Selection and validation of reference genes for qPCR analysis of differentiation and maturation of THP-1 cells into M1 macrophage-like cells

Ren, Guoqiang; Juhl, Morten; Peng, Qiuyue; Fink, Trine; Porsborg, Simone Riis

Published in:
Immunology & Cell Biology

DOI (link to publication from Publisher):
10.1111/imcb.12590

Publication date:
2022

Document Version
Publisher's PDF, also known as Version of record

Immunology & Cell Biology 2022; **100**: 822–829
www.wileyonlinelibrary.com/journal/icb

SHORT COMMUNICATION

OPEN

Selection and validation of reference genes for qPCR analysis of differentiation and maturation of THP-1 cells into M1 macrophage-like cells

Guoqiang Ren[1], Morten Juhl[2], Qiuyue Peng[1], Trine Fink[1] & Simone Riis Porsborg[1]

1 Regenerative Medicine Group, Department of Health Science and Technology, Aalborg University, Fredrik Bajers Vej 3B, 9220, Aalborg, Denmark
2 Cardiology Stem Cell Centre, The Centre for Cardiac, Vascular, Pulmonary and Infectious Diseases, Copenhagen University Hospital Rigshospitalet, Denmark

Keywords
Interferon-γ, lipopolysaccharide, M1 macrophage-like cells, reference gene, RT-qPCR, THP-1

Correspondence
Simone Riis Porsborg, Regenerative Medicine Group, Department of Health Science and Technology, Aalborg University, Fredrik Bajers Vej 3B, 9220 Aalborg, Denmark.
E-mail: sriis@hst.aau.dk

Received 6 August 2022;
Revised 19 September 2022;
Accepted 29 September 2022

doi: 10.1111/imcb.12590

Immunology & Cell Biology 2022; **100**: 822–829

Abstract

For cell-based assays studying monocytes and macrophages, the immortalized monocyte cell line THP-1 is widely used and stimulated with phorbol 12-myristate 13-acetate, lipopolysaccharide (LPS) and/or interferon-γ (IFN-γ), after which it differentiates and polarizes into proinflammatory M1-like macrophages. For the quantification of this and the effect of different factors affecting these processes, the expression levels of various maturation markers are determined using reverse transcription-quantitative PCR. For this purpose, stably expressed reference genes are crucial. However, no studies evaluating the stability of reference genes in THP-1 cells stimulated with LPS and IFN-γ have been performed. Therefore, this paper describes the selection of the most used reference genes [*RPL37A* (ribosomal protein L37a), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *UBC* (ubiquitin C), *B2M* (0β2-microbulin), *ACTB* (β-actin) and *PPIA* (cyclophilin A)], the *in silico* primer design, the analysis and the validation of these in accordance with the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines and more recent recommendations for the validation of the stability of reference genes. Using the RefFinder platform, including the four most popular algorithms for reference gene validation, the Delta CT, BestKeeper, NormFinder and geNorm, we find the reference genes *GAPDH* and *UBC* to be the most stable. Furthermore, we demonstrate that the normalization of gene expression data using the least stable reference genes, *ACTB* and *B2M*, dramatically affects the interpretation of experimental data. Taken together, it is vital to validate the stability of reference genes under the specific experimental conditions used when utilizing the THP-1 monocyte model system.

INTRODUCTION

When developing standardized cell-based assays, such as potency assays, the use of immortalized cell lines rather than primary cells is an attractive option to avoid interdonor variation. Consequently, when studying factors that may affect monocyte and macrophage function and biology, the spontaneously immortalized monocyte cell line THP-1 is recognized as a valid alternative to primary monocytes and macrophages.[1,2]

To stimulate the differentiation of THP-1 monocytes into macrophage-like cells, phorbol 12-myristate 13-acetate (PMA) is commonly used. To further promote the polarization toward a macrophage M1 phenotype, the THP1 macrophage-like cells can be stimulated with lipopolysaccharide (LPS) and/or interferon-γ (IFN-γ).[3,4]

The differentiation and polarization processes are often evaluated by following the transcription of maturation marker genes using reverse transcription-quantitative PCR. This is a rapid and sensitive technique for quantifying low-

abundance messenger RNA expression and determining differences in expression levels by scaling expression data from genes of interest (GOIs) to one or more reference genes.[5] As the reference genes form the baseline against which the GOI is measured, they must be stably expressed across all experimental conditions. Interestingly, although LPS and IFN-γ have been extensively used in the polarization of THP-1–derived macrophages toward M1 macrophages, we have only been able to identify studies on selecting reference genes for the initial PMA-induced differentiation, not for the subsequent polarization. Furthermore, most of these studies are of an older date, and the design and selection of primers do not strictly adhere to the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines nor to more recent recommendations for validation of stability of reference genes.[6,7]

In this paper, we describe the selection, *in silico* primer design, analysis and validation of suitable reference genes when studying the differentiation and polarization of THP-1 cells into M1 macrophage-like cells. In addition, we demonstrate the suitability of this model to assess the anti-inflammatory properties of human adipose-derived stem cells (ASCs).

