Degree project

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Upregulation of CLIC1 gene in THP1 cells stimulated by lipopolysaccharides

Bachelor Degree Project in Bioscience

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Abstract

The NLRP3 inflammasome is a multiprotein complex that controls caspase-1 activation and the development of the pro-inflammatory cytokines IL-1 and IL-18, as well as pyroptosis. The NLRP3 inflammasome in murine macrophages is normally triggered by pathogen-associated molecular patterns as well as a variety of structurally unrelated stimuli. The exact mechanism of NLRP3 activation by such a diverse set of stimuli is unknown, although many signaling processes have been proposed, including cytosolic efflux and the inflow of certain ions. As a result, numerous studies have suggested that anion channels play a role in NLRP3 inflammasome formation, although no direct evidence of their participation has been found. This thesis project aims to measure the expressions level of CLIC1 before and after LPS treatment. The measurement was done with the help of reference genes. Using qPCR, potential reference genes were tested for their stability and evaluated by GenEx. The study identified GUSB and TBP as the most stable genes. Using the delta delta cq method, data from qPCR were normalized by reference genes. The results from this analysis showed an upregulation in the expression levels of CLIC1. These results showed that CLIC1 an anion channel plays a role in the activation and formation of the NLRP3 inflammasome and other inflammatory disorders such as oxidative stress. The study identified GUSB and TBP as reference genes in LPS stimulated THP-1 macrophages and an upregulation in the expression levels of CLIC1, leading to speculation that LPS stimulation leads to the translocation of CLIC1 to the plasma membrane and suggests the possibility to target CLICs to treat NLRP3-driven diseases.

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Abbreviation

Abbreviation	Description
AIM	Absent-in-melanoma
ALR	Absent-in-melanoma (AIM)-like receptors(ALR),
ASC	Associated speck-like protein containing a CARD
CLIC	Chloride intracellular channel
DAMPS	Damage-associated molecular patterns
GST	Glutathione-S-transferase
IL-1β	Interleukin-1β
IPC	Interplate calibrator
iPS	Induced pluripotent stem
LPS	Lipopolysaccharide
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NF-κB	Nuclear factor B
NLR	Nucleotide-binding and oligomerization domain (NOD)-like receptors
NOD	Nucleotide-binding and oligomerization domain
PBMC	Peripheral blood mononuclear cell
PAMPS	Pathogen-associated molecular patterns
PRR	Pathogen recognition receptors
ROS	Reactive oxygen species

Introduction

The inflammasome, discovered by Fabio Martinon in 2001 (Martinon et al., 2002), is a multiprotein complex that acts as a platform for caspase-1 activation, caspase-1-dependent proteolytic maturation, and interleukin-1 release (Gross et al., 2011). Interleukin-1β (IL-1β), a pro-inflammatory cytokine, is an endogenous pyrogen produced by activated macrophages and monocytes and is the cause of chronic and acute inflammation. IL-1β functions in the production of systemic and local responses to infection, injury, and immunological challenges. It also plays a role in febrile reactions (Dinarello et al., 1999). The inflammasome complex is also part of the innate immune system that works by sensing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). PAMPS are signals derived from extracellular pathogens, whereas DAMPS are signals derived from damaged host cells and extracellular matrix; these signals are derived via pathogen recognition receptors (PRR) (Man & Kanneganti, 2015). Depending on the signals sensed, different PRRs will oligomerize and interact with a specific caspase and an adaptor protein, causing caspase activation. The activation of caspase leads to the maturation of pro-inflammatory cytokines (IL-1β and IL-18) for PAMPs or pyroptosis for DAMPs (Amin et al., 2017). The inflammasome complex can be broken into sub-molecular components, with each element having different response patterns and functions. These components include; one sensor molecule, which may include one of these; NLRP1, NLRP2, NLRP3, NLRC4, AIM2, pyrin, or the adaptor ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain or CARD). The adaptor ASC is associated with all inflammasomes, and its role is to recruit pro-caspase-1 into the inflammasome. The adaptor ASC is pivotal for the activation and perpetuation of inflammation.

PRR is a sensor receptor, which plays a role in the activation or the assembling of the inflammasome complex (Man & Kanneganti, 2015). PRR can be classified into two groups based on their localization. The transmembrane pattern recognition receptors, which include Toll-like receptors and C-type lectin. The second is cytoplasmic pattern recognition receptors, including NLR and other receptors like the ALR (Amin et al., 2017). NLR is a sensory receptor that is involved in the formation of an inflammasome. It consists of an effector domain called the N-terminal mainly for interacting with proteins, a central NACHT domain which mediates self-oligomerization, and a C-terminal domain that controls activator sensing and ligand binding. So far, around 22 NLRs have been found in the human body, with several, such as NLRP1 and NLRP3, linked to the creation of inflammasomes (Di Virgilio, 2013). Pyrin is a pattern recognition receptor, which functions as a regulator in inflammasome signaling through phosphorylation. Phosphorylation of serine keeps the pyrin complex inactive (Stutz et al., 2017). The inflammasome can be divided into two separate groups based on the caspase involved. These groups are; the canonical-inflammasome which directly triggers caspase 1, and the non-canonical inflammasome, which initiates inflammasome using other caspases (Amin et al., 2017). In the canonical inflammasome pathway, when pattern recognition receptors such as NRL, pyrin, and ASC detect DAMPs and PAMPs, they employ ASC into a homotypical pyrin-pyrin domain interaction, results in pro-caspase 1 binding with ASC, thus activating the inflammasome causing the secretion and pyroptosis of IL1β/IL18. In NLRP3, inflammasome direction activation may occur due to Caspase 8 directly binding to ASC (Amin et al., 2017).

