Degree project

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Expression of the chloride channel CLCC1 is down-regulated after 24 hours in LPS-primed THP-1 monocyte-like cell line

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Abstract

Inflammation is the body's response to infection or injury and is mediated by the innate immune system. The NLRP3 inflammasome is a multi-protein complex that is a major contributor to many inflammatory disorders. Emerging evidence suggests the involvement of the Endoplasmic reticulum stress with the NLRP3 inflammasome. The endoplasmic reticulum stress is a series of stress signals that can activate the unfolded protein response and usually accompanies inflammation and eventually causes cell death. Recently, a localized endoplasmic reticulum micro-protein called the chloride clic like-1 channel was found to be involved in the endoplasmic reticulum homeostasis. Recent evidence suggests the involvement of endoplasmic reticulum stress in the inflammation pathways of the NLRP3 inflammasome. The relationship between the ER and the NLRP3 inflammasome has not been clearly described. This study aimed at investigating the expression levels of the microprotein CLCC1 to shed a light on the relationship between the endoplasmic reticulum stress and the NLRP3 inflammasome. The expression levels of CLCC1 were analyzed by qPCR in cultured monocytes under different time points of Lipopolysachaaride immuno-stimulation. The stability of expression in candidate reference genes was investigated for normalization purposes. This study reported the regulation of CLCC1 as a novel finding under prolonged LPS exposure of monocytes and stable reference genes such as GUSB and ACTB were identified. The relationship between CLCC1 and NLRP3 inflammasome priming by LPS indicated that CLCC1 is regulated and may be involved in the inflammatory mechanisms of endoplasmic reticulum stress and NLRP3 inflammasome inflammatory diseases, contributing to a potential therapeutic target in the endoplasmic reticulum and inflammasome related diseases.

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List of Abbreviations

Abbreviation	Description
ASC	adaptor molecule apoptosis-associated speck-like
CARD	Caspase recruitment domain
DAMP	Danger-associated molecular patterns
ERS	Endoplasmic reteciulum stress
IL-18	Interleukin-1 beta
IL-1B	Interleukin-18
LPS	Lipopolysaccharide
PAMP	Pathogen-associated molecular patterns
PRR	Pattern recognition receptors
PYD	Pyrin domain
ROS	Reactive oxygen species
TLR	Toll-like receptors

Introduction

Inflammation is defined as the body's repose to an injury or infection and it is mediated via the innate immune system, which acts as the first line of acute host defense against pathogens. This idea was named the "stranger theory" or the "self-non-self" theory (Tauber, 1994). Later on, a researcher named Polly Matzinger suggested a new theory called the ''danger theory'' and it explained that damaged cells or tissues release alarming signals similar to those activated by pathogens (Matzinger, 1994). These danger signals get recognized by pattern recognition receptors (PRR) which in return activate the response of the immune cells with the absence of pathogens (sterile inflammation). PRR's are mainly expressed in the innate immune system cells like neutrophils, macrophages/monocytes, and dendritic cells which can recognize two types of molecular patterns, (1) PAMPS: pathogen-associated molecular patterns and (2) DAMPS: danger-associated molecular patterns. Several types of PRR's exist, including toll-like receptors (TLR's), retinoic acid-inducible gene (RIG)-I-like receptors, C-type leucine receptors (CLR's), and nucleotide-binding oligomerization domain-like receptors (NLR's) (Karasawa & Takahashi, 2017). These pattern recognition receptors act as an early alarming mechanism to start the immune response.

Inflammasomes are protein complexes located in the cytosol and are responsible for immune responses regarding infections and cellular damage. The formation of these complexes results in the cleavage of inflammatory cytokines such as Il-1B and IL-18 into their bioactive or mature form via caspase-1 (He et al., 2016). However, several types of inflammasomes have been observed and are defined by the NLR category, such as NLRP1, NLRP3, NLRP4, NLRP6, and NLRP12.

One member of the NLR family is the NLR pyrin domain containing 3 (NLRP3) that has been extensively observed and recognizes several types of DAMP's while toll-like receptors can recognize microbial ligands or PAMP's and indirectly prepare the NLRP3 inflammasome for formation (Karasawa & Takahashi, 2017). First explained by Martinon (2002), the NLRP3 inflammasome is made up of NLR pyrin domain containing 3 (NLRP3) protein, making up the NLRP3 inflammasome with a cysteine protease caspase-1 (with catalytic domains p10 & p20), and the adaptor molecule apoptosis-associated speck-like protein (ASC), (Figure 1). The NLRP3 contains leucine-rich repeats (LRR's), NACHT domain, and a pyrin domain (PYD), while ASC consists of a PYD and a caspase recruitment domain CARD (Takahashi, 2019). The activation of NLRP3 is described in a two-step manner, the priming step and the activation step. The NLRP3 inflammasome has been recognized to promote a form of cell death known as pyroptosis (Yang et al., 2019).

A recent study hypothesized that extra-cellular lipopolysaccharides (LPS) from Gram-negative bacteria bind to the CARD domains of the pro-caspase-1 via the toll-like receptor 4 and triggered the oligomerization and activation of caspase-1 which leads to the activation of what is called the non-canonical inflammasome pathway and IL-1b secretion (He et al., 2016). Additionally, a process called the alternative NLRP3 activation indicated the formation of an active NLRP3 inflammasome is not always a two-step process, stimulation from LPS alone can lead to upregulation and NLRP3 inflammasome detection in THP-1 cells without any treatment other than LPS (Gritsenko et al., 2020).

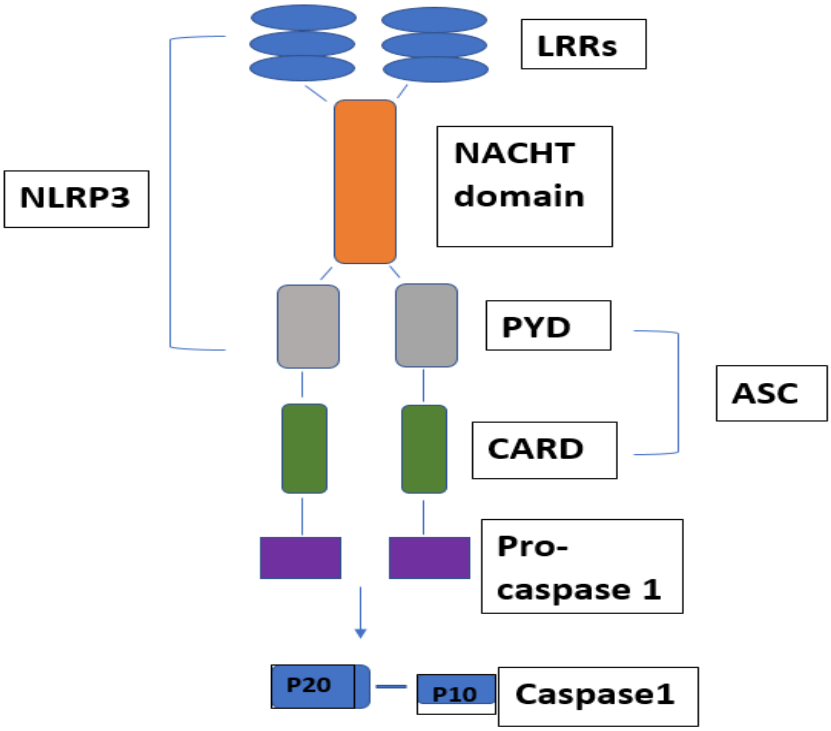

Figure 1. LRRs and the NACHT domain make up the NLRP3, the PYD and CARD domain forms the ASC, while p10 and p20 catalytic domains cleave IL-1B into its mature form.