RESULTS AND DISCUSSION

Validation of primers

Primers for the six putative reference genes were evaluated for their robustness, specificity and efficiency. The temperature gradient analysis revealed that for annealing temperatures between 59°C and 65°C, the difference in Cq value was within one cycle (< 1) for all primer sets (Figure 1a). The similar Cq values across the temperature gradient indicated that the reactions were robust, and thus less susceptible to thermal variations. For the reactions with no template control, none of the primers yielded a product before 36 cycles, indicating that the primer dimer formation was not a by-product in any of the reactions with a complementary DNA (cDNA) template (data not shown).

The robustness and repeatability of the reactions were confirmed in the analysis of the PCR efficiency, which demonstrated that the efficiency of the PCRs was consistently high across different samples and experiments (Figure 1b). Finally, the melt curve analysis indicated a high specificity of each reaction with a single peak for each primer pair (Figure 1c). Of note, each of the graphs shown in panel c consists of 10 or more different melt curves that are so similar that they overlap to a degree where the individual traces are not discernable.

The validation of primers for the marker genes revealed an equally narrow range of Cq values across different temperatures, no primer dimer formation in the no template controls and efficiencies of 1.905 and 1.874 for *TNFA* (tumor necrosis factor-a) and *CCL2* (C–C motif chemokine ligand 2), respectively (data not shown).

Comparison of expression levels of reference genes

The distribution of Cq (mean of technical duplicates) for all genes and experimental conditions were compared (Figure 2). In general, *B2M* (β2-microbulin) was the most abundant, with Cq values between 16.4 and 18.22. The *UBC* (ubiquitin C) was the least expressed, with Cq values between 21.42 and 22.3, revealing a roughly 30-fold difference in expression levels between these genes. Comparison of the triplicate values within each gene demonstrated a relatively small interassay variation for all genes but *ACTB* (β-actin), indicating that *ACTB* would be an unsuitable reference gene under these experimental conditions.

Ranking of candidate reference gene

To rank the stability of the reference genes across all experimental conditions, RefFinder takes into account the four different software algorithms, namely, the Delta CT, NormFinder, BestKeeper and geNorm.

The Delta CT method is based on a comparison of the relative expression of pairs of genes within each sample. A stable ΔCt indicates that the genes are either stably expressed or that both genes are regulated in a similar manner. By comparing all genes, the genes can be ranked according to their stability across all conditions.[8] NormFinder ranks the candidate reference genes in relation to their stability across the samples.[9] The geNorm algorithm is based on the stability of the geometric mean of the putative reference genes during stepwise exclusion of the least stable gene, leading to a ranking of genes from most to least stable.[10] The BestKeeper algorithm ranks the candidate genes using a pairwise correlation analysis of all genes.[11] Finally, the RefFinder algorithm enables the ranking of the reference genes based on the four previously described algorithms[12] and has been validated in several studies.[13,14]

When using the RefFinder algorithm on data from all experimental conditions, we found that the most stably expressed genes in descending order were *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *UBC*, *RPL37A* (ribosomal protein L37a) and *PPIA* (cyclophilin A), whereas *B2M* and *ACTB* were the least stably expressed (Figure 3a). When looking at the individual algorithms, we found variations in the ranking of the four most stable genes, but all algorithms found that *B2M* was the second most unstable and *ACTB* the most unstable gene (Figure 3b). Based on this analysis,