In the non-canonical inflammasome pathway, activation of inflammasome may result in two ways. Promotion of caspase 8 by microbes may result in the maturation of IL1β. The second method is of activation is set in motion by lipopolysaccharide(LPS) from Gram-negative bacteria or priming activates caspase 4 and 5, resulting in cleavage of gasdermin D mediating pyroptosis activating NLRP3 inflammasome (Amin et al.,2017) as seen in Figure 1. Regulation and expression of the NLRP3 inflammasome, an interleukin-1 family cytokine-activating protein complex, has

been related to a host of metabolic disorders, including obesity, atherosclerosis, and type 2 diabetes, and other diseases (De Nardo & Latz, 2011), with macrophages the primary cells expressing the inflammasome gene. Macrophages are present in all mammalian tissues and play an essential role in immune system activation, tissue repair, and the ability to perform phagocytosis (Kalagara et al., 2016).

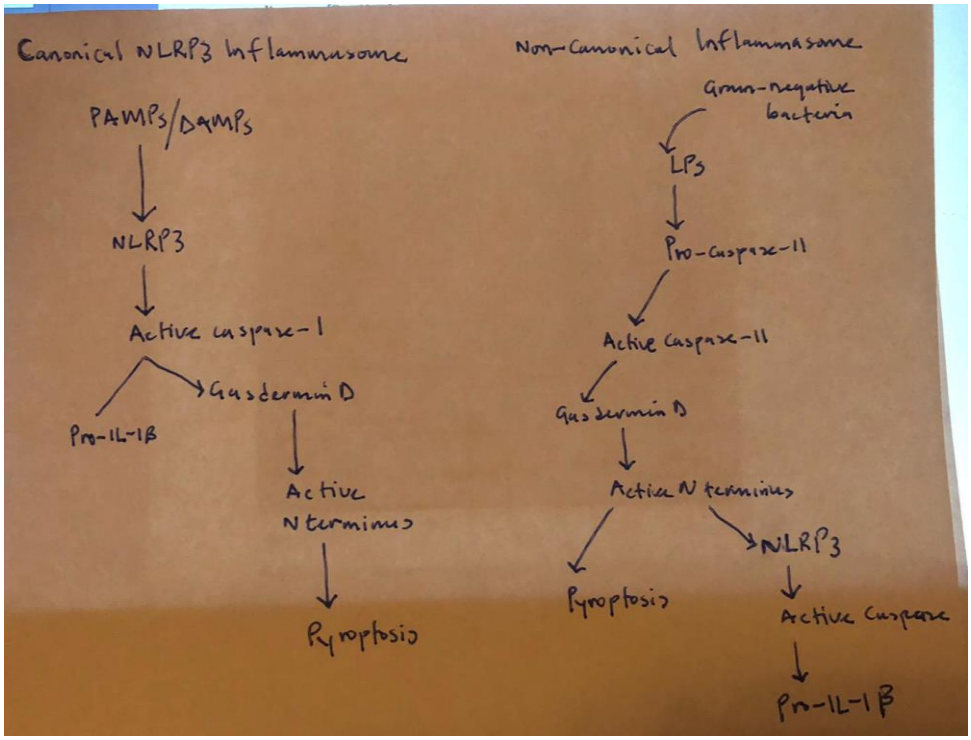

Figure 1. The Non-Canonical and Canonical NLRP3 Inflammasome Pathways. Activation Mechanisms LPS internalization into the cytosol via transfection or infection causes non-canonical NLRP3 inflammasome activation (right), which results in the cleavage of Gasdermin -D by caspase-11/4/5 and causes pyroptosis. In the canonical pathway(left), human monocytes are activated in response to LPS and employ ASC into a homotypical pyrin-pyrin domain interaction, results in pro-caspase 1 binding with ASC, thus activating the inflammasome causing the secretion and pyroptosis of IL1β.