The priming signal for the NLRP3 inflammasome is the initiation stage for activation by the expression of NLRP3 and it is induced by endogenous molecules, TLR ligands like (LPS), tumor necrosis, and Il-1B via the nuclear factor Kappa B. NF-KB promotes the release of pro-inflammatory cytokines and is considered a key transcription factor in innate immunity as well as adaptive immunity (Zhang et al., 2017). The activation of the NLRP3 inflammasome includes various cellular and molecular steps that have been proposed to trigger activation, including potassium efflux, extracellular ATP, calcium signaling, viral RNA, reactive oxygen species, mitochondrial dysfunction, and lysosomal rupture (He et al., 2016). The exact process of NLRP3 activation has not been fully interpreted yet. Several common pathways exist for activation of the NLRP3 inflammasome such as cationic efflux within and at the extracellular space of the cell as well as the involvement of other organelles like lysosomes, mitochondria, and endoplasmic reticulum (Sutterwala, Haasken & Cassel, 2014). Recently, the endoplasmic reticulum stress (ERS) was found to be a factor in activating the NLRP3 inflammasome through NF-KB activation, calcium metabolism, and reactive oxygen species (ROS) (Li et al., 2020).

The Endoplasmic reticulum is the intracellular organ that involves secreting proteins, synthesis of lipids, maintaining intracellular calcium homeostasis, and membrane biosynthesis. ER stress occurs due to improper protein folding accumulation in the lumen (Kaufman, 2004). Cells undergo a series of signaling pathways activated by ER stress as a defensive response to maintain protein homeostasis named the unfolded protein response UPR. Activator proteins involved are inositol-requiring enzyme 1a (IRE1a), double-stranded RNA-dependent PKR-like ER kinase (PERK), and activation of transcription factor-6 (ATF-6) (Zhang et al., 2016). The transcription of ER stress-related genes such as XBP1, ATF4 & the transcription factor CCAAT enhancer-binding protein CHOP have been reported in LPS injected mice, suggesting that LPS can induce ER stress-

related genes (Endo et al., 2005).

Moreover, calcium storage situates in the endoplasmic reticulum in eukaryotic cells, when ER stress occurs, this leads to a huge influx of calcium ions shuttled into the mitochondria by the mitochondria-associated membranes which in turn damages the mitochondria due to calcium overload (Gómez-Suaga et al., 2018). This phenomenon causes excessive mitochondrial ROS production that can activate the NLRP3 inflammasome complex (Gong et al., 2018). The release of calcium is considered a potent trigger of the NLRP3 inflammasome during ERS (Li et al., 2020). Additionally, NF-kB promotes the secretion of Il-1B during ERS that involves ROS generation as well as the activation of thioredoxin interacting protein (TXNIP) which is an NLRP3 ligand that is sensitive to ROS and can activate caspase-1 and the NLRP3 in a ROS dependent manner (Hu et al., 2018).

However, a recent study indicated the regulation of ER stress through a 54- amino acid micro-protein named (PIGBOS) that showed dependent interaction with another micro-protein chloride channel CLIC like 1 (CLCC1) at the ER-mitochondrial contact sites (Chu et al., 2020). Interestingly, when knocking out the PIGBOS gene in mice, the loss of PIGBOS led to the activation of UPR and heightened ER stress. The chloride channel CLIC like 1 is a putative chloride channel transmembrane protein situated in the ER and Golgi apparatus with a high permeability to anions, especially chloride (Nagasawa et al., 2001). Additionally, the knockdown of CLCC1 in the mouse model has been shown to cause ER stress and increased sensitivity to chemically induced ER stressors, suggesting that CLCC1 has a crucial role in maintaining ER homeostasis (Jia et al., 2015).

As mentioned earlier, ER stress is involved in activating the NLRP3 inflammasome by intersecting with common inflammatory pathways such as oxidative stress, calcium homeostasis, and NF-KB transcription factor. Recent evidence has shown that ER stress exacerbates and accompanies inflammation (ERS-induced inflammation) and is the pathological basis of many diseases such as diabetes, inflammatory bowel disease, atherosclerosis, cancer, alcoholic liver diseases, and rheumatoid arthritis (Li et al., 2020). However, the exact mechanisms and relationship of how ER stress exacerbates NLRP3 derived inflammation have not been completely described and remain largely unexplored (Chong et al., 2021). Considering the complex relationship between ER stress and the NLRP3 inflammasome, the investigated question in this study focused on the involvement of the ER micro-protein CLCC1 in the context of NLRP3 inflammasome priming by exposure of the immunostimulant LPS. Thus, in efforts to shed light on the relationship and mechanisms between the NLRP3 inflammasome activation and ER stress, this study aimed at investigating gene expression levels of the chloride channel (CLCC1). The primary objective of this study was to test the regulation of CLCC1 in LPS primed THP-1 monocytes. Gene expression levels of CLCC1 were measured via qPCR before and after LPS stimulation in THP-1 monocytes at different time points. The IL-1B gene expression levels were measured as a positive control for LPS priming. Moreover, to achieve robust and accurate data normalization for relative quantification of target genes, the objectives of this study included investigating potential reference genes by testing the stability of expression via qPCR in LPS unprimed and primed THP-1 monocytes. Data analysis was performed by using sophisticated statistical software such as GeNorm and NormFinder to determine the stability of the selected reference genes.

Materials and Methods

THP-1 Growth Conditions

THP 1 cells were cultured in RPMI 1640 with L-glutamine 0.3g/L, 10% heat-inactivated FBS, 10 mM HEPES, 1 mM sodium pyruvate, 0.45% glucose and 100 U/ml penicillin-streptomycin at 37°C and 5% CO2. Zeocin (200µg/ml) (Invivogen) was added to the culture medium as per the manufacturer's instructions. Cells were differentiated by the addition of 100 nM PMA for 72 hours, washing twice with culture media, and incubating at 37°C and 5% CO2 for 48 hours in fresh media. Priming was conducted by adding 500ng/ml LPS. Total RNA was extracted with the RNeasy Mini Kit (Qiagen) after four LPS treatments at (4 hours, 8 hours, and 24 hours) respectively. A total of twelve total RNA samples from three biological replicates of each time point were used.

cDNA synthesis

Total RNA concentration and purity were measured by DS11 spectrophotometer (DeNovix). RNA samples were purified from genomic DNA by DNase treatment using the DNA-free™ kit (Applied Biosystems) according to the protocol's instructions. Complementary DNA was generated via the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) without the RNase inhibitor. The reverse transcription reaction program was run as per protocol recommendation using the MJ Research PTC-200 Thermal cycler (Bio-Rad). An equivalent of 0.5 µg of total RNA per 20 µL reaction was reverse transcribed from four biological samples done in triplicates. No Reverse Transcription-reaction controls were included to check for genomic contamination done in triplicates. The generated cDNA samples were divided into ten aliquots of 50 ng each stored in the freezer for later use in qPCR and diluted in nuclease-free water.

qPCR

Potential reference genes (TUBB, B2M, RRN18s, and TBP) in the SYBR Human reference gene panel (TATAA Bio-center) and target genes (CLCC1 and IL-1B) were tested for efficiency of amplification to be in an acceptable range by performing 10-fold serial dilutions of five standards ranging from 100 ng to 0.01 ng. qPCR was performed with three technical replicates and 200 nM primer concentration. Average (Cq) values were plotted against the logarithmic value of the sample dilution factor. Efficiency was calculated according to the qPCR efficiency calculator of Thermo Fisher Scientific. qPCR was performed by using the following, SYBR Select Master Mix kit (Applied Biosystems) and AriaMx Real-time PCR System (Agilent) with an amplification profile of 95°C for 2 minutes, followed by 95°C for 15 seconds of 40 cycles, 60°C for 1 minute and a melt curve analysis (step and hold) as per protocol recommendation. Potential reference genes and target genes were analyzed for expression by qPCR in triplicates from four biological replicates in each sample. The qPCR final reaction volume was set to 10 µL with 1 ng of input cDNA and (200nM) primer concentration with including no-template controls in triplicates. Interplate calibrator was run in triplicates to compensate for variations between qPCR runs. Il-1B and CLCC1 (Table 1) primers were obtained from (Thermo Fisher Scientific). CLCC1 primers were selected from qPrimer database and blasted in the NCBI human genome database to check for different splice variants.

