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ORIGINAL ARTICLE

Reference gene selection and validation for mRNA expression analysis by RT-qPCR in murine M1- and M2-polarized macrophage

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Abstract

Murine bone marrow-derived macrophages (M0) and M1- and M2-polarized macrophages are being widely used as a laboratory model for polarized macrophages related molecular mechanism analysis. Gene expression analysis based on reference gene normalization using RT-qPCR was a powerful way to explore the molecular mechanism. But little is known about reference genes in these cell models. So, the goal of this study was to identify reference genes in these types of macrophages. Candidate reference genes in murine bone marrow-derived and polarized macrophages were selected from microarray data using Limma linear model method and evaluated by determining the stability value using five algorithms: BestKeeper, NormFinder, GeNorm, Delta CT method, and RefFinder. Finally, the selected stable reference genes were validated by testing three important immune and inflammatory genes (NLRP1, IL-1β, and TNF-α) in the cell lines. Our study has clearly shown that Ubc followed by Eef1a1 and B2m respectively were recognized as the three ideal reference genes for gene expression analysis in murine bone marrow-derived and polarized macrophages. When three reference genes with strong different stability were used for validation, a large variation of a gene expression level of IL-1β, TNF-α and NLRP1 were obtained which provides clear evidence of the need for careful selection of reference genes for RT-qPCR analysis. Normalization of mRNA expression level with Ubc rather than Actb or Gusb by qPCR in macrophages and polarized macrophages is required to ensure the accuracy of the qPCR analysis.

Keywords Reference gene · RT-qPCR · Macrophages · Polarization

Abbreviations

BMDM or M0	Bone marrow-derived macrophage
RT-qPCR	Real-time quantitative PCR
M1	Classically activated macrophages
M2	Alternatively activated macrophages
RG	Reference gene
STDEV	Standard deviation

Introduction

Macrophages derived from the hematopoietic system are found in all tissues of our body, these large phagocytes exhibit high plasticity and great functional diversity depending on different microenvironment [1, 2]. Not only do macrophages play pivotal roles in innate and adaptive immune responses [3], but they also have crucial roles in organ development, tissue repair and homeostasis, cancer progression and therapy [4–7]. Based on specifically microenvironmental stimuli, macrophages can be induced into two different phenotypic and functional cell types, classically activated macrophages (M1) and alternatively activated macrophages (M2) [8]. It is well acknowledged that macrophages exhibit phenotypic plasticity in vitro. IFN-γ or LPS induces M1 macrophages associated with a pro-inflammatory phenotype while IL-4 or IL-13 induces an

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alternative activation program in M2 macrophages associated with an anti-inflammatory and tissue remodelling phenotype [7, 9]. A large number of reports have generally indicated that macrophage M1/M2 polarization is a key determinant of multiple disease development and regression such as cancer, allergic and chronic inflammation, infection, metabolic diseases, atherosclerosis which might be served as novel diagnostic and therapeutic strategies [7, 10]. Most importantly, after different forms of macrophage polarization and activation, studies in the fields of different molecular changes of cytokines, chemokines and signalling molecules using gene expression analysis to understand the M1/M2 macrophage targeting molecular regulation mechanisms of diseases play a key role in macrophage biology and their behaviour [11].

RT-qPCR is one of the most widely used tools to detect and quantify mRNA levels because of its high sensitivity and accuracy with the potential for high throughput, which allows quantification of low mRNA concentrations [12]. RT-qPCR includes RNA extraction from samples, reverse transcription to prepare cDNA using purified RNA as template and quantitative PCR (qPCR, quantitatively measure the amplification of cDNA using fluorescent dyes). At present, there are a lot of superior quality kits for RNA extraction, cDNA preparation, and qPCR. Indeed, the three determinants (RNA and cDNA quantity, and qPCR efficiency) need to be normalized. However, these are not enough to compare RT-qPCR data directly. Normalization of RT-qPCR data and target gene expression level with suitable endogenous reference genes (RGs) is required because the selection of ideal RGs has an important impact on the experimental outcomes [13–15]. The suitable RGs should be stable in cells and tissues or under different experimental conditions. But increasing evidence has shown that RGs expression levels often vary under different experimental settings [14–19]. Thus, it is necessary to meticulously evaluate and validate the RGs expression for each experimental situation [20].

To date, there are many studies have reported on the selection of suitable RGs in different macrophage cell types [14, 15, 17–19]. However, no optimal RGs have been identified and validated for the mRNA level normalization between polarized macrophages. In view of the roles of polarized macrophages in physiology and pathology as well as the importance of studying their precise molecular regulatory mechanisms, our aim is to identify the most stable RGs in polarized macrophages from C57BL/6 mice using NormFinder [21], GeNorm [22], BestKeeper software [23], delta CT method [24] and RefFinder methods [25, 26].