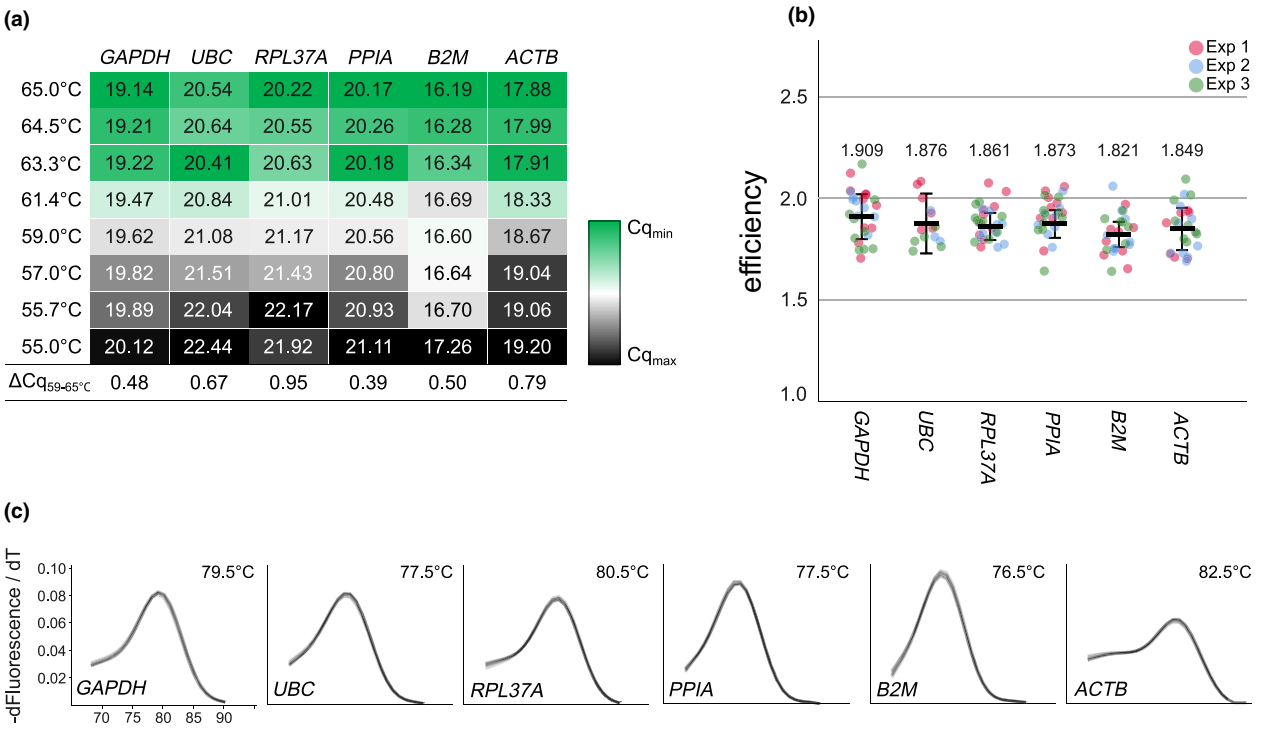

Figure 1. Validation of the primers for the six reference genes. **(a)** The effect of annealing temperature, ranging from 55°C to 65°C, on the Cq value of each reference gene. The delta-Cq is calculated for the desired range of robustness (i.e. 59–65°C). **(b)** Efficiencies of the reference gene primers across experiments. Colors represent different experiments (n = 3). Mean ± standard deviation depicted by line and error bars. **(c)** First derivatives of melting curves. The peak temperatures are listed for the individual plots. *ACTB*, β-actin; *B2M*, β2-microbulin; *CCL2*, C–C motif chemokine ligand 2; Cq, quantification cycle; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *PPIA*, cyclophilin A; *RPL37A*, ribosomal protein L37a; *TNFA*, tumor necrosis factor-α; *UBC*, ubiquitin C.

GAPDH and *UBC* were selected as the top choice of reference genes for this experiment.

Furthermore, we employed the RefFinder algorithm on data from the individual stimulation groups (Figure 3c). In cells stimulated with PMA, LPS and LPS combined with IFN-γ, the most stable genes were *UBC* and *B2M*. For cells stimulated with IFN-γ only, the most stable genes were *B2M* and *RLP37A*. The least stable reference gene identified between all groups was consistently *ACTB*. These results from the analysis of individual stimulation groups, where different genes scored better in other groups, underscore the importance of validating the choice of reference genes for each specific experimental setup.

Effect of reference genes on the relative expression of GOIs

To demonstrate the impact and importance of validating and combining the most reliable reference genes for data normalization of measured Cq values, we analyzed the relative expression of two GOIs, *TNFA* and *CCL2*, in our experimental setup. When normalizing the expression of *TNFA* and *CCL2* to the most stable reference genes, *GAPDH* and *UBC*, we saw a robust and statistically significant upregulation in cells cultured with LPS + IFN-γ compared with the other conditions. When normalizing to the least stable reference genes, *ACTB* and *B2M*, the results were overall less robust, and the difference was not significant when comparing the LPS group with the LPS + IFN-γ group (Figure 4a, b). The upregulation of GOIs was thus masked by variations in the reference gene expression, which underscores the necessity to validate reference genes for each experimental condition.

Effect of reference genes on the evaluation of the immunosuppressive effect of ASC-conditioned medium

ASCs are considered to be potent suppressors of an immune response, as they secrete a high amount of immune-suppressive cytokines, such as interleukin 10 and transforming growth factor-β1.[15] We used conditioned medium (CM) from ASCs in the THP-1 model system to assess if the anti-inflammatory properties could be detected in this experimental setup.

When comparing THP-1 cells stimulated with LPS + IFN-γ alone or combined with CM, we observed a

reduction in *TNFA* and *CCL2* expression in the CM group (Figure 4c, d). The reduction was most apparent when normalizing to the most stable reference genes, and for *TNFA*, the decrease was not statistically significant when normalizing to the least stable reference genes.