Table 1. Primer details for CLCC1 and IL-1B.

Primer Pair	Forward primer(5'->3')	Reverse primer(3'->5')	Primer Bank/Qprimer ID
CLCC1(1)	AAACATGGAAGTGGCGATTCG	GTCTCAACTGAGTGTACCAACG	115270969c1
CLCC1(2)	ACCCAGTCATAAATCACCTGTT	GTGAACCTTCTGTATTCCCTGA	ENST00000369968.6_700-999
CLCC1(3)	ACCGAAAGAAAGCAGTACTGAA	GTGAACCTTCTGTATTCCCTGA	ENST00000369968.6_850-1100
CLCC1(4)	ACATTCACCACATTTGTAACGG	CTGGAATTTCCTTCATGAGTGC	ENST00000369968.6_150-449
IL-1B	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA	27894305c1

Statistical Analysis

The algorithms GeNorm and Normfinder in GenEx 6.0 software were used to determine the expression stability of candidate reference genes by the input of raw Cq values. Relative gene expression (fold change) of CLCC1 and IL-1B was calculated by the delta-delta Cq method in Microsoft Excel software by normalizing the target genes with the geometric mean of the suitable reference genes with the average delta Cq of the calibrator sample (0H LPS). Statistical significance between groups was tested by one-way ANOVA with Tuckey's post-hoc test for multiple comparisons using the software SPSS 21.0 and p-values lower than 0.05 were considered significant.

Results

Genes such as (B2M and RRN18s) in the TATAA reference gene panel were designed within an exon and therefore may amplify genomic amplification. No RT controls showed no amplification of genomic DNA in the assay for all genes analyzed including potential reference genes. All No template controls showed no amplification and thus no contamination was observed. Primer efficiencies can be seen (Appendix Table 1). Raw data for statistical analysis from One-way ANOVA are presented in (Supplementary File 2) in the appendix. Fold change calculations for both target genes (CLCC1(4) and IL-1B) are included in the appendix (see supplementary file 1).

Validation of Reference Genes

The two most stable pairs of genes in the panel according to GeNorm were GUSB and TBP with an M-value of 0.65. The least stable genes were TUBB & PPIA with an M-value of 1.0 and 1.2

respectively (Figure 2). One gene (HRP) in the reference gene panel out of the twelve was excluded from further analysis due to efficiency of amplification being outside the acceptable range before testing stability of expression. All M-values generated are shown in (see Appendix Table 2).

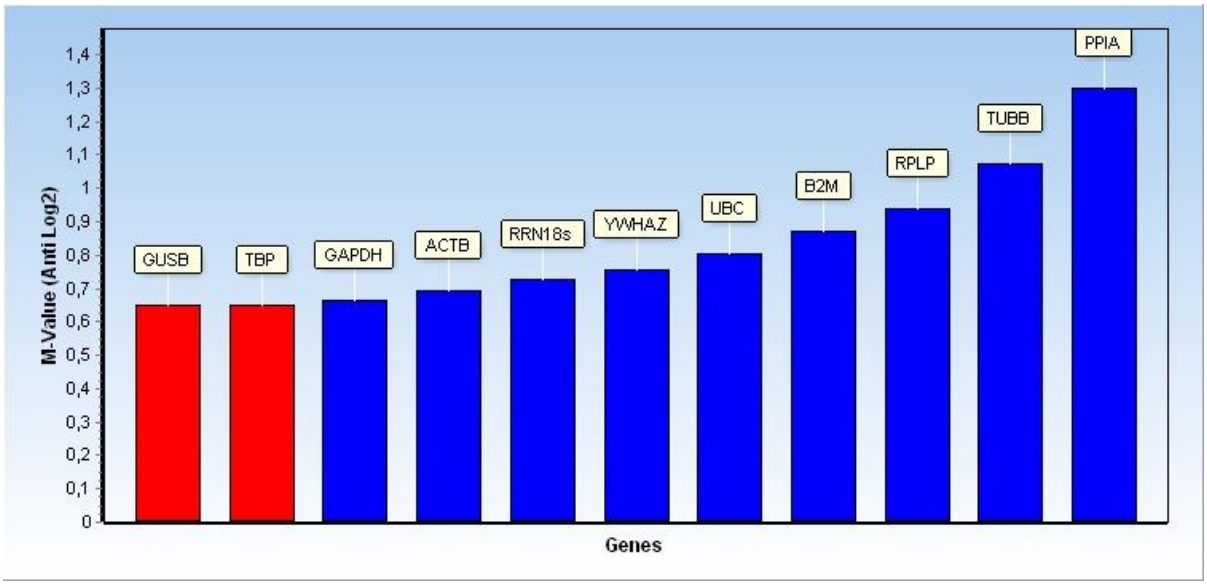

Figure 2. A GeNorm Bar chart for the stability of reference genes after LPS stimulation at 0, 4, 8, and 24 hours. Candidate genes are ranked from the highest to the lowest stability according to the M-value from left to right.

The two most stable genes according to the findings from NormFinder were GUSB & ACTB with a stability value of 0.42 and 0.47 respectively (Figure 3). While the two least stable genes were B2M and RPLP with a stability value of 0.94 and 1. Genes with a stability value (SD) higher than 1 (TUBB & PPIA) were excluded from the NormFinder analysis due to higher variability of expression. Genes with high variability influence the overall of the NormFInder analysis along with the number of candidate genes analyzed, therfore genes with SD higher than 1 were excluded. After ticking the inter and intra group variation option in NormFinder, GUSB and ACTB were confirmed to be the best combination of reference genes with a stability value (SD) of 0.19 (See Appendix Figure 1).

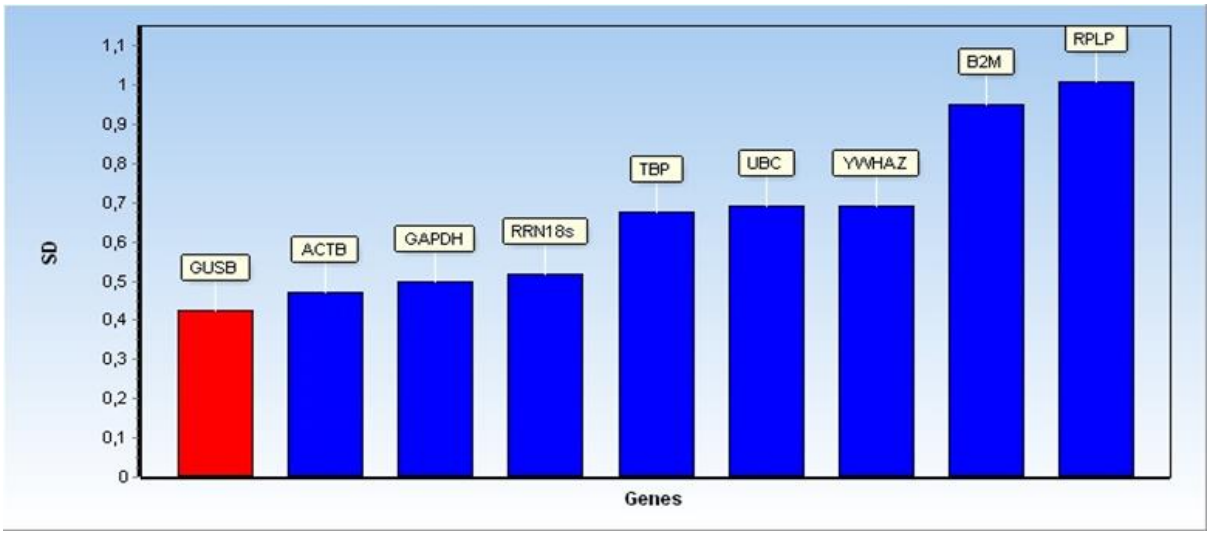

Figure 3. Bar chart from NormFinder for the candidate reference genes tested across all LPS treatments. Candidate genes are ranked from the highest stability (low value) to the lowest stability (high value) from left to right.