Methods

Murine bone marrow-derived macrophage isolation

The murine bone marrow-derived macrophages were isolated by a previously reported method with slight modification [27]. Briefly, bone marrow was flushed from fresh femurs and tibiae of 8–10-week-old healthy wild type C57BL/6 mice obtained from Nanjing medical university (Nanjing, China) after CO_2 euthanasia. The single-cell suspended BM cells were cultured in RPMI-1640 medium (Invitrogen) containing 10% fetal bovine serum, 50 nM β-mercaptoethanol, and 50 ng/mL M-CSF (Peprotech) at the concentration of 2×10^6 cells/mL at 37 °C under 5% CO_2. Bone marrow macrophage medium was refreshed on day 3 and mature M0-macrophages were generated on day 7.

All animal care and procedures were per the ethical standards approved by Jiangsu Society for Animal Welfare, China (Acceptance number: XZMC20130226) and Science and Technology Department of Jiangsu Province, China (Acceptance number: SYXK(SU)-2015-0030 and SCXK(SU)-2015-0009).

Polarization of murine macrophages

M1-polarization was induced by 100 ng/mL LPS (Sigma) and 20 ng/mL recombinant murine IFNγ (Peprotech), whereas M2-polarization by 20 ng/mL IL-4 (Peprotech) overnight as previously described [28]. There were eight repeats for each cell type including M0 macrophage.

Characterization of murine bone marrow-derived macrophage and polarization

It was reported in a previous study that macrophage markers were CD11b and F4/80 [29]. M1 macrophage markers were CD80 and CD86, and M2 macrophage markers were CD206 and CD163 [30]. In our study, macrophages were stained with CD11b-FITC, F4/80-APC, CD80-PE or CD206-FITC. Using flow cytometry, M0, M1-Mφ, and M2-Mφ cells were stained with $CD11b^+$ $F4/80^+$, $F4/80^+$ $CD80^+CD206^{low}$, and $F4/80^+CD80^{low}$ $CD206^+$ respectively [31, 32].

RT-qPCR

RNA was isolated from M0, M1 and M2-polarized macrophages using the TRIZOL-chloroform extraction method [20]. Genomic DNA was removed using RNase-free DNase I (Beyotime). The extracted RNA concentration and purity were calculated by NanoDrop 2000 spectrophotometer (Thermo USA). The integrity was also evaluated using 1%

Agarose gel electrophoresis. All cDNA samples were synthesized from 500 ng isolated RNA samples using PrimeScript RT Reagent Kit (Perfect Real Time) from Takara and kept at − 20 °C until ready for use. Melting temperatures of all genes were designed at 60 °C. Primers of reference genes and target genes were selected in consideration of different intracellular biological functions, purchased from Thermo scientific and the detail information was listed in Table 1. The specificity and efficiency of all reference gene primers were checked [20]. RT-qPCR was done using light cycler®480 and SYBR Green master mix. 10 µL of 2 × supermix SYBR Green 1 Master, 1 µL forward and reverse primers (10 nM), 8 µL of distilled water and 1 µL of cDNA were all included in the PCR reaction tube. The RT-qPCR runs were comprised of 1 min- predenaturation at 95 °C, followed by 40 cycles of three-step PCR including denaturing phase (95 °C for 20 s), annealing phase (60 °C for 15 s) and extension phase (72 °C for 15 s). After PCR, the melting curve and the CT values were obtained from the Light Cycler software. Samples were measured in technical duplicates.

Selection of candidate reference genes from microarray data

The microarray data having been deposited into the NCBI Gene Expression Omnibus database (GEO ID: GSE69607) was discussed in our study [33]. In this database, the same M0, M1, and M2 from C57BL/6J mice were used for microarray analysis. The data were normalized with the RMA algorithm using GenePattern software. The differential expression analysis in this microarray experiment was provided by a freely available bioinformatics tool Limma linear model method [34]. The expression levels of 14 commonly used reference genes were compared and the cut-off criteria to identify the differentially expressed candidate reference genes in M0, M1 and M2 were set to P-value < 0.05 implemented in Limma and $\log_2$|Fold change| ≥ 0.585 [35]. On

Table 1 Summary of 12 housekeeping genes and target genes evaluated in this study