NLRP3 may be triggered by various agonists with different properties or structures from various sources, including viruses, environmental stress, metabolic dysregulation, and tissue harm (Tang et al., 2017). While mitochondrial dysfunction, reactive oxygen species (ROS) formation, lysosome destruction, or disruption of intracellular ion homeostasis have all been proposed as mechanisms for NLRP3 activation by the above agonists, it is still unclear how these signaling events orchestrate NLRP3 inflammasome activation (Tang et al., 2017). Since some NLRP3 activators decrease intracellular potassium concentration, increasing extracellular potassium concentration prevents inflammasome activation by all tested NLRP3 activators, potassium efflux has been suggested to play an essential role in NLRP3 inflammasome activation (Pétrilli et al., 2007). Other studies have shown that several non-specific chloride channel inhibitors block NLRP3 inflammasome activation, and incubation of macrophages in the chloride-free medium increases ATP-induced caspase-1 activation and IL-1 activity, indicating that chloride current can play a role in NLRP3 inflammasome activation. However, it is unknown how chloride current controls NLRP3 inflammasome activation (Compan et al., 2012).

The chloride intracellular channel (CLIC) protein family comprises six evolutionarily conserved proteins (CLIC1–CLIC6) linked to membrane remodeling, intracellular trafficking, vacuole development, and actin reorganization (Domingo-Fernández et al., 2017). In reaction to increased cytoplasmic oxidation or pH changes, cytoplasmic soluble CLIC proteins insert into cellular membranes, acting as anion channels that reduce cationic fluctuations (Domingo-Fernández et

al., 2017). CLIC1 has been found to cross the phagosomal membrane and interact with NADH oxidase to control phagosomal acidification. In a study conducted by Raquel Domingo-Fernández et al. (2017), CLIC1 was shown to be involved in the priming phase of LPS-induced IL-1b and modulating the NLRP3 signal 2. In their study, knocking down CLIC1 and CLIC4 resulted in a significant reduction in ASC specks' development, which led to the activation of NLRP3 (Domingo-Fernández et al., 2017). Their results also showed that CLIC1 and CLIC4 are NLRP3 and IL-1b regulators, making them potential therapeutic candidates for reducing inflammation (Domingo-Fernández et al., 2017).

Based on the above studies and bearing the fact that anion channels function in the activation of inflammasome lead to the research question as to what is the role of CLIC1 in the activation of NLRP3 inflammasome in LPS stimulated macrophages. This thesis project aims to measure the expression level of CLIC1 before and after stimulation with LPS. The primary objective of this study was measuring the regulation of CLIC1 in THP-1 monocytes by analyzing the expression levels of CLIC1 using qPCR at different time points before and after LPS stimulation. However, in other to this, reference gene or genes are needed to normalize the target gene. Research has been undertaken to identify potential reference genes in various systems, such as rat oligodendrocytes and bovine muscular tissues (Kalagara et al., 2016). Reference genes have also been studied for mouse monocyte under conditions of laminin stimulation (Kalagara et al., 2016). However, there are limited studies based on the stability of potential reference genes in LPS-treated macrophages as of writing.

Methods

THP 1 cells were cultured in RPMI 1640 with L-glutamine of concentration of 0.3g/L, 10% heat-inactivated FBS, 10 mM HEPES, 1mM sodium pyruvate, 0.45% glucose and 100 U/ml penicillin-streptomycin at 37°C and 5% CO_2. Zeocin (200µg/ml) (Invivogen) was added to the culture medium. Cells were differentiated by the addition of 100nM PMA for 72 hours, washing twice with culture media, and incubating at 37°C and 5% CO2 for 48 hours in fresh media. Priming is conducted by adding 500ng/ml LPS. Following the RNeasy Mini Kit from Qiagen, total RNA was isolated from unprimed and primed THP-1 cells at different time points (0 hours, 4 hours, 8 hours, and 24 hours) after the addition of LPS. In total, 12 samples, four time-points with three biological replicates, of THP-1 isolated total RNA were used. The concentration and purity of the RNA were measured using the DS11 spectrophotometer (Denovix).

Reverse Transcription

cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Reverse transcription reactions without RNase were carried out using 500 ng of total RNA in a final volume of 20 µl of the reaction mix without RNase. The thermal cycling conditions were as follows: 10 min at 25 °C, 120 min at 37 °C, 5 min at 85 °C, and indefinitely at 4 °C. A no RT reaction was also performed to act as a control for genomic contamination.