IL-1B Expression Analysis

IL-1B is considered a well-known marker of inflammasome priming and inflammation. As a positive control for LPS priming, IL-1B was analyzed by performing qPCR upon various LPS time points after LPS treatment. The fold change of IL-1B (Table 2) after the delta delta Cq method ($2^{-(ddcq)}$) indicated that on average, IL-1B was upregulated 19.4 folds after 4 hours of LPS treatment, 23.06 folds after 8 hours, and 17.20 folds after 24 hours respectively in comparison with the calibrator sample (0 hours LPS) (see Appendix Table 3). P-values were (0.00008) at 4 hours, (0.00005) at 8 hours, and (0.0001) at 24 hours when compared to the unprimed sample group as shown in (Figure 4).

Table 2. Fold changes of IL-1B after LPS stimulation.

Sample	Fold Change	Average Fold Change (FC)
NO LPS	1.802	1
NO LPS	1.232	
NO LPS	0.4500	
4H LPS	22.608	19.49
4H LPS	17.137	
4H LPS	18.729	
8H LPS	23.954	23.06
8H LPS	25.435	
8H LPS	19.806	
24H LPS	12.169	17.20
24H LPS	21.831	
24H LPS	17.608	

Table 2 indicates groups of LPS treatment with the corresponding fold change of each biological replicate (n=3) and the average fold change of each group.

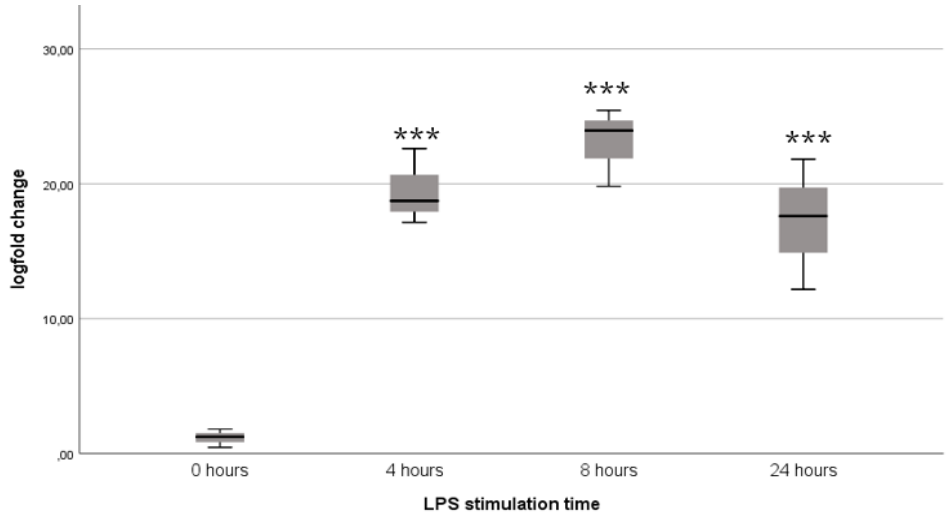

Figure 4. Box plot showing relative log fold change of IL-1B at 0, 4, 8, and 24 hours after LPS stimulation (n=3 in each group). Statistical significance was determined by one-way ANOVA following Tukey's post-hoc test. Asterisks resemble P-value significance P ≤ 0.05 (*), P ≤ 0.01 (**), P ≤ 0.001 (***).

CLCC1 Expression Analysis

CLCC1 has been proposed to regulate ER stress in mice and showed an important interaction at the ER-mitochondrial contact sites that were critical for maintaining ER homeostasis. After performing qPCR to check the efficiency of CLCC1 primer pairs (see Table 1). Primer pair CLCC1(4) was selected due to having the highest efficiency (106%) within the desired range and was selected for expression analysis (Figure 5). Further expression analysis by qPCR for CLCC1 indicated that CLCC1 was differentially expressed after 24 hours of LPS and downregulated by 2.2 folds on average (Table 3) in comparison with the calibrator sample (0 hours). Results were normalized with the appropriate reference genes (Appendix Table 4) with the delta-delta Cq method ($2^{-(ddcq)}$) to ensure accuracy. The One-way ANOVA analysis for multiple comparisons between the 0 hours group and 24 hours group indicated a (p-value: 0.023). The 4 and 8 hour time points showed no significant difference to the control (Figure 6).

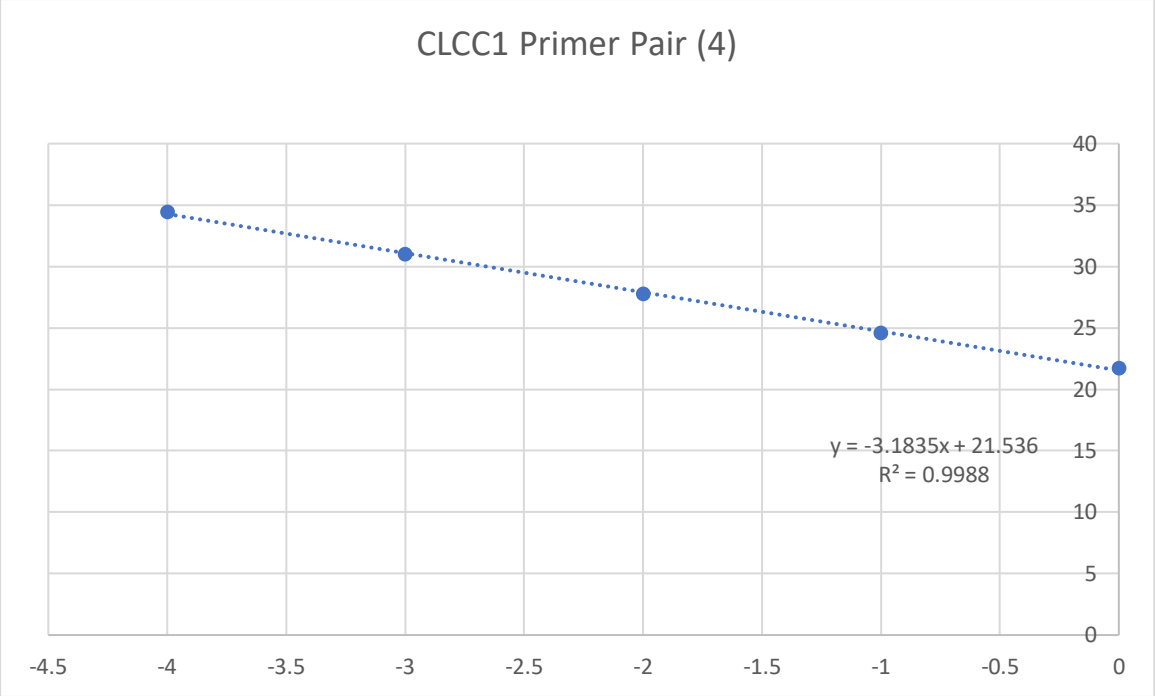

Figure 5. The efficiency of CLCC1(4) with the average Cq values plotted on the Y-axis and the logarithmic value of the sample dilution factor on the X-axis. Slope=-3.1835, R=0.99.

Table 3. Exact values for Fold changes of CLCC1(4) expression analysis after LPS stimulation.