Symbol	Official full name	Accession numbers	Primer sequence (forward/reverse)	Products size (bp)
Actb	Beta-actin	NM_007393	F:atgtggatcagcaagcagga R:aagggtgtaaaacgcagctca	99
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	NM_001001303	F:catggccttccgtgttccta R:gcggcacgtcagatcca	55
Ubc	Ubiquitin C	NM_019639.4	F:ccagtgttaccaccaagaag R:acccaagaacaagcacaagg	94
Eef1a1	Elongation factor 1 alpha 1 eukaryotic translation	NM_010106	F:tccgattacgacgatgttga R:agtcgccttggacgttctt	125
B2m	Beta-2 microglobulin	NM_009735	F:ttcagtatgttcggcttccc R:tggtgcttgtctcactgacc	103
Rplp0	60S acidic ribosomal protein P0	NM_007475	F:ccgatctgcagacacacact R:accctgaagtgctcgacatc	91
Ywhaz	tyrosine 3-monooxygenase /tryptophan 5-monooxygenase activation protein, zeta polypeptide	NM_011740	F:ctttctggttgcgaagcatt R:ttgagcagaagacggaaggt	148
Hmbs	hydroxymethylbilane synthase	NM_013551	F:cagggtacaaggctttcagc R:cggagtcatgtccggtaac	149
Gusb	β-glucuronidase	NM_010368	F:actcctcactgaacatgcga R:ataagacgcatcagaagccg	96
Ppia	Cyclophilin A/Peptidyl prolyl isomerase A	NM_008907	F:cagtgctcagagctcgaaagt R:gtgttcttcgacatcacggc	109
Alas 1	δ-Aminolevulinate synthase	NM_020559	F:gtctgtgccatctgggactc R:ctgtccacatcagctgtcca	119
Hprt 1	Hypoxanthine phosphoribosyltransferase1	NM_013556	F:cataacctggttcatcatcgc R:tcctcctcagaccgctttt	95
Nlrp1	NLR family pyrin domain containing 1	NM_033004	F:ccactgagctactatgcagtaca R:acaacatcttcacaccaccatc	202
IL-1β	Interleukin 1 beta	NM_008361	F:gaaatgccaccttttgacagtg R:tggatgctctcatcaggacag	116
TNF-α	Tumor necrosis factor, alpha	NM_013693.3	F:gacagtgacctggactgtgg R:gagacagaggcaacctgacc	132

Official full name, accession numbers, official full name, primer sequences and product sizes are shown

the contrary, the candidate stable reference gene was identified as P-value > 0.05 and $\log_2$|Fold change| < 0.585.

Statistical analysis of the stability of the reference genes

Four software; geNorm [22], NormFinder [21] and bestkeeper [23] together with the comparative delta CT method [24] were used to assess the reference genes stability. The basic principles of the four software and How the most reference genes filtered by the software were described by our previous study [20]. RefFinder-comprehensive ranking method were used for over ranking the above four methods according the introduction of previous research [36, 37]

Validation of reference genes

After reference genes selection, gene expression based on the selected most stable reference genes, the least stable reference genes, and the most usually used reference genes (Actb) were detected to validate the reference genes using the $2^{-\Delta\Delta Ct}$ methods.

Results

Characterization of murine bone marrow-derived macrophage phenotype

Macrophage progenitors adhered to the cell dish and could not be washed away on day 3. The murine bone marrow-derived macrophages (M0) were induced using 50 ng/mL M-CSF-contained medium for 7 days. During the 7 days culture, the mature M0-macrophages were observed as adherent flattened cells with larger size (Fig. 1a). M1-Mφ were induced with 20 ng/mL LPS and 20 ng/mL IFNγ overnight. The morphological features were similar to M0–Mφ (Fig. 1a). M2–Mφ were induced with 20 ng/mL IL-4 overnight causing the cells to be rounded and more loosely attached (Fig. 1a), which is similar to M2–Mφ.

In our study, flow cytometric analysis was employed to identify and quantify bone marrow-derived macrophages and their polarized forms using CD11b-FITC, F4/80-APC, CD80-PE or CD206-FITC antibodies. The results demonstrated that M0, M1, and M2 cells respectively amounted to 99.8%, 99.2% and 92% of the total number of cells stained (Fig. 1b). We also compared MFI of CD80-PE and CD206-FITC in M1 and M2 cells. The results showed that the CD80 expression level in M1 was higher than in M2 while CD206 expression level in M2 is higher than in M1 (Fig. 1c) which suggested that M1 was CD80 + CD206low and M2 was CD80low CD206+,consistent with previous reports [31, 32].

Selection and characteristics of candidate reference genes

To identify candidate RGs, we compared the gene expression levels of 14 commonly used RGs. Genes with low expression levels were excluded (as the underline value shown in Table 2). After filtration, 12 genes except Tbp and Tfrc were selected as the candidate reference genes for qPCR. Six genes including Actb, Eef1a1, B2m, Rplp0, Hmbs, and Ppia were the most stable RGs based on the database of a published microarray data and a Limma linear model method, which suggested that the candidate stable reference gene should be identified at P-value > 0.05 and log2|Fold change| < 0.585 [34, 35]. All data was shown in Table 2.