qPCR

qPCRs were carried out on the Agilent AriaMx System using the Syber Select Master Mix (Applied Biosystem). CLIC1 primers were designed using NCBI/Primer-BLAST and qprimer software and purchased from Thermo Fisher Scientific. The sequences for these primers can be seen in Table 1. Primers for the potential reference genes were obtained from the TATAA Biocenter (Tataa Biocenter). The panel contains twelve potential reference genes that were divided evenly between 2 other colleagues, where each student received four. Those analyzed in this project included UBC, GAPDH, YWHAZ, and RPLP. To identify the stabled expressed gene, a comparison was made between all twelve genes tested by the students, with IPC used to cancels out any difference. Three out of the twelve potential reference genes were discarded as they amplified genomic DNA and were not linear. The primer pairs for both the potential reference genes and target gene were tested for efficiency and linearity by qPCR. The linearity and efficiency test was done by performing a 10-fold serial dilution with DNA amounts ranging from 50 ng, 5 ng, 0.5 ng, 0.05 ng, and 0.005 ng. The dilutants were then run in triplicates on the Agilent AriaMx System using the Syber Select Master Mix (Applied Biosystems). The cq values obtained from the qPCR were plotted against the log of dilution value to show linearity. The sequences of the CLIC1 primers tested for linearity can be seen in Table 1 below. The sequences of the primer selected and used to analyze gene expression was CLIC1 forward (5'- CCCATTCTCCCAGAGACTGTTC -3') and reverse (5'- TGTCTCGGTCCGCCTTTTGG -3'). qPCR was performed using the technical replicates on the Agilent AriaMx with 2 ng of input cDNA and (200nM) primers. The qPCR final reaction volume was adjusted to 10µl. The reactions followed the default PCR thermal-cycling profile that is; 50°C for 2 min followed 95°C for 2 min, followed by 40 cycles at 95°C for 15 secs and 60°C for 1 min. Any difference between the plates was canceled out by using an interplate calibrator (IPC). Normalization with the IPC was done in Excel, and the difference was subtracted from the plates.

Table 1. Designed CLIC1 primer pairs. Primer pair 2 was selected and used expression evaluation.

Primer pair	Forward primer(5'->3')	Reverse primer(5'->3')
1	TTGACACCAAAAGGCGGACC	GTTGGTGTCTGTGTGCACTTC
2	CCCATTCTCCCAGAGACTGTTC	TGTCTCGGTCCGCCTTTTGG
3	GTTGACACCAAAAGGCGGA	CACTGCCTCCAGAAATTCCTCA
4	CAGCACTCAATGACAATCTGGAG	GCTCGTTGCCATCCAAAAAC
5	ACACATAGTACAGGTGGTGTG	CTGGACAGGTGGAAGCGAAT

Statistical Analysis

GenEx version 6.0 was used in analyzing the expression stability of candidate reference genes. The algorithms GeNorm and NormFinder were used on efficiency corrected Cq values to determine the most stable reference gene. The delta-delta cq method was used to calculate the expression level of CLIC1 before and after stimulation. SPSS was used to determine any significant difference between the groups using one-way ANOVA with Tukey's post-hoc test for multiple comparisons. a $P < 0.05$ significance level.

Results

The data from the potential reference gene study derived from the qPCR runs were examined to see whether any of the genes could be used for normalization. The algorithms NormFinder and GeNorm (using raw data/Cq-values) in GenEx were used to identify one or several potential reference genes. The expression values of these reference genes were used to normalize the data of the gene of interest. Normalization calculation can be seen in Table 2 of the appendix. GenEx was used to identify the most stable reference gene using the algorithms NormFinder and GeNorm.

NormFinder Results

The overall average value for stability was 0.66. GUSB was the gene with a stable expression, as seen in Figure 2, with a standard deviation value of 0.43. The next best was ACTB, with a standard deviation of 0.47. RPLP and B2M were the least stable genes with stability values of 1.01 and 0.95, respectively.

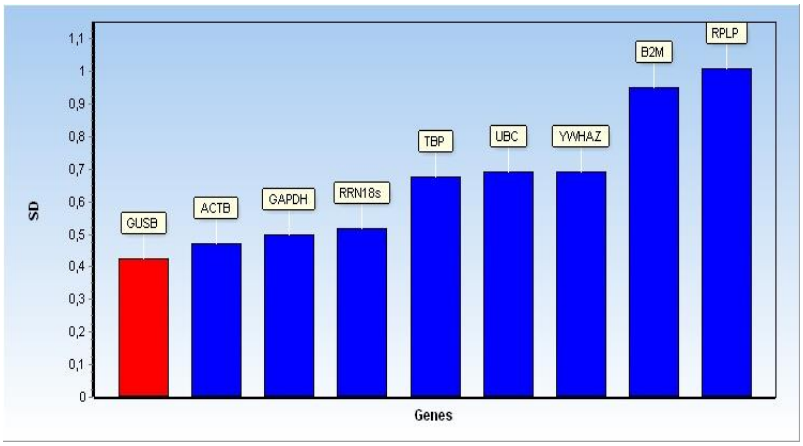

Figure 2. A bar chart showing the results based on NormFinder analysis of reference genes. Standard deviation values were determined between control cell data sets and four LPS treatment sets. Genes are

arranged in descending order of stability from left to right, with GUSB being more stable and RPLP least stable.

GeNorm Results

GUSB and TBP were the combined most stable genes, as seen in Figure 3, with M-values of 0.65. The third-best was GAPDH, with an M-value of 0.66. TUBB and PPIA were the least stable expressed genes with M- values of 1.01 and 1.3, respectively.