Sample	Fold Change	Average Fold Change (FC)
NO LPS	0.73448	1
NOLPS	1.39088	
NO LPS	0.97887	
4H LPS	0.78999	0.67
4H LPS	0.56129	
4H LPS	0.67364	
8H LPS	0.82932	0.81
8H LPS	0.84473	
8H LPS	0.77148	
24H LPS	0.32226	0.45
24H LPS	0.68591	
24H LPS	0.36838	

Table 3 indicates groups of LPS treatment with the corresponding fold change of each biological replicate (n=3) and the average fold change of each group.

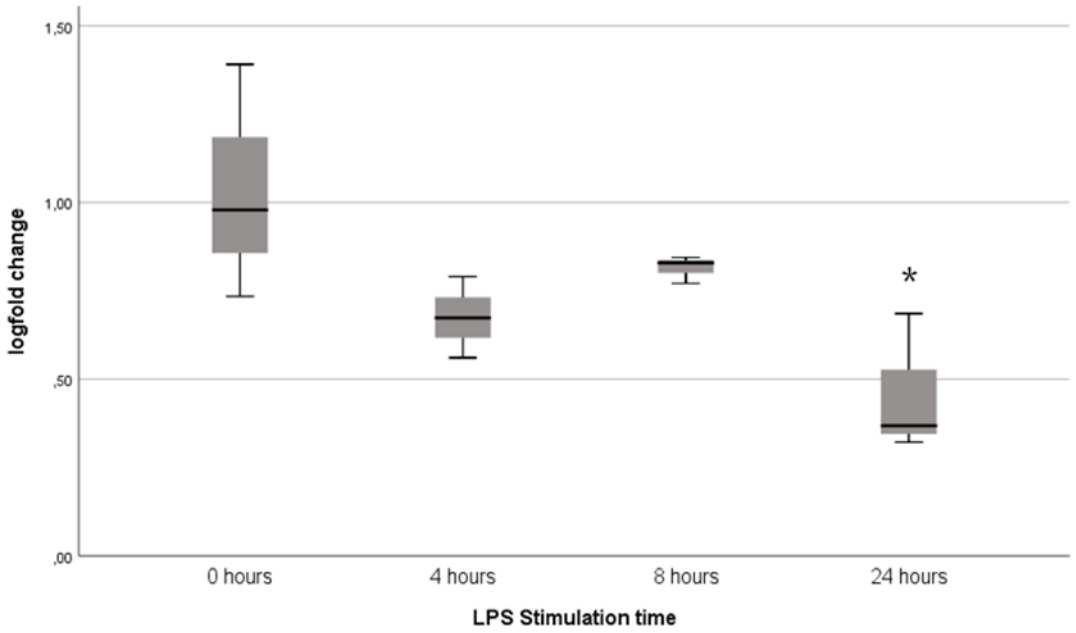

Figure 6. Box plot showing relative log fold change of CLCC1 at 0, 4, 8, and 24 hours after LPS stimulation (n=3 in each group). Statistical significance was determined by one-way ANOVA following Tukey's post-hoc test. Asterisks resemble P-value significance $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***).

Discussion

Emerging evidence has described ERS-induced inflammation as the pathological cause of various inflammatory diseases. The intersection and involvement of ERS with inflammatory pathways have attracted attention in seeking novel therapeutic targets, especially concerning the NLRP3 inflammasome complex. The exact underlying mechanisms of the ERS involvement in NLRP3 inflammatory pathways have not been fully described. This present study primarily aimed at investigating a chloride channel micro-protein (CLCC1) located in the endoplasmic reticulum in relation to the NLRP3 inflammasome. The involvement of this novel gene in the context of NLRP3 inflammasomes has not been studied before, which provides a potential in both providing a better understanding and discovering novel therapeutic targets in ERS-induced NLRP3 inflammation. This is the first study to report a novel finding of the regulation of CLCC1 expression in relation to the NLRP3 inflammasome model in LPS primed and cultured THP-1 monocytes. This study reported the downregulation by 2.1 folds of CLCC1 when THP-1 monocytes were exposed to LPS treatment for 24 hours, indicating the regulation of CLCC1 in relation to NLRP3 inflammasome priming with LPS.

Initially, to acquire reliable and accurate results in the relative quantification of target genes in qPCR experiments, it is essential for data normalization purposes to determine stable reference genes. Four reference genes were tested for stability of expression in a reference gene panel by qPCR and results were joined with the rest of the tested genes in the panel in the same fashion for a robust analysis. The software NormFinder and GeNorm were used in the analysis. Both software generated similar results. However, GeNorm suggested GUSB & TBP as the best combination of stably expressed genes (Figure 2), whilst NormFinder suggested GUSB & ACTB as the best pair of reference genes (Figure 3). These slightly different findings are dependent on the method used to evaluate candidate reference genes, GeNorm calculates overall expression

variation based on an M-value, a lower M-value indicates higher stability, while NormFinder, in addition to calculating the overall expression variation, this software accounts inter and intragroup variation of samples into consideration (Piehler et al., 2010). For example, GeNorm ranked TBP as the most stable gene along with GUSB (Figure 2), while NormFinder ranked GUSB & ACTB as the most stable genes (Figure 3). The variation in the generated was mostly due to the different algorithms in testing stability of expression in the software (Hibbeler et al., 2008). In a similar qPCR experiment done by Piehler et al. (2010) in LPS stimulated monocytes isolated from human peripheral blood mononuclear cells (PBMC), ACTB was assigned as the most stable reference gene using NormFinder, but showed poor stability using GeNorm. This finding is consistent with the results of this study using NormFinder, noting that TBP showed low stability in NormFinder but was assigned as one of the best genes in GeNorm, these findings suggest that NormFinder is considered more consistent concerning studies with multiple experimental conditions. Another similar study was done by Maeß et al. (2010) in LPS stimulated THP-1 PMA differentiated monocytes showed that ACTB was ranked as one of the most stable genes, ruling out that it is appropriate to use in THP-1 monocytes qPCR experiments. The study by Maeß also found B2M as a poor reference gene that showed low stability in NormFinder. Kalagara et al. (2016) showed that GADPH was generally considered the least stable reference gene in LPS-stimulated mouse-derived macrophages, while UBC showed the highest stability. These findings agree with the generated results of this study, as GADPH was assigned as a highly variable gene. Moreover, based on a study by Sundaram et al. (2019) in validating reference genes showed that NormFinder is considered fairly better in analyzing overall variation among different groups in comparison GeNorm and the CV analysis method. Additionally, their study suggested that when highly variable genes were included in a set of analyzed reference genes in NormFinder, this affected the analysis of highly stable genes significantly and introduced biased results due to the algorithms construct.

In the case of relative quantification, the accuracy of results depends heavily on the selected reference genes that maintain stability and remain unchanged throughout the whole experimental conditions (Adams, 2020). Reference genes act as internal controls for the reaction and have a different sequence than that of the target gene, for a gene to be considered an ideal reference gene it should be (1) unaffected by experimental factors and maintains stable expression levels, (2) having minimum variability of expression levels between different physiological conditions, a selected reference gene also should preferably have a similar threshold cycle with the target gene (Chervoneva et al., 2010). However, an ideal reference gene or a group of reference genes should be determined for each experiment instead of using any of the common reference genes such as GADPH, B-actin, or B-2 microglobulin. Even though these genes are expected to be stably expressed, they may fail to do so in specific experimental conditions due to transcription variability (Tanaka et al., 2017). According to the (MIQE) guidelines of minimum information for publication of quantitative real-time PCR experiments, normalization with a single reference gene is not considered acceptable and is rarely justified, unless the expression is invariable under the desired conditions (Bustin et al., 2009).

Concerning IL-1B results, qPCR was performed on IL-1B as a positive control to verify that the NLRP3 priming process occurred in THP-1 macrophages by normalizing IL-1B as a target gene with the geometric mean of GUSB and ACTB using the delta delta Cq (2^-(ddcq)). Relative gene expression results showed a significant upregulation of IL-1B after LPS stimulation for 4, 8, and 24 hours respectively (Figure 4). It has been observed by Bauernfeind er al. (2019) that macrophages show no signs of NLRP3 activation when induced with activators solely, while the

pretreatment with microbial ligands such as LPS, causes the secretion of IL-1B strongly and enhanced NLRP3 expression by priming the inflammasome complex via the NF-kB pathway, hence the results from IL-1B expression in this study confirmed that the priming of THP-1 cells was conducted successfully.