Primer expression level and specificity detection

To further screen stable reference genes for qPCR analysis, we designed and synthesized 12 primers with high efficiency [20]. Firstly, we detected the expression levels and specificity of the 12 RGs using SYBR Green I Master [38]. As shown in Fig. 2a, different genes had different CT values between 14.2 and 31.9 in different samples. Actb had the highest expression level while Hmbs expression level was the lowest. The result is similar to the above microarray data. The specificity of all the primers was high with unique single-peak amplification according to the melting curve as shown in (Fig. 2b).

Identification of the most stable RGs

Bestkeeper

In our experiment, the mean Ct values ranged from 15.68 (Actb) to 29.7 (Hmbs) in all M0, M1 and M2 samples (Table 3). STD [±CP], CV% and Pearson coefficient [R] are usually used to evaluate gene stability and the STD [±CP] is the index of the bestkeeper, most commonly used to compare the stability of the selected RGs [23]. Genes with the lowest STD [±CP] values have less variation and vice versa in gene expression. Our results showed that the gene stability was different in M0, M1 and M2 macrophages. In M0, Rplp0, Hprt1 and Ywhaz were ranked as the most stable RGs. Gapdh, Hmbs and Eef1a1 were stably expressed in M1 while Alsasl, Gapdh, and Eef1a1 were the most stable in M2. In all the cell types, Rplp0 with (STD [± CP] = 0.51) and Hprt1with (STD [± CP] = 0.55) followed by Ywhaz with (STD [± CP] = 0.59) were highly stable, while Gusb with (STD [± CP] = 2.26)