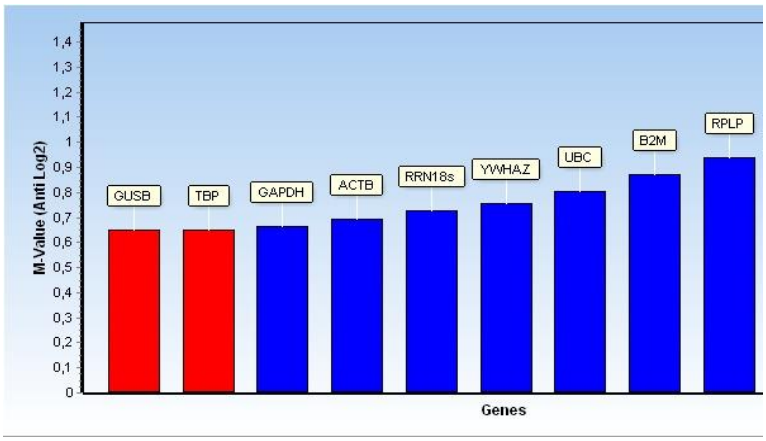

Figure 3. A bar chart showing the results from GeNorm analysis of potential reference genes by treatment. Stability values were determined between control cell data sets and four LPS treatment protocols. Genes are arranged in descending order of stability from left to right, with GUSB and TBP having stable expressions and the expression of PPIA being unstable.

Expression of CLIC1

The linearity and specificity of the five primer pairs for CLIC1 were tested, and a linearity curve was generated, as seen in Figure 4. All CLIC1 primer pairs were shown to be linear and had efficiency numbers ranging from 93.43% for primer pair 1 to 100.42 % for primer pair 5. The desired primer range was from 90-110%. The expression of CLIC1 was normalized using a combination of GUSB and TBP as reference. The cq values of CLIC1 were normalized with those of the reference genes GUSB and TBP, and the expression level of CLIC1 was measured. The results showed an upregulation in the expression levels of CLIC1, as seen in Figure 5. The results showed a 3-fold increase after 8 and 24 hours but showed no significant difference after 4 hours of LPS stimulation, as seen in Figure 5. The normalization calculations were done using the delta-delta method and can be seen in Table 2 of the appendix. Using one-way ANOVA on SPSS, a multiple comparisons test was performed with a significance level of 0.05. The results from Tukey's post hoc test showed a difference between 0h LPS and 8h LPS with a p-value of 0.019 and between 0H LPS and 24h LPS with a p-value of 0.008. The results also showed a significant difference between the 4H LPS and the 24H LPS with a p-value of 0.039.

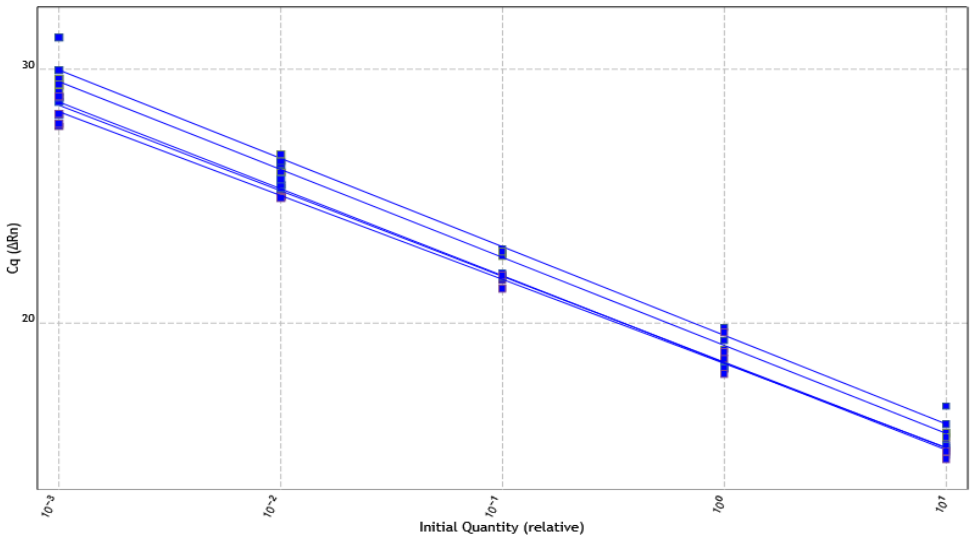

Figure 4. shows results for the linearity analysis for CLIC1. The efficiency values for the primers ranged from 93.43% to 100.42% of the primers for CLIC1.