Whilst results for the expression CLCC1 as a target gene were normalized with the geometric mean of GUSB and ACTB and fold change was calculated using the delta-delta Cq approach (2^-(ddcq)). It has been observed that CLCC1 was differentially expressed and downregulated by 2.1 folds after LPS stimulation for 24 hours (Figure 6). This was considered a novel finding in the research on NLRP3 inflammasome in THP-1 monocytes based on the fact that CLCC1 has never been studied in the context of this study. Results indicated that CLCC1 was negatively regulated upon prolonged stimulation of LPS (24 hours) based on the One-way ANOVA statistical analysis with post-hoc for multiple comparisons of groups (See supplementary file 2). Findings from other studies in mouse models indicated that acute knockdown of CLCC1 showed heightened sensitivity of cultured cells to chemically induced ER stressors suggesting that CLCC1 has an important role in maintaining ER homeostasis (Jia et al., 2015). The transcription factor CCAATenhancer-binding protein CHOP and secretion of IL-1B was shown to be overexpressed in the lungs of LPS treated mice by an unknown mechanism, CHOP is believed to be involved in ER stress and induces apoptosis (Nishitoh, 2011). A study done by Endo et al. (2005) showed that the treatment of mice with LPS caused ER stress and led to the UPR pathway activation and induction of CHOP in addition to the transcription of ERS-related genes (ATF4 and XBP1). Similarly, LPS treated/cultured macrophages induced the ERS-CHOP pathway in addition to caspase-11 which is considered important for the formation of the NLRP3 pro-caspase-1 for cleaving IL-1B into its mature form, concluding that ERS-CHOP is involved in the pathogenesis of LPS-induced inflammation in the lungs (Endo et al., 2006). In addition, the administration of LPS in the livers of obese mice in a study by Lebeaupin et al (2015) resulted in the activation of ER stress known pathways (IRE1a & PERK) in addition to the CHOP pathway, eventually leading to the NLRP3 activation and hepatocyte pyroptosis. However, It is also important to note that ERS involvement in the NLRP3 is not always involved in the classical UPR branches (PERK, IRE1a & ATF6) when THP-1 monocytes were LPS stimulated as explained by Menu et al., (2012). For example, Kim et al. (2013) have reported a direct link between ER stress and NLRP3 activation, by indicating that ER stress can solely without any priming stimuli such as LPS participate in the priming and activation of the NLRP3 inflammasome through the NF-KB signaling pathway, in which causes the cleavage of pro-IL-1B into its mature form via caspase-1. Additionally, the same study by Kim et al. has shown that ER stress can also activate the NLRP3 inflammasome by induction of the ROS NLRP3 ligand (TXNIP), concluding that oxidative stress is also involved in the process. These results together rule out the direct involvement of ERS in activating the NLRP3 inflammasome. The results obtained from this study proposed that CLCC1 may have an important role such as being a common event in the priming of NLRP3 that eventually might give a better understanding of ERS-induced inflammation. This is because CLCC1 was observed to be downregulated after prolonged LPS exposure while ERS and the NLRP3 inflammasome were shown to be directly related, although the exact underlying mechanisms of this complex relationship are still an area of research.

Nevertheless, one of the limitations of this study included that should be taken into consideration is that there was little to no research on the analyzed CLCC1 gene, especially in the context of inflammasomes and most importantly, the NLRP3 model. This limits the conclusions drawn since there is no sufficient information in the literature to make definitive answers. An important

limitation was that the study design of this research utilized THP-1 monocytes that were only primed with the immunostimulant LPS without the inclusion of activation factors such as ATP or potassium ions, that together form an active NLRP3 inflammasome. Hence, this limited the overview of CLCC1 in the context of a complete and activated NLRP3 inflammasome. Another limitation was that the significance of the CLCC1 role in maintaining ER homeostasis has not been assessed extensively an is considered putative. However, the strengths of this study included a robust and careful methodological approach in the analysis of candidate reference genes, which is considered essential for obtaining reliable results in every qPCR experiment.

Overall, the findings of this study suggested that treatment of THP-1 macrophages with LPS significantly reduced the expression of CLCC1 on a transcriptional level by 2.1-folds following LPS stimulation of THP-1 monocytes. CLCC1 has been shown to be crucial for ER homeostasis and it can be hypothesized that CLCC1 is being affected by the treatment of LPS and is involved in the priming process of NLRP3 and IL-1B secretion. Thus, further research is required to shed light on the novel involvement of CLCC1 in ERS-induced inflammation and more specifically, the NLRP3 involvement. Hence, in efforts to better understand ERS-induced inflammatory diseases, this novel finding may provide the potential novel therapeutic target in the dysregulation of the immune system that results in exacerbated inflammatory effects due to the ER involvement witnessed in various diseases such as inflammatory bowel disease, rheumatoid arthritis, and atherosclerosis.

Conclusion

The results obtained from analyzing candidate reference genes suggested GUSB and TBP in GeNorm analysis as the best pair of stable genes, while NormFinder suggested GUSB and ACTB as the two most stable genes of the twelve analyzed. Relative gene expression results of IL-1B confirmed the successful LPS priming of the macrophages. The target gene CLCC1 has been observed to be differentially expressed and downregulated after prolonged LPS treatment, thus concluding that CLCC1 is downregulated after LPS priming and may be involved in the priming mechanisms of the NLRP3 inflammasome. Recommended studies on this gene in the future can include the observation of CLCC1 knockdown in the context of ERS-induced inflammation and especially the NLRP3 inflammasome, also to approve the findings of this study it could be useful to analyze this gene on the protein level rather than just the transcriptional level. Future directions could also include analyzing the CLCC1 gene under LPS stimulation in addition to inflammasome activation factors or known ERS inducers such as tunicamycin.

Ethical Considerations

THP-1 cells are immortalized monocyte-like cell lines. Nevertheless, it is crucial to point out differences between THP 1 cells and primary monocytes. THP-1 cells are usually studied as a model representing human blood peripheral monocytes (primary monocytes). Unlike primary monocytes, THP-1 cells are cultured and used in vitro studies. Due to this fact, no ethical considerations were made regarding the cultured cells used in this study. Moreover, no gender-related information was included in this study, thus no ethical considerations were considered in this regard. In terms of environmental impact, a RISK assessment was made before conducting this study to assess the chemical hazards and their risks to the environment. Safety

measurements were taken into consideration and the SYB kit used in this study was carefully handled and disposed of according to guidelines, thus no environmental harm was associated with this study. The software KLARA was used to assess the threats included in the materials used in this study.

References

Adams, G., 2020. A beginner's guide to RT-PCR, qPCR, and RT-qPCR. The Biochemist, 42(3), pp.48-53.

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

Bosshart, H. and Heinzelmann, M., 2016. THP-1 cells as a model for human monocytes. Annals of Translational Medicine, 4(21), pp.438-438.

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

Chervoneva, I., Li, Y., Schulz, S., Croker, S., Wilson, C., Waldman, S. and Hyslop, T., 2010. Selection of optimal reference genes for normalization in quantitative RT-PCR. BMC Bioinformatics, 11(1).