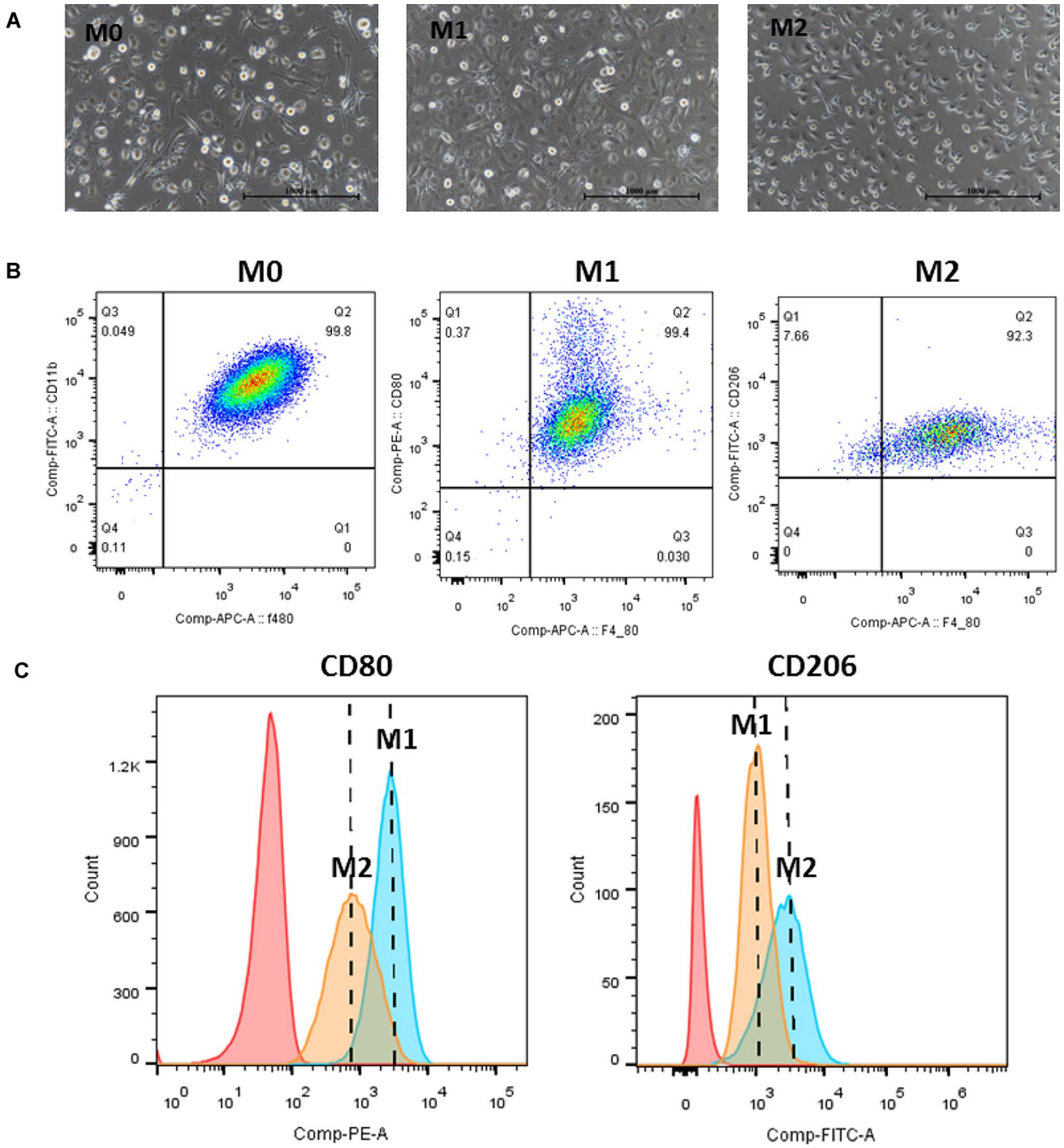

Fig. 1 Characterization of murine bone marrow-derived macrophage phenotype Morphological and cell surface marker expression (CD11b-FITC, F4/80-APC, CD80-PE or CD206-FITC.) changes observed by microscopic (**a**) and flow cytometric analyses (**b, c**)

having the highest value, indicating it had the lowest stability (as shown in Table 4).

GeNorm analysis

GeNorm grades the candidate gene stability according to the calculated M-value. M-value ≤ 1.5 was considered a stable expression of RGs. Genes with the lowest M-value were considered as the most stable genes. As seen in

Table 2 Identification of M0-M1-M2 specific reference genes from microarray data using Limma linear model method

Symbol	M0 expression (mean ± SD)	M1 expression (mean ± SD)	M2 expression (mean ± SD)	LogFC (M0 VS M1)	P-value (M0 VS M1)	LogFC (M0 VS M2)	P-value (M0 VS M2)	logFC (M1 VS M2)	P-value (M2 VS M1)
Actb	19,620.21 ± 558.65	18,498.04 ± 875.05	19,944.85 ± 186.94	0.09	0.33800	− 0.02	0.80301	− 0.11	0.35456
Gapdh	12,405.66 ± 365.68	14,273.75 ± 466.23	12,311.11 ± 465.92	− 0.20	0.03503	0.01	0.90923	0.21	0.08577
Ubc	5056.65 ± 545.89	4983.13 ± 769.53	3927.72 ± 222.10	0.03	0.83648	0.36	0.01350	0.33	0.04317
Eef1a1	17,004.44 ± 326.89	16,610.23 ± 700.76	17,601.12 ± 709.11	0.03	0.68857	− 0.05	0.61312	− 0.08	0.47653
B2m	16,742.41 ± 495.82	17,702.04 ± 422.78	15,887.40 ± 384.90	− 0.08	0.35187	0.08	0.44410	0.16	0.18924
Rplp0	11,045.81 ± 393.15	10,326.33 ± 173.77	11,107.88 ± 1033.34	0.10	0.26878	− 0.01	0.95807	− 0.10	0.40005
Ywhaz	1287.79 ± 462.64	1351.10 ± 216.24	2184.22 ± 189.16	− 0.13	0.60115	− 0.83	0.01169	− 0.70	0.00075
Hmbs	451.83 ± 29.16	452.44 ± 36.69	418.49 ± 61.24	0.00	0.99316	0.12	0.35347	0.12	0.39888
Gusb	4670.78 ± 398.07	2773.90 ± 256.60	3730.59 ± 161.90	0.75	0.00002	0.32	0.01586	− 0.43	0.00558
Ppia	13,988.93 ± 414.40	12,541.33 ± 473.74	13,776.74 ± 467.02	0.16	0.08854	0.02	0.82121	− 0.14	0.25488
Tbp	79.29 ± 7.02	89.21 ± 12.47	89.74 ± 1.48	− 0.16	0.20014	− 0.18	0.12521	− 0.02	0.89682
Alas1	854.97 ± 59.00	2342.17 ± 52.18	1674.10 ± 35.20	− 1.46	0.00000	− 0.97	0.00001	0.48	0.00128
Hprt	3276.17 ± 443.13	1672.07 ± 52.04	2205.30 ± 149.98	0.96	0.00000	0.56	0.00193	− 0.40	0.00567
Tfrc	58.01 ± 15.03	60.36 ± 12.44	278.97 ± 6.23	− 0.07	0.71959	− 2.30	0.00000	− 2.23	0.00000

Low expression levels of genes are labled with underline values

Table 5, most of the genes were stable in M0, M1, and M2 except Gusb, which meant that it is not suitable as a control gene for qPCR analysis. The results obtained from the GeNorm resembled those got from BestKeeper. After a combination, the two most stable genes were Ubc and B2m. All pairwise variation was shown in Fig. 3b to determine the optimal number of the candidate genes. According to [22], he recommends a cut-off value of 0.15, that genes with a $V \leq 0.15$ should be included. Therefore, based on the V value, the two most stable RGs ($V_2/V_{3)}$ of this dataset would be adequate for accurate normalization (Tables 6, 7, 8).