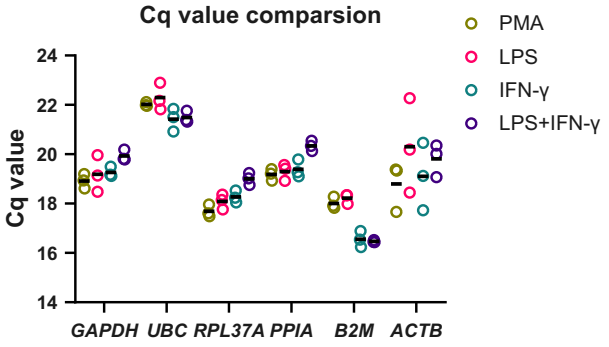

Figure 2. The effect of reference genes on the variability of Cq values across stimulation groups. Plotted are the range of Cq values obtained from the four different stimulation conditions used through reverse transcription-quantitative PCR (n = 3): the horizontal line identifies the mean Cq value of each reference gene. *ACTB*, β-actin; *B2M*, β2-microbulin; *CCL2*, C–C motif chemokine ligand 2; Cq, quantification cycle; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; IFN-γ, interferon-γ; LPS, lipopolysaccharide; *PPIA*, cyclophilin A; PMA, phorbol 12-myristate 13-acetate; *RPL37A*, ribosomal protein L37a; *TNFA*, tumor necrosis factor-α; *UBC*, ubiquitin C.

These data indicate the importance of evaluating potential reference genes to elucidate biological responses under the experimental paraments chosen.

In conclusion, robust, specific and efficient primers for six widely used reference genes were designed and ranked in order of stability across various THP-1 differentiation and polarization protocols using five different software algorithms. The RefFinder algorithm found the descending order of stability of the genes to be *GAPDH*, *UBC*, *RPL37A*, *PPIA*, *B2M* and *ACTB*. Only when normalizing the expression of the two GOIs, *TNFA* and *CCL2*, to the most stable reference genes, *GAPDH* and *UBC*, did we see a robust and statistically significant upregulation in cells cultured with LPS + IFN-γ compared with LPS alone. This demonstrates the impact and importance of validating reference genes for each experimental condition and combining the most reliable reference genes for data normalization of measured Cq values.

METHODS

Cell culture and production of CM

THP-1 cells (American Type Culture Collection) were grown in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum, 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (all from Gibco, Waltham, MA, USA) in T25 flasks (Greiner Bio-One, Esslingen, Germany) at

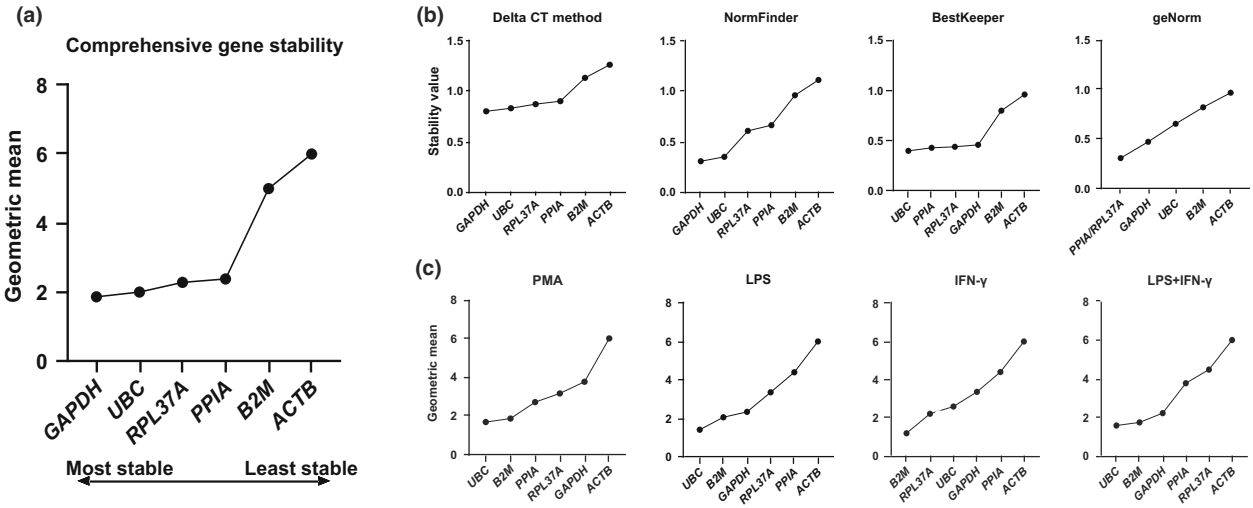

Figure 3. Stability of the six reference genes. **(a)** Ranking of the reference genes for the stimulation groups combined as calculated by the R-based software packages, which is based on RefFinder; The reference gene is ranked based on the geometric mean values of the comparative, the ΔCt method, the NormFinder, the BestKeeper and the geNorm algorithms (n = 24). **(b)** Comparison of stability ranking by stability value of the four distinct algorithms, described previously, across stimulation conditions (n = 24). **(c)** Comparison of stability ranking for each stimulation condition using RefFinder (n = 6). *ACTB*, β-actin; *B2M*, β2-microbulin; *CCL2*, C–C motif chemokine ligand 2; Cq, quantification cycle; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; IFN-γ, interferon-γ; LPS, lipopolysaccharide; *PPIA*, cyclophilin A; PMA, phorbol 12-myristate 13-acetate; *RPL37A*, ribosomal protein L37a; *TNFA*, tumor necrosis factor-α; *UBC*, ubiquitin C.