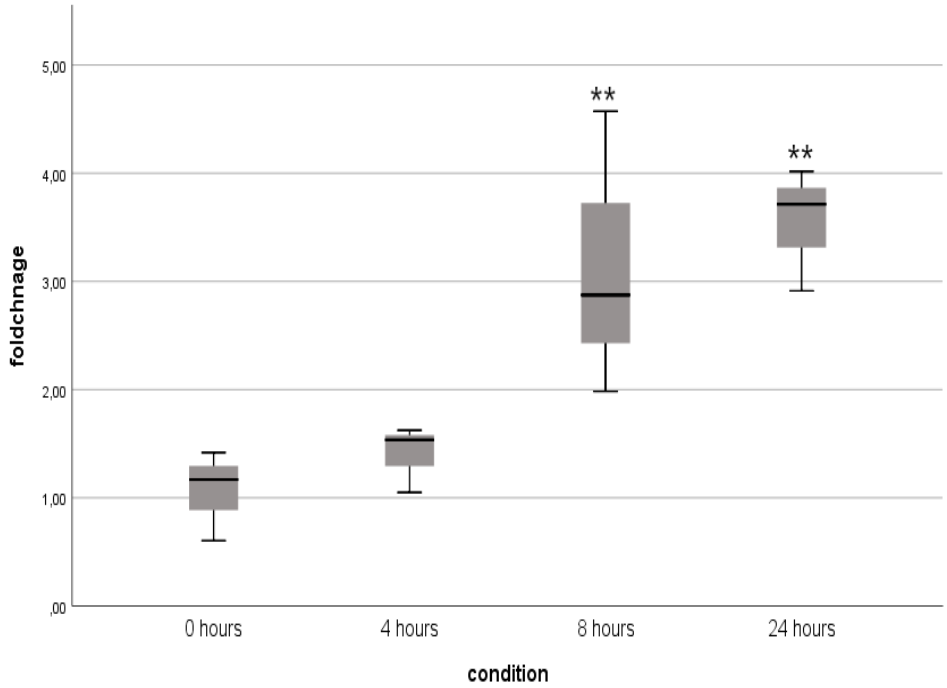

Figure 5. Box plot showing CLIC1 expression levels stimulated with LPS at various time points. The upper line of the box represents the 75th percentile and the lower line the 25th percentile of the log2 FC values. The bold horizontal line inside the box represents the median. The whiskers represent the maximum and minimum log2 FC values. One-way ANOVA with Tukey's post-hoc test was used to evaluate statistical significance. P-value significance is indicated by asterisks: $P \leq 0.05$ (*), $P \leq 0.01$ (**), and $P \leq 0.001$ (***).

Discussion

Macrophages have been widely researched because of their relevance in a wide range of standard and pathological processes (Kalagara et al., 2016). However, the evaluation of reference genes used for qPCR data normalization in macrophage research has received less attention (Kalagara et al., 2016). Some studies have analyzed and identified reference genes for THP-1 macrophages, but however extensive research showed no published papers have specifically analyzed reference genes for THP-1 macrophages when stimulated by LPS-stimulated. Previous research has demonstrated that common reference genes such as GAPDH and ACTB have generally been shown to contain many pseudogenes that could distort the observed expression levels (Kalagara et al., 2016). To ensure stability in the cell type of interest and under the specific experimental circumstances to be investigated, reference genes should be examined individually for each experimental system. Even if equivalent cell models are utilized, a comparison with previously published research involving monocytes and macrophages emphasizes the necessity of individual validation of reference genes. Armin P. Piehler et al., (2010), for example, verified reference genes in primary human monocytes isolated from peripheral blood after being activated with LPS (Piehler et al., 2010). Piehler and colleagues discovered PPIB and B2M to be the most stably expressed genes in primary human monocytes, which was in contrast to our findings where GUSB and TBP are the most stably expressed gene in our THP-1 monocyte maturation model, but RPLP and B2M are unsuitable reference genes for LPS-stimulated THP-1 macrophages. This was in contrast with a study by Marten Maeß et al., (2010), which, when using THP-1 macrophages induced by phorbol 12-myristate 13-acetate (PMA), found ACTB to be the most stably expressed gene. However, similar results were obtained by Michele Zampieri et al., (2010), like those seen in this thesis project when analyzing for potential reference genes using peripheral blood mononuclear cell (PBMC), with GUSB being the most stably expressed gene. The results were obtained using the algorithms GeNorm and NormFinder.

NormFinder is an algorithm in GenEx which computes raw stability values for genes depending on the stability of various genes. This algorithm employs a model-based method in which all expression levels are compared using analysis of variance, and all genes and specimen findings are used to calculate predicted expression values. The genes with the least deviation from the estimated values are identified using a stability metric. When selecting the reference gene, the gene with a lower stability value is deemed more stable and selected as the reference gene. NormFinder adds this value by combining the estimation of target reference gene category expression variations. The data entered was linearly corrected with the efficiency values of the primers, and the equation Q = E Ct (min)- Ct (sample) was utilized for this calculation. Ct (min) refers to an assay's lowest Ct value, whereas Ct (sample) relates to the sample in the issue.