NormFinder

The principle of NormFinder ranking stability of RGs is based on stability values. Gene with the lowest stability value is considered to remain constant, while those with the highest stability values are the least stable RGs. After NormFinder software analysis, Ubc (Stability value = 0.076 and 0.504) in M0 and M1 cells, Gapdh (Stability value = 0.084) in M2 cells were the most stable genes. After the combination of all the data in the three cell lines, Eef1a1 (Stability value = 0.422) was considered to be the most stably expressed RG (Fig. 4).

The comparative delta-Ct method

The delta-Ct method determines the stability of RGs according to the average of STDEV within the samples [39]. Those with lower STDEV value between samples is considered as the most stable RGs. Consistent with NormFinder analysis, the delta-Ct method also identified the Eef1a1 and Ubc as the most stable RGs. Meanwhile, Gusb was recognized as the least stable.

The overall ranking of reference gene stability

We co-ranked the four methods and calculated the geometric mean using the RefFinder-comprehensive ranking method to screen the most stable RGs [14, 26]. The geometric mean values were used to rank stable genes. Finally, it ranked Ubc and Eef1a1 as the two most stable, and Gusb and Rplp0 were considered as the least stable RGs in all M0, M1 and M2 cells.

Validation of reference genes

To validate the performance of the above selected RGs, we determined the gene expression levels of three genes from the inflammatory signaling pathways, NLRP1, IL-1β and TNF-α using the most and least stable reference and Actb according to their stability in M0, M1, and M2 cells. The results showed strong variation in fold changes when different reference genes were used in M1 and M2 cells, which indicated that normalization of RT-qPCR data with an