Chu, Q., Martinez, T., Novak, S., Donaldson, C., Tan, D., & Vaughan, J. et al. (2020). Regulation of the ER stress response by a mitochondrial micro protein. The FASEB Journal, 34(S1), 1-1. https://doi.org/10.1096/fasebj.2020.34.s1.04094

Endo, M., Mori, M., Akira, S., & Gotoh, T. (2006). C/EBP Homologous Protein (CHOP) Is Crucial for the Induction of Caspase-11 and the Pathogenesis of Lipopolysaccharide-Induced Inflammatio. The Journal Of Immunology, 176(10), 6245-6253. https://doi.org/10.4049/jimmunol.176.10.6245

Endo, M., Mori, M., Akira, S., & Gotoh, T. (2006). C/EBP Homologous Protein (CHOP) Is Crucial for the Induction of Caspase-11 and the Pathogenesis of Lipopolysaccharide-Induced Inflammatio. The Journal Of Immunology, 176(10), 6245-6253. https://doi.org/10.4049/jimmunol.176.10.6245

Endo, M., Oyadomari, S., Suga, M., Mori, M., & Gotoh, T. (2005). The ER Stress Pathway Involving CHOP Is Activated in the Lungs of LPS-Treated Mice. The Journal Of Biochemistry, 138(4), 501-507. https://doi.org/10.1093/jb/mvi143

Gómez-Suaga, P., Bravo-San Pedro, J., González-Polo, R., Fuentes, J., & Niso-Santano, M. (2018). ER–mitochondria signaling in Parkinson's disease. Cell Death & Disease, 9(3). https://doi.org/10.1038/s41419-017-0079-3

Gong, T., Yang, Y., Jin, T., Jiang, W., & Zhou, R. (2018). Orchestration of NLRP3 Inflammasome Activation by Ion Fluxes. Trends In Immunology, 39(5), 393-406. https://doi.org/10.1016/j.it.2018.01.009

Gritsenko, A., Yu, S., Martin-Sanchez, F., Diaz-del-Olmo, I., Nichols, E., & Davis, D. et al. (2020). Priming Is Dispensable for NLRP3 Inflammasome Activation in Human Monocytes In Vitro. Frontiers In Immunology, 11. https://doi.org/10.3389/fimmu.2020.565924

He, Y., Hara, H., & Núñez, G. (2016). Mechanism and Regulation of NLRP3 Inflammasome Activation. Trends in Biochemical Sciences, 41(12), 1012-1021. doi: 10.1016/j.tibs.2016.09.002

Hibbeler, S., Scharsack, J., & Becker, S. (2008). Housekeeping genes for quantitative expression studies in the three-spined stickleback Gasterosteus aculeatus. BMC Molecular Biology, 9(1).

https://doi.org/10.1186/1471-2199-9-18

Hibbeler, S., Scharsack, J., & Becker, S. (2008). Housekeeping genes for quantitative expression studies in the three-spined stickleback Gasterosteus aculeatus. BMC Molecular Biology, 9(1). https://doi.org/10.1186/1471-2199-9-18

Hu, X., Li, D., Wang, J., Guo, J., Li, Y., & Cao, Y. et al. (2018). Melatonin inhibits endoplasmic reticulum stress-associated TXNIP/NLRP3 inflammasome activation in lipopolysaccharide-induced endometritis in mice. International Immunopharmacology, 64, 101-109. https://doi.org/10.1016/j.intimp.2018.08.028

Jia, Y., Jucius, T., Cook, S., & Ackerman, S. (2015). Loss of Clcc1 Results in ER Stress, Misfolded Protein Accumulation, and Neurodegeneration. Journal Of Neuroscience, 35(7), 3001-3009. https://doi.org/10.1523/jneurosci.3678-14.2015

Kalagara, R., Gao, W., Glenn, H., Ziegler, C., Belmont, L., & Meldrum, D. (2016). Identification of stable reference genes for lipopolysaccharide-stimulated macrophage gene expression studies. Biology Methods and Protocols, 1(1). doi: 10.1093/biomethods/bpw005

Karasawa, T., & Takahashi, M. (2017). Role of NLRP3 Inflammasomes in Atherosclerosis. Journal of Atherosclerosis and Thrombosis, 24(5), 443-451. doi: 10.5551/jat.rv17001

Kaufman, R. (2004). Regulation of mRNA translation by protein folding in the endoplasmic reticulum. Trends In Biochemical Sciences, 29(3), 152-158. doi: 10.1016/j.tibs.2004.01.004

Kim, S., Joe, Y., Jeong, S., Zheng, M., Back, S., & Park, S. et al. (2013). Endoplasmic reticulum stress is sufficient for the induction of IL-1β production via activation of the NF-κB and inflammasome pathways. Innate Immunity, 20(8), 799-815. https://doi.org/10.1177/1753425913508593

Kim, S., Joe, Y., Jeong, S., Zheng, M., Back, S., & Park, S. et al. (2013). Endoplasmic reticulum stress is sufficient for the induction of IL-1β production via activation of the NF-κB and inflammasome pathways. Innate Immunity, 20(8), 799-815. https://doi.org/10.1177/1753425913508593

Lebeaupin, C., Proics, E., de Bieville, C., Rousseau, D., Bonnafous, S., & Patouraux, S. et al. (2015). ER stress induces NLRP3 inflammasome activation and hepatocyte death. Cell Death & Disease, 6(9), e1879-e1879. https://doi.org/10.1038/cddis.2015.248

Lebeaupin, C., Proics, E., de Bieville, C., Rousseau, D., Bonnafous, S., & Patouraux, S. et al. (2015). ER stress induces NLRP3 inflammasome activation and hepatocyte death. Cell Death & Disease, 6(9), e1879-e1879. https://doi.org/10.1038/cddis.2015.248

Li, W., Cao, T., Luo, C., Cai, J., Zhou, X., Xiao, X., & Liu, S. (2020). Crosstalk between ER stress, NLRP3 inflammasome, and inflammation. Applied Microbiology And Biotechnology, 104(14), 6129-6140. https://doi.org/10.1007/s00253-020-10614-y

Maeß, M., Sendelbach, S., & Lorkowski, S. (2010). Selection of reliable reference genes during THP-1 monocyte differentiation into macrophages. BMC Molecular Biology, 11(1), 90. https://doi.org/10.1186/1471-2199-11-90

Maeß, M., Sendelbach, S., & Lorkowski, S. (2010). Selection of reliable reference genes during THP-1 monocyte differentiation into macrophages. BMC Molecular Biology, 11(1), 90. https://doi.org/10.1186/1471-2199-11-90

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

Matzinger, P. (1994). Tolerance, Danger, and the Extended Family. Annual Review Of Immunology, 12(1), 991-1045. doi: 10.1146/annurev.iy.12.040194.005015

Menu, P., Mayor, A., Zhou, R., Tardivel, A., Ichijo, H., Mori, K., & Tschopp, J. (2012). ER stress activates the NLRP3 inflammasome via an UPR-independent pathway. Cell Death & Disease, 3(1), e261-e261. https://doi.org/10.1038/cddis.2011.132

Menu, P., Mayor, A., Zhou, R., Tardivel, A., Ichijo, H., Mori, K., & Tschopp, J. (2012). ER stress activates the NLRP3 inflammasome via an UPR-independent pathway. Cell Death & Disease, 3(1), e261-e261. https://doi.org/10.1038/cddis.2011.132

Nagasawa, M., Kanzaki, M., Iino, Y., Morishita, Y., & Kojima, I. (2001). Identification of a Novel Chloride Channel Expressed in the Endoplasmic Reticulum, Golgi Apparatus, and Nucleus. Journal Of Biological Chemistry, 276(23), 20413-20418. https://doi.org/10.1074/jbc.m100366200

Nishitoh, H. (2011). CHOP is a multifunctional transcription factor in the ER stress response. Journal Of Biochemistry, 151(3), 217-219. https://doi.org/10.1093/jb/mvr143

Piehler, A., Grimholt, R., Ovstebo, R., & Berg, J. (2010). Gene expression results in lipopolysaccharide-stimulated monocytes depend significantly on the choice of reference genes. BMC Immunology, 11(1), 21. https://doi.org/10.1186/1471-2172-11-21