NormFinder identified GUSB as the reference gene in LPS-stimulated macrophages, as shown in Figure 2. This was in line with the results from GeNorm, which found GUSB and also TBP as the best two reference genes, as shown in Figure 3. TBP was also found to be stably expressed in a study by Kensuke Okamura et al., (2020), in human induced pluripotent stem (iPS) cells, which was somewhat similar to the results obtained from this project. However, in Kensuke Okamura et al., (2020), study, GUSB was found to be unstable and not a suitable reference gene.

GeNorm ranks the reference gene based on their M-value. The M-value, a measure of relative expression stability and is used to rank candidate reference genes. Unstable reference genes with high M-values are on the right, while the best reference genes are on the left (Helle mans & Vandesompele, 2014). The average M-value for the best pair of reference genes indicates how well they work together to standardize the data. When analyzing reference targets with genomic DNA as input, or when reference targets are highly stably expressed, average M-values < 0.2 are typical. When evaluating potential reference targets on a homogenous collection of samples, M-

values between 0.2 and 0.5 are expected (Hellemans & Vandesompele, 2014). When assessing potential reference targets on a diverse group of samples, M-values in the 0.5–1 range are predicted. This was the expected range in this study.

CLIC1 expression was normalized using the geometric mean of GUSB and TBP. The results showed significant upregulation at 8 hours and 24 hours following stimulation with LPS, as shown in Figure 5. This was somewhat expected as CLIC1 is commonly overexpressed in oxidative stress-related diseases (Xu et al., 2016). Some studies have shown that chloride efflux mediated by CLICs activates the NLRP3 inflammasome by acting downstream of potassium efflux and mitochondrial damage, indicating that a chloride efflux is an upstream event proximal to NLRP3 inflammasome assembly (Tang et al., 2017). Results from Tang et al., (2017), showed that chloride efflux mediated by CLICs could control NLRP3 inflammasome assembly. CLICs activity and expression inhibition inhibited NLPR3–NEK7 interaction, NLRP3–ASC complex formation, and ASC oligomerization, indicating that CLICs and intracellular chloride regulate NEK7–NLRP3 contact to alter NLRP3 inflammasome activation (Tang et al., 2017).

The result in this study showed that CLIC1 was affected by LPS stimulation in the THP-1 macrophages, as a change could be seen in the expression level across the various time points. Previous studies have shown that on a protein level, in response to LPS stimulation, CLIC1 is translocated into the nucleus and plasma membrane (Li et al., 2020). As a result of this translocation, nuclear factor B (NF-κB) and interferon regulatory factor-3 are activated to control the expression of pro-IL-1. The translocation also results in siRNAs of CLIC1 inhibiting the NLRP3 inflammasome activation and IL-1 release (Li et al., 2020). Other studies have shown that CLICs function downstream of ROS, enhancing CLIC membrane translocation and chloride efflux. As a result, the signaling cascade preceding the activation of the NLRP3 inflammasome seems to be potassium efflux-mitochondrial damage-CLICs-chloride efflux (Tang et al., 2017). CLICs can inhibit the interaction of NEK7 and NLRP3, influencing ASC (apoptosis-associated speck-like protein containing a CARD) oligomerization. This corresponds with the results obtained in this experiment, as there was a 3-fold increase in the expression of CLIC1 in our investigation, as seen in Figure 5, which confirms that the CLIC1 gene is upregulated in LPS-primed macrophages. However, these results did not correspond to those obtained by Domingo-Fernández et al. (2017), which showed no significant change in the expression level of CLIC1 in LPS stimulated bone marrow-derived macrophages. Further research found that ASC oligomerization (formation of a speck) is chloride channel-dependent but inactive in the absence of K+ efflux since NEK7 can only connect with NLRP3 to activate the inflammasome in the presence of a K+ efflux (Li et al., 2020). Raquel Domingo-Fernández et al. (2017) demonstrated that preconditioning LPS-stimulated macrophages with high extracellular NaCl reduces inflammation by decreasing IL-1 post-transcriptional activation. Based on these findings, an assumption can be made that LPS stimulation would have similar effects to CLIC1, causing it to translocate and inhibit the activation of NLRP3 and the release of IL-1.

In a previous study, the shortened variant of Aβ25–35 enhanced CLIC1 chloride conductance in cortical microglia and produced microglial neurotoxicity; the CLIC1 ion channel blocker IAA-94 protected cell damage and decreased free radical production (Xu et al., 2016). Furthermore, when RAW 264.7 macrophages were exposed to heat-inactivated *Candida albicans* cells, their proteomics revealed lower CLIC1 expression, which lowered TNF-α and p-ERK levels via an anti-inflammatory response. These findings show that CLIC1 regulates macrophage function via its ion channel activity, and hence CLIC1 is a promising target for drug development (Xu et al., 2016). CLIC1 has the potential to be both a diagnostic and therapeutic target for a variety of malignancies (Gururaja Rao et al., 2020). In a broader scheme, the results obtained from this study, that is, CLIC1 being overexpressed and affected by LPS, could help in other CLIC1 related studies and

NLRP3 related diseases such as endoplasmic reticulum stress, as it could act as a base for further research.