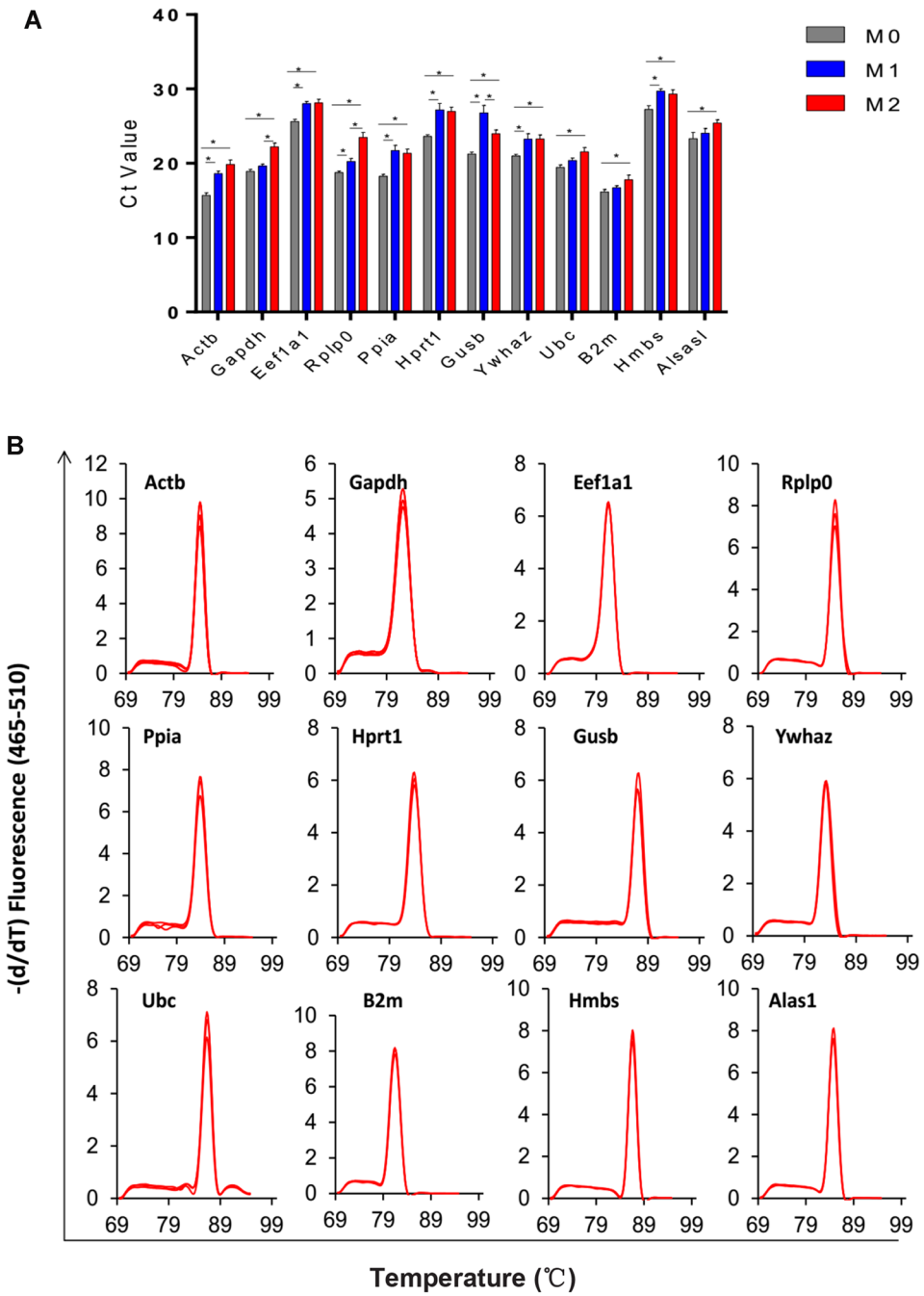

Fig. 2 Primer specificity and expression level analysis. **a** Ranges of Ct values of the 12 pre-selected reference genes in M0, M1, and M2 Plotted as boxes are the ranges of Ct values, with the included horizontal line identifying the mean (n = 8). The blue boxes represent M0 and the green boxes represent M1, and the red boxes represent M2. Bars represent the mean ± SD. *p < 0.05. **b** The images represent the melting curve of the 12 reference genes amplicons after the RT-qPCR reactions from one of 8 independent experiments. (Color figure online)

unstably expressed RG could impact upon the significance of gene expression study.

Discussion

RT-qPCR is most usually used to detect gene expression levels and predict possible gene functions in different experimental conditions. Recently, more and more researchers have found out that the traditional RGs such as Actb and Gapdh are not always stable in a lot of experimental situations making them unsuitable to be used as reference genes to normalize RT-qPCR results [20]. Moreover, an increasing number of RT-qPCR studies were validated by most reference gene selection although there are a great number of studies still lacking reliable RGs selection for quantitative gene expression analysis. Macrophages with polarization plasticity (M0, M1, and M2) belonging to the immune system play a key role in many normal physiological conditions and disease processes. They largely perform their functions through the regulation of encoding cytokines or expression of other genes. Some studies have reported on

Table 3 Descriptive statistical analysis of candidates' RGs by bestkeeper

Group	Gene names	Geomean	Ar Mean	Min [CP]	Max [CP]	STD [± CP]	CV %	Pearson coefficient [R]	P-value
M0 (N = 8)	Actb	15.68	15.7	14.25	16.36	0.68	4.33	0.965	0.001
	Gapdh	18.89	18.91	17.69	19.69	0.6	3.19	0.988	0.001
	Eef1a1	25.6	25.61	24.22	26.44	0.68	2.66	0.973	0.001
	Rplp0	18.73	18.74	18.06	19.42	0.51	2.73	0.749	0.033
	Ppia	18.23	18.24	16.93	18.86	0.62	3.42	0.889	0.003
	Hprt1	23.59	23.6	22.43	24.08	0.55	2.34	0.912	0.002
	Gusb	21.22	21.23	19.89	21.99	0.64	3.03	0.826	0.012
	Ywhaz	20.96	20.97	19.79	21.43	0.59	2.81	0.932	0.001
	Ubc	19.44	19.46	17.92	20.42	0.71	3.62	0.988	0.001
	B2m	16.14	16.16	14.92	17.54	0.62	3.81	0.983	0.001
	Hmbs	27.23	27.26	25.65	30.35	0.84	3.08	0.88	0.004
	Alsasl	23.22	23.31	21.42	29.02	1.43	6.12	0.75	0.032
M1 (N = 8)	Actb	18.61	18.63	17.58	20.01	0.73	3.9	0.676	0.065
	Gapdh	19.62	19.63	18.48	20.5	0.57	2.92	0.532	0.175
	Eef1a1	28	28.01	27.27	29.69	0.62	2.22	0.731	0.039
	Rplp0	20.2	20.23	19.02	21.67	1.07	5.28	0.001	0.996
	Ppia	21.64	21.72	19.55	25.02	1.55	7.15	0.901	0.002
	Hprt1	27.08	27.17	25.06	31.32	1.95	7.16	0.834	0.01
	Gusb	26.64	26.77	24.15	31.79	2.27	8.46	0.873	0.005
	Ywhaz	23.19	23.26	21.61	26.76	1.63	7	0.905	0.002
	Ubc	20.35	20.37	19.11	21.98	0.73	3.57	0.896	0.003
	B2m	16.69	16.71	16.01	17.98	0.66	3.92	0.834	0.01
	Hmbs	29.7	29.71	28.99	31.72	0.61	2.04	0.681	0.063
	Alsasl	23.99	24.04	22.99	28.48	1.11	4.62	0.823	0.012
M2 (N = 8)	Actb	19.77	19.84	17.64	22.37	1.46	7.36	0.996	0.001
	Gapdh	22.18	22.22	20.55	24.45	1.19	5.35	0.993	0.001
	Eef1a1	28.08	28.11	26.33	30.11	1.21	4.32	0.994	0.001
	Rplp0	23.4	23.47	20.03	25.17	1.59	6.77	0.263	0.528
	Ppia	21.29	21.34	19.48	23.82	1.33	6.25	0.992	0.001
	Hprt1	26.94	26.98	25.07	29.32	1.34	4.98	0.988	0.001
	Gusb	23.95	23.99	21.99	25.75	1.25	5.22	0.984	0.001
	Ywhaz	23.23	23.28	21.59	25.84	1.25	5.35	0.984	0.001
	Ubc	21.5	21.56	19.75	24.16	1.31	6.09	0.994	0.001
	B2m	17.71	17.78	15.93	21.2	1.35	7.59	0.969	0.001
	Hmbs	29.29	29.32	27.44	31.94	1.23	4.21	0.966	0.001
	Alsasl	25.41	25.43	23.98	26.41	1.05	4.11	0.922	0.001
Combination (N = 24)	Actb	17.93	18.06	14.25	22.37	1.71	9.48	0.957	0.001
	Gapdh	20.18	20.25	17.69	24.45	1.35	6.64	0.84	0.001
	Eef1a1	27.2	27.24	24.22	30.11	1.28	4.68	0.954	0.001
	Rplp0	20.69	20.82	18.06	25.17	2.02	9.71	0.644	0.001
	Ppia	20.33	20.44	16.93	25.02	1.76	8.63	0.924	0.001
	Hprt1	25.82	25.92	22.43	31.32	1.87	7.23	0.898	0.001
	Gusb	23.84	24	19.89	31.79	2.26	9.43	0.753	0.001
	Ywhaz	22.43	22.5	19.79	26.76	1.47	6.53	0.913	0.001
	Ubc	20.41	20.46	17.92	24.16	1.11	5.45	0.933	0.001
	B2m	16.83	16.88	14.92	21.2	1.06	6.26	0.863	0.001
	Hmbs	28.72	28.76	25.65	31.94	1.41	4.89	0.881	0.001
	Alsasl	24.19	24.26	21.42	29.02	1.58	6.53	0.731	0.001