Sundaram, V., Sampathkumar, N., Massaad, C., & Grenier, J. (2019). Optimal use of statistical methods to validate reference gene stability in longitudinal studies. PLOS ONE, 14(7), e0219440. https://doi.org/10.1371/journal.pone.0219440

Sundaram, V., Sampathkumar, N., Massaad, C., & Grenier, J. (2019). Optimal use of statistical methods to validate reference gene stability in longitudinal studies. PLOS ONE, 14(7), e0219440. https://doi.org/10.1371/journal.pone.0219440

Sutterwala, F., Haasken, S., & Cassel, S. (2014). Mechanism of NLRP3 inflammasome activation. Annals of The New York Academy of Sciences, 1319(1), 82-95. doi: 10.1111/nyas.12458

Takahashi, M. (2019). Role of NLRP3 Inflammasome in Cardiac Inflammation and Remodeling after Myocardial Infarction. Biological and Pharmaceutical Bulletin, 42(4), 518-523. doi: 10.1248/bpb. b18-00369

Tanaka, A., To, J., O'Brien, B., Donnelly, S., & Lund, M. (2017). Selection of reliable reference genes for the normalization of gene expression levels following time course LPS stimulation of murine bone marrow derived macrophages. BMC Immunology, 18(1). doi: 10.1186/s12865-017-0223-y

Tauber, A. (1994). The immune self: theory or metaphor? Immunology Today, 15(3), 134-136. doi: 10.1016/0167-5699(94)90157-0

Yang, Y., Wang, H., Kouadir, M., Song, H., & Shi, F. (2019). Recent advances in the mechanisms of NLRP3 inflammasome activation and its inhibitors. Cell Death & Disease, 10(2). doi: 10.1038/s41419-019-1413-8

Zhang, J., Zhang, K., Li, Z., & Guo, B. (2016). ER Stress-induced Inflammasome Activation Contributes to Hepatic Inflammation and Steatosis. Journal Of Clinical & Cellular Immunology, 7(5). https://doi.org/10.4172/2155-9899.1000457

Zhang, Q., Lenardo, M., & Baltimore, D. (2017). 30 Years of NF-κB: A Blossoming of Relevance to Human Pathobiology. Cell, 168(1-2), 37-57. https://doi.org/10.1016/j.cell.2016.12.012

Appendix

Table 1 Appendix. All primer efficincies for potential reference genes and target genes.

Primer	Efficiency (%)
ACTB	105.6
GADPH	98.8
GUSB	100.2
PPIA	100.6
RPLP	93.1
TBP	102.7
UBC	108.7
B2M	104.2
RRN18s	106.0
TUBB	95
YWHAZ	119
HRP	102.4
IL-1B	99.15
CLCC1(1)	119
CLCC1(2)	116
CLCC1(3)	106
CLCC1(4)	

Table 1 Appendix. Primer efficiencies are present on the right side of the table in percentages, values in red indicate a percentage outside the acceptable range (90-110%).

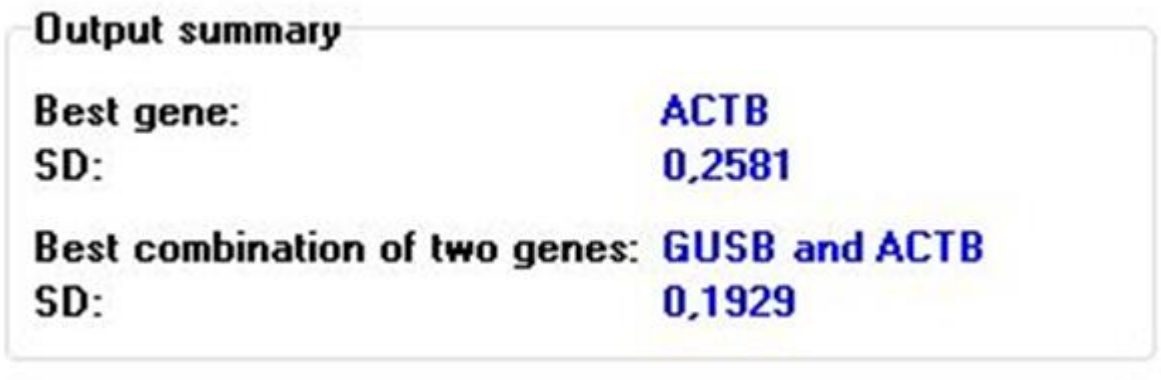

Figure 1 Appendix. Best combination of genes (NormFinder) when applying inter and intra groups into consideration in GenEx.

Table 2. M-values for the selected potential reference genes in GeNorm analysis.

Gene Name	M-Value
PPIA	1.299623413
TUBB	1.07286861
RPLP	0.936895921
B2M	0.873558404
UBC	0.80479767
YWHAZ	0.756925703
RRN18s	0.727416116
ACTB	0.690736491
GAPDH	0.6661741
TBP	0.651971433
GUSB	0.651971433

Table 2. Fold changes calculation using the delta-delta Cq method for IL-1B.

Sample	Cq1	Cq2	Cq3	Average ΔCq	ΔCq	ΔΔCq	2^-(ΔΔCq)	Average Fold Change

NO LPS	21.9	21.62	21.75	21.75	2.473	-0.85036	1.8030	1
NO LPS	23.24	23.26	23.28	23.26	3.195	-0.30156	1.2325	
NO LPS	23.74	23.81	23.91	23.82	4.475	1.15192	0.4500	
4H LPS	18.36	18.44	18.51	18.43	-1.175	-4.49881	22.6087	19
4H LPS	18.47	18.44	18.61	18.50	-0.775	-4.09905	17.1370	
4H LPS	18.96	19.26	19.21	19.14	-0.903	-4.22728	18.7300	
8H LPS	18.39	18.73	18.74	18.62	-1.258	-4.58223	23.9546	23
8H LPS	19.11	18.69	18.9	18.9	-1.345	-4.66879	25.4358	
8H LPS	19.03	18.91	18.87	18.93	-0.984	-4.30792	19.8068	
24 LPS	20.32	20.26	19.78	20.12	-0.281	-3.60521	12.1696	17
24 LPS	19.67	19.68	19.59	19.64	-1.124	-4.44832	21.8312	
24 LPS	19.44	19.51	19.69	19.54	-0.814	-4.13819	17.6084	

Table 4. Fold change calculation for CLCC1(4) using the delta-delta Cq method.

Sample	**Cq1**	**Cq2**	**Cq3**	**Average ΔCq**	**ΔCq**	**ΔΔCq**	**2^-(ΔΔCq)**	**Average Fold Change**
NO LPS	29.93	29.63	29.89	29.817	10.533	0.44520	0.7345	1
NO LPS	29.85	29.45	30.25	29.850	9.612	-0.4760	1.3909	
NO LPS	29.29	29.44	29.66	29.463	10.119	0.03081	0.9789	
4H LPS	30.04	29.97	30.11	30.040	10.428	0.34008	0.7900	0.67
4H LPS	30.51	30.22	29.88	30.203	10.921	0.83318	0.5613	
4H LPS	30.70	30.82	30.59	30.705	10.658	0.56994	0.6736	
8H LPS	30.54	29.83	30.34	30.237	10.358	0.26999	0.8293	0.81
8H LPS	30.78	30.41	30.54	30.577	10.331	0.24344	0.8447	
8H LPS	30.57	30.17	30.41	30.383	10.462	0.37430	0.7715	
24 LPS	31.96	32.48	31.93	32.123	11.722	1.63368	0.3223	0.45
24 LPS	31	32.15	31.06	31.403	10.632	0.54390	0.6859	

24 LPS	32	31.82	31.85	31.90	11.529	1.44070	0.3684

Supplementary file 1.

Supplementary file 1.xlsx

Supplementary file 2.

Supplementary file 2.xlsx