37°C in a humidified incubator with 5% CO_2. Human ASCs, established as described previously,[16] were maintained in alpha-minimum essential medium supplemented with 10% fetal bovine serum and 100 units mL^{-1} penicillin and 100 µg of streptomycin (all from Gibco).

To prepare the CM from the ASCs, passage 3 ASCs were seeded at 8000 cells per cm^2 in T75 culture flasks (Greiner Bio-One) and cultured until 60% confluence. At this time point, the ASCs were washed three times with phosphate-buffered saline (Gibco) and 15 mL Roswell Park Memorial Institute 1640 medium was added. After 48 h, the media was collected, centrifuged at 300*g* for 10 min and the supernatant frozen at −80°C.

Differentiation of THP-1 cells into M1 macrophages

First, the optimal concentration of LPS for induction of THP-1 monocyte differentiation into macrophages was determined. The aim was to identify conditions that resulted in a robust increase in expression of prototypical markers, yet as low as possible to avoid potential off-target effects.[3,17] In brief, after PMA stimulation, THP-1 monocytes were incubated with a 10-fold titration of LPS ranging from 0.1 to 1000 ng mL^{-1} for 24 h in combination with 20 ng mL^{-1} IFN-γ, after which the cell morphology was monitored by phase contrast microscopy (data not shown) and the transcriptional levels of *TNFA* and *CCL2* were determined by quantitative PCR (Supplementary figure 1). As a concentration of 10 ng mL^{-1} was well within the range of inducing a response, yet still on the lower end, this was chosen for subsequent experiments.

For differentiation of THP-1 monocytes into macrophages, 500 000 cells were seeded in a 12-well suspension culture plate (Greiner Bio-One) in 1 mL media; to this mixture, 10 ng mL^{-1} PMA (Sigma-Aldrich, Burlington, VT, USA) was added. After 48 h of PMA stimulation, the media was changed after being washed with phosphate-buffered saline two times,

