Nigericin	< 0.001
DMSO	0.1 µM SB216763 + LPS and Nigericin	< 0.001
DMSO	1 µM SB216763 + LPS and Nigericin	< 0.001
DMSO	10 µM SB216763 + LPS and Nigericin	< 0.001
LPS and Nigericin	0.1 µM SB216763 + LPS and Nigericin	0.706
LPS and Nigericin	1 µM SB216763 + LPS and Nigericin	0.691
LPS and Nigericin	10 µM SB216763 + LPS and Nigericin	0.237
0.1 µM SB216763 + LPS and Nigericin	1 µM SB216763 + LPS and Nigericin	0.984
0.1 µM SB216763 + LPS and Nigericin	10 µM SB216763 + LPS and Nigericin	0.420
1 µM SB216763 + LPS and Nigericin	10 µM SB216763 + LPS and Nigericin	0.432