Despite the completion of the project, it wasn't without its limitations. One of these being the inability to analyze the target gene on a protein level. That's is performing analysis such as Western Blotting to measure the expression of CLIC1 on a protein level, as to better see if CLCI1 is also upregulated on the protein or the is simply no change. Analysis on the protein level would provide the pathways, mechanism, and various effects of CLIC1 in THP-1 macrophages. Another limitation is that the THP-1 macrophages used were primed with LPS without the presence of activation components such as ATP or potassium ions, which together create an active NLRP3 inflammasome. The study's benefits, on the other hand, included a strong and rigorous scientific approach in the assessment of possible reference genes, which is considered important for reliable results in every qPCR experiment.

Conclusion

The results from the reference gene analysis identified GUSB and TBP as the best combination with the GeNorm algorithm and GUSB with NormFinder as the optimal reference from the TATAA panel of reference genes for this experiment. The results also reveal that upon stimulation by LPS, CLIC1 is being upregulated in THP-1 macrophages, leading to speculation that LPS stimulation leads to the translocation of CLIC1 to the plasma membrane and suggests the possibility to target CLICs to treat NLRP3-driven diseases. It would be interesting to evaluate the CLIC4 gene in the future, as it has also been linked to the activation of the NLRP3 inflammasome.

Ethical Aspect

In this study, the experiment used cell lines, which in this case were THP1 macrophages. Cell lines are animal cell cultures that can be repeatedly and indefinitely propagated. THP-1 is a naturally immortalized monocyte-like cell line originating from childhood peripheral blood acute monocyte leukemia. The THP-1 cells, including their genetically modified derivatives, are proper instruments for studying monocyte structure and function in health and disease. Due to these reasons, no ethical consideration was made, as the project made use of precultured cells. The study also had no gender-based data or analysis made, and thus no consideration was made. The results from the experiment have a social impact as it benefits the general public, as a better understanding of CLIC1 expression level would go a long way in curing disease. To test for an environmental impact, a risk assessment was performed refers to determining hazards and risk factors that have the potential to cause harm. Analyze and measure the risk associated with the threat, and select the safest ways to mitigate the threat or handle the danger if it cannot be avoided. The risk assessment, in this case, was performed using the KLARA software, and the Kit analyzed was the SYBR select master mix and resulted in no environmental threats.

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Appendix

Table 2. Normalization and expression level calculation of CLIC1 gene with the reference genes. The results show a 3-fold increase.

		CLIC1							
replicates	sample	cq1	cq2	cq3	avg cq	deltacq	ddcq	2^ddcq	avg fold
1	0H	21.74	21.7	21.78	21.74	2.456487	-0.5048	1.41893	1.063478
2	0H	22.975	22.94	23.01	22.975	2.736953	-0.22434	1.168241	
3	0H	23.035	23.07	23	23.035	3.690433	0.729142	0.603263	
avd dct		2.961291							
1	4H	22.13	21.86	21.63	21.87333	2.261373	-0.69992	1.624412	1.402731
2	4H	22.18	22.17	22.175	22.175	2.892801	-0.06849	1.048618	
3	4H	22.39	22.29	22.49	22.39	2.342901	-0.61839	1.535161	
1	8H	21.31	21.03	21.61	21.31667	1.437951	-1.52334	2.874558	3.143471
2	8H	20.41	21.14	21.49	21.01333	0.768059	-2.19323	4.573287	
3	8H	21.895	21.91	21.88	21.895	1.973922	-0.98737	1.982566	
1	24H	21.61	22.39	21.46	21.82	1.418305	-1.54299	2.91397	3.548014
2	24H	21.61	21.85	21.72	21.72667	0.955194	-2.0061	4.016939	
3	24H	21.24	21.62	21.43	21.43	1.068653	-1.89264	3.713134	

Table 3. results from ANOVA showing significant difference between the groups.

Multiple Comparisons

Dependent Variable: Dcq

Tukey HSD

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0H	4H	0.46227	0.39993	0.668	-0.8184	1.743
	8H	1,56798*	0.39993	0.019	0.2873	2.8487
	24H	1,81391*	0.39993	0.008	0.5332	3.0946
4H	0H	-0.46227	0.39993	0.668	-1.743	0.8184
	8H	1.10571	0.39993	0.093	-0.175	2.3864
	24H	1,35164*	0.39993	0.039	0.0709	2.6323
8H	0H	-1,56798*	0.39993	0.019	-2.8487	-0.2873
	4H	-1.10571	0.39993	0.093	-2.3864	0.175
	24H	0.24593	0.39993	0.924	-1.0348	1.5266
24H	0H	-1,81391*	0.39993	0.008	-3.0946	-0.5332
	4H	-1,35164*	0.39993	0.039	-2.6323	-0.0709
	8H	-0.24593	0.39993	0.924	-1.5266	1.0348

*** The mean difference is significant at the 0.05 level.**