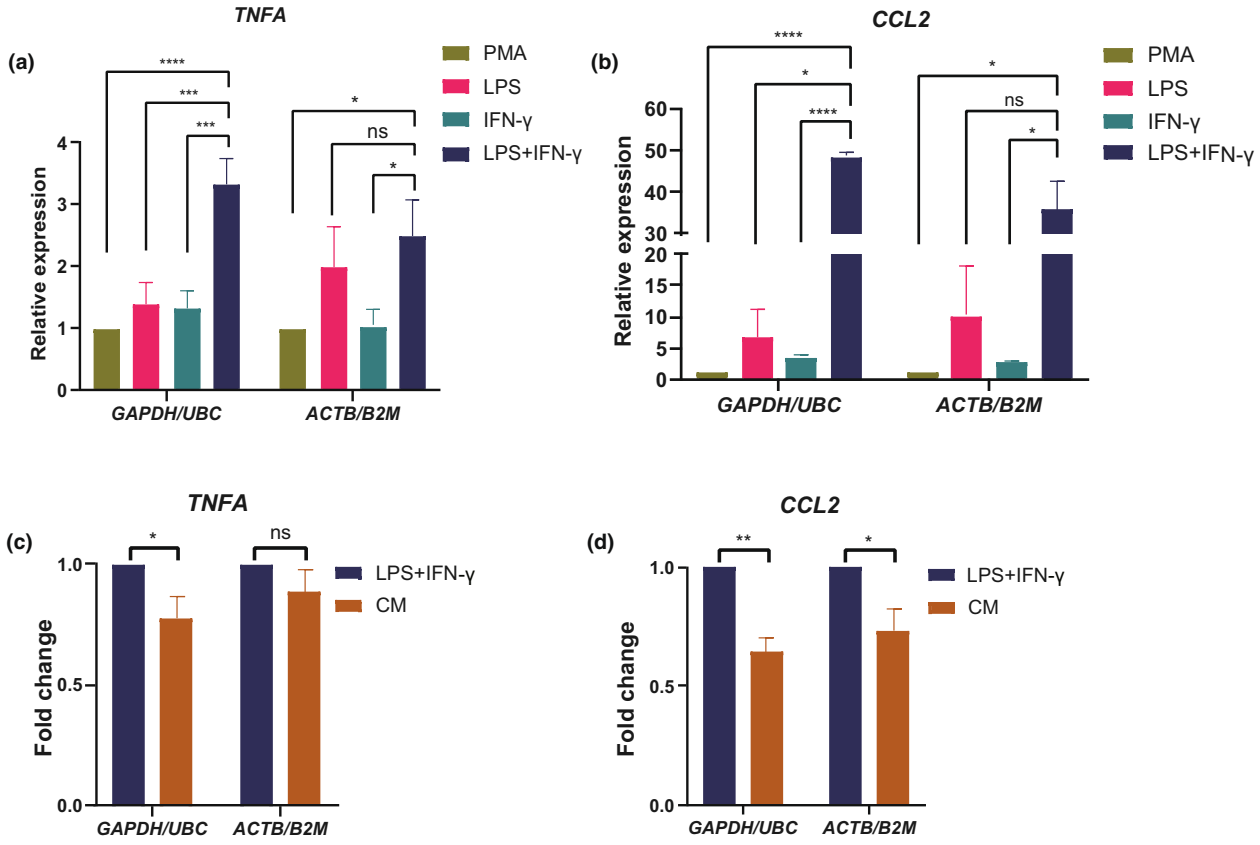

Figure 4. The effect of reference genes on the relative gene expression of genes of interest (GOIs) when polarizing THP-1 cells into M1 macrophage-like cells and investigating the effect of adipose-derived stem cell–conditioned media (CM) on the polarization of macrophages. Fold change of the GOI, **(a)** *TNFA* and **(b)** *CCL2*, expression levels of the M1 macrophage-like cells, as compared with the phorbol 12-myristate 13-acetate (PMA)–stimulated macrophage-like cells, calculated using the most (*GAPDH/UBC*) and least stable reference genes (*ACTB/B2M*). Fold change in the expression of the GOI, **(c)** *TNFA* and **(d)** *CCL2*, as compared with the LPS + IFN-γ–stimulated group. For each GOI, the effect of the different stimulation protocols on the expression levels, and the significant difference of these, are shown ($n = 3$). The error bars represent means ± standard deviation. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. *ACTB*, β-actin; *B2M*, β2-microbulin; *CCL2*, C–C motif chemokine ligand 2; Cq, quantification cycle; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; IFN-γ, interferon-γ; LPS, lipopolysaccharide; ns, not significant; *PPIA*, cyclophilin A; *RPL37A*, ribosomal protein L37a; *TNFA*, tumor necrosis factor-α; *UBC*, ubiquitin C.

and the cells were exposed to 10 ng mL^{-1} LPS (Sigma-Aldrich) and/or 20 ng mL^{-1} IFN-γ (PeproTech, Rocky Hill, CT, USA) in Roswell Park Memorial Institute 1640 medium, or ASC-derived CM supplemented with 10 ng mL^{-1} LPS and 20 ng mL^{-1} IFN-γ for 24 h, after which they were harvested for RNA isolation (Figure 1).

RNA isolation and cDNA synthesis

Total RNA was isolated from cell lysates using the Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. After RNA was collected, the quantity and quality were assessed using the NanoDrop2000 (Thermo Fisher, Waltham, MA, USA). Ratios of absorbance at 260/280 (acceptable value range: 1.8–2.0) and 260/230 (acceptable value range: 2.0–2.2) were determined to confirm that all samples were suitable for reliable reverse transcription-quantitative PCR analysis. For each experiment, the RNA concentration was adjusted across all samples to yield similar concentrations. Based on the adjusted RNA, cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions.

Selection of potential reference genes and marker genes

We performed a review of the literature to identify commonly used reference genes in the study of THP-1 cells and chose to analyze the following six putative reference genes: *RPL37A*, *GAPDH*, *UBC*, *B2M*, *ACTB* and *PPIA*.[18–22]

To follow the maturation and polarization of THP-1 cells, we selected two target genes, *TNFA* and *CCL2*, that are hallmark markers of M1 macrophage polarization.[23–25]

In silico primer design

Primers for the selected reference genes were designed using the Primer-BLAST NCBI tool[26] using the criteria listed in Table 1.

The absence of primer dimers and hairpins was confirmed using AutoDimer.[27] A prediction of secondary structures on products was performed by the mfold web server[28] with ionic conditions of 50.0 mM Na^+ and 1.5 mM Mg^{++}, identical to the default settings of Primer-BLAST. All secondary structures had melting temperatures below 60°C. Primers that conformed to the design criteria were purchased from LGC Biosearch Technologies (Broxbourne, Hertfordshire, UK). The primers are listed in Table 2.

Temperature-dependent performance of primers

First, the performance of the primers across a range of temperatures was determined using the CFX Connect Real-Time PCR cycler (Bio-Rad), where a temperature gradient block allows for a temperature gradient from the top to the bottom rows. For each primer pair, two sets of eight reactions were prepared with either pooled cDNA template from PMA-stimulated THP-1 cells or no template control, respectively, and each set was loaded onto adjacent columns of a 96-well PCR plate.

Table 1. Criteria for the PCR primer design.

Product size	70–150 bp
Primer length	Optimum 20 bases; 18–25 allowed
GC content	50% ± 20%
Tm	Optimum 63°C; 62–65°C allowed
Tm differences	≤ 1 within pairs; ≤ 3 between multiple pairs
Poly-X repeats	≤ 4
Exon–exon span OR Separated by intron	Yes (intron > 500 bp)
Specificity stringency	At least four total mismatches to unintended targets, including two mismatches within the last 4 bp at the 3′ end
Max GC of terminal 5 bases	3
Self-complementarity	< 6
Off-target products	Only products > 500 bp

bp, base pairs; GC, guanine–cytosine content; Tm, melting temperature.

Each reaction comprised 10 µL of SYBR Green SuperMix Universal (Bio-Rad), 0.5 µL of forward primer (5 µM), 0.5 µL of reverse primer (5 µM), 9 µL of cDNA template (1/30 dilution of reverse transcription reaction) or 9 µL of H_2O for the no template controls. The PCR amplification profile consisted of 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and a gradient of temperatures from the top to the bottom row ranging from 55°C to 65°C for 30 s, and ending with a melting curve from 65°C to 95°C in 0.5°C increments.

Quantitative PCR

Quantitative PCR was performed using the CFX Connect Real-Time PCR Detection System (Bio-Rad). For each cDNA sample and each primer pair, duplicate reactions were performed, as described in the previous section. The PCR protocol consisted of an initial activation of the polymerase at 95°C for 3 min, followed by 40 cycles of a denaturation step at 95°C for 10 s and a combined annealing and extension step at 60°C for 30 s, followed by a melting curve analysis.

Reverse transcription-quantitative PCR data processing and reference gene stability

To assess Cq (quantification cycle) values, the baseline threshold based on the automatic calculation of the CFX Manager Software was set for all experiments, and a Cq value was obtained in the single threshold mode of the CFX Manager Software. Relative quantities of all samples were determined by taking their individual efficiency into account.[29]

Efficiencies were determined using LinRegPCR,[30] which, based on linear regression analysis of raw fluorescence data, enables the determination of the PCR efficiency of each reaction to overcome limitations associated with conventional standard curves.[29,31]

Table 2. Candidate reference and target genes.

Target	Identifier	Sequences	Tm	Unintended products	Product size (bp)	Secondary structures on products
PPIA	NM_021130.5	TGCTGGACCCAACACAAATGGT	62.95	Related products (PPIAL4)	74	Tm ≤ 56.2
		CATGCTTGCCATCCAACCACTCA	63.81			
RPL37A	NM_000998.5	AGCTGTGGGGATCTGGCACT	63.41	No	85	Tm ≤ 54.3
		CGTGACAGCGGAAGTGGTATTGTA	62.91			
UBC	NM_021009.7	TTCCGTCGCAGCCGGGATTT	64.93	No	70	Tm ≤ 48.1
		TGCATTGTCAAGTGACGATCACAGC	64.22			
B2M	NM_004048.4	GGAGGCTATCCAGCGTACTCCA	63.31	No	107	Tm ≤ 50.7
		CGGATGGATGAAACCCAGACACA	62.75			
ACTB	NM_001101.5	GCCTCGCCTTTGCCGATCC	64.02	No	72	Tm ≤ 44.7
		GCGCGGCGATATCATCATCCA	63.32			
GAPDH	NM_002046.7	ATGCCTCCTGCACCACCAACT	64.39	Transcript variants 2, 3, 4, 7	97	Tm ≤ 54
		ATGGCATGGACTGTGGTCATGAGT	64.23			
TNFA	NM_000594.4	GGGACCTCTCTCTAATCAGCCCT	62.62	No	70	Tm ≤ 57
		GGCTTGTCACTCGGGGTTCG	63.38			
CCL2	NM_002982.4	AATCACCAGCAGCAAGTGTCCC	63.36	No	133	Tm ≤ 58.7
		CGGAGTTTGGGTTTGCTTGTCC	62.47			

ACTB, β-actin; *B2M*, β2-microbulin; *CCL2*, C–C motif chemokine ligand 2; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *PPIA*, cyclophilin A; *RPL37A*, ribosomal protein L37a; Tm, melting temperature; *TNFA*, tumor necrosis factor-α; *UBC*, ubiquitin C.

Finally, the stability of expression of the reference genes was analyzed on the web-based RefFinder platform,[12] using Cq values from the CFX Manager Software.

Normalization of GOIs to reference genes

For each time point and experimental condition, the expression levels of the GOIs were divided by the geometric mean of two reference genes.[6,10]

Statistical analysis

All data are presented as mean ± standard deviation. For a comparison between two groups, Student's paired *t*-test was used. When comparing more than two groups, a paired one-way ANOVA together with Tukey *post hoc* test was used. *P*-values < 0.05 were considered statistically significant. Statistical analysis was performed using GraphPad Prism 9 (GraphPad Prism, San Diego, CA, USA).

ACKNOWLEDGMENTS

All authors have read and agreed to the published version of the manuscript. This project was funded by the Diabetesforeningen. The first author is supported by China Scholarship Council and S.C. Van Fonden.

AUTHOR CONTRIBUTIONS

Guoqiang Ren: Conceptualization; data curation; formal analysis; investigation; methodology; project administration; software; validation; visualization; writing – original draft; writing – review and editing. **Morten Juhl:** Conceptualization; methodology; project administration; software; supervision; visualization; writing – review and editing. **Qiuyue Peng:** Investigation; methodology; visualization. **Trine Fink:** Conceptualization; project administration; resources; supervision; writing – original draft; writing – review and editing. **Simone Porsborg:** Conceptualization; funding acquisition; project administration; resources; supervision; writing – original draft; writing – review and editing.

CONFLICT OF INTEREST

All authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

1. Auwerx J. The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation. *Experientia* 1991; **47**: 22–31.
2. Cassol E, Alfano M, Biswas P, Poli G. Monocyte-derived macrophages and myeloid cell lines as targets of HIV-1 replication and persistence. *J Leukoc Biol* 2006; **80**: 1018–1030.
3. Genin M, Clement F, Fattaccioli A, Raes M, Michiels C. M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide. *BMC Cancer* 2015; **15**: 577.
4. Baxter EW, Graham AE, Re NA, *et al.* Standardized protocols for differentiation of THP-1 cells to macrophages with distinct M(IFNγ+LPS), M(IL-4) and M(IL-10) phenotypes. *J Immunol Methods* 2020; **478**: 112721.

5. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 2000; **25**: 169–193.
6. Bustin SA, Benes V, Garson JA, *et al*. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009; **55**: 611–622.
7. Bustin S, Huggett J. qPCR primer design revisited. *Biomol Detect Quantif* 2017; **14**: 19–28.
8. Silver N, Best S, Jiang J, Thein SL. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Mol Biol* 2006; **7**: 33.
9. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* 2004; **64**: 5245–5250.
10. Vandesompele J, De Preter K, Pattyn F, *et al*. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; **3**: RESEARCH0034.
11. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--excel-based tool using pair-wise correlations. *Biotechnol Lett* 2004; **26**: 509–515.
12. Xie F, Xiao P, Chen D, Xu L, Zhang B. miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. *Plant Mol Biol* 2012; **80**: 75–84.
13. Köhsler M, Leitsch D, Müller N, Walochnik J. Validation of reference genes for the normalization of RT-qPCR gene expression in *Acanthamoeba* spp. *Sci Rep* 2020; **10**: 10362.
14. De Spiegelaere W, Dern-Wieloch J, Weigel R, *et al*. Reference gene validation for RT-qPCR, a note on different available software packages. *PLoS One* 2015; **10**: e0122515.
15. Ceccarelli S, Pontecorvi P, Anastasiadou E, Napoli C, Marchese C. Immunomodulatory effect of adipose-derived stem cells: the cutting edge of clinical application. *Front Cell Dev Biol* 2020; **8**: 236.
16. Zachar V, Rasmussen JG, Fink T. Isolation and growth of adipose tissue-derived stem cells. *Methods Mol Biol* 2011; **698**: 37–49.
17. Hirose K, Iwabuchi K, Shimada K, *et al*. Different responses to oxidized low-density lipoproteins in human polarized macrophages. *Lipids Health Dis* 2011; **10**: 1.
18. Maeß MB, Sendelbach S, Lorkowski S. Selection of reliable reference genes during THP-1 monocyte differentiation into macrophages. *BMC Mol Biol* 2010; **11**: 90.
19. Jiang MX, Hong X, Bin LB, *et al*. Expression profiling of TRIM protein family in THP1-derived macrophages following TLR stimulation. *Sci Reports* 2017; **7**: e42781.
20. Kulkarni NN, Gunnarsson HI, Yi Z, *et al*. Glucocorticoid dexamethasone down-regulates basal and vitamin D3 induced cathelicidin expression in human monocytes and bronchial epithelial cell line. *Immunobiology* 2016; **221**: 245–252.
21. Green ID, Pinello N, Song R, *et al*. Macrophage development and activation involve coordinated intron retention in key inflammatory regulators. *Nucleic Acids Res* 2020; **48**: 6513–6529.
22. Kaur H, Moreau R. Curcumin steers THP-1 cells under LPS and mTORC1 challenges toward phenotypically resting, low cytokine-producing macrophages. *J Nutr Biochem* 2021; **88**: 108553.
23. Huang Y, Tian C, Li Q, Xu Q. *TET1* knockdown inhibits *Porphyromonas gingivalis* LPS/IFN-γ-induced M1 macrophage polarization through the NF-κB pathway in THP-1 cells. *Int J Mol Sci* 2019; **20**: 2023.
24. Tsai CF, Chen GW, Chen YC, *et al*. Regulatory effects of quercetin on M1/M2 macrophage polarization and oxidative/antioxidative balance. *Nutrients* 2021; **14**: 67.
25. Lee MKS, Moore XL, Fu Y, *et al*. High-density lipoprotein inhibits human M1 macrophage polarization through redistribution of caveolin-1. *Br J Pharmacol* 2016; **173**: 741–751.
26. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 2012; **13**: 134.
27. Vallone PM, Butler JM. AutoDimer: a screening tool for primer-dimer and hairpin structures. *Biotechniques* 2004; **37**: 226–231.
28. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 2003; **31**: 3406–3415.
29. Ruijter JM, Barnewall RJ, Marsh IB, *et al*. Efficiency correction is required for accurate quantitative PCR analysis and reporting. *Clin Chem* 2021; **67**: 829–842.
30. RDML-LinRegPCR ¦ GEAR. https://www.gear-genomics.com/rdml-tools/linregpcr.html (accessed 6 Apr2022).
31. Ruijter JM, Ramakers C, Hoogaars WMH, *et al*. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res* 2009; **37**: e45.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.