Table 4 Overall rankings of candidate genes in all samples by Bestkeeper

Ranking	M0		M1		M2		Combination	
	Genes	std dev [± CP]	Genes	std dev [± CP]	Genes	Std Dev [± CP]	Genes	Std Dev [± CP]
1 211	Rplp0	0.51	Gapdh	0.57	Alsasl	1.05	B2m	1.06
2	Hprt1	0.55	Hmbs	0.61	Gapdh	1.19	Ubc	1.11
3	Ywhaz	0.59	Eef1a1	0.62	Eef1a1	1.21	Eef1a1	1.28
4	Gapdh	0.6	B2m	0.66	Hmbs	1.23	Gapdh	1.35
5	Ppia	0.62	Actb	0.73	Gusb	1.25	Hmbs	1.41
6	B2m	0.62	Ubc	0.73	Ywhaz	1.25	Ywhaz	1.47
7	Gusb	0.64	Rplp0	1.07	Ubc	1.31	Alsasl	1.58
8	Actb	0.68	Alsasl	1.11	Ppia	1.33	Actb	1.71
9	Eef1a1	0.68	Ppia	1.55	Hprt1	1.34	Ppia	1.76
10	Ubc	0.71	Ywhaz	1.63	B2m	1.35	Hprt1	1.87
11	Hmbs	0.84	Hprt1	1.95	Actb	1.46	Rplp0	2.02
12	Alsasl	1.43	Gusb	2.27	Rplp0	1.59	Gusb	2.26

Table 5 Overall ranking of candidate genes in all samples by GeNorm analysis

Ranking	M0		M1		M2		Combination	
	Gene name	Stability value	Gene name	Stability value	Gene name	Stability value	Gene name	Stability value
1 211	Hprt1 | Ywhaz	0.091	Ubc | B2m	0.301	Ppia | Ubc	0.13	Ubc | B2m	0.352
1	Ywhaz		B2m		Ubc		B2m	
3	Ppia	0.123	Actb	0.331	Ywhaz	0.144	Gapdh	0.647
4	Actb	0.167	Gapdh	0.388	Hprt1	0.156	Eef1a1	0.78
5	Gapdh	0.182	Eef1a1	0.444	Gapdh	0.17	Actb	0.869
6	Eef1a1	0.195	Hmbs	0.58	Eef1a1	0.2	Hmbs	0.944
7	Ubc	0.205	Rplp0	0.765	Gusb	0.231	Ywhaz	1.024
8	Gusb	0.23	Alsasl	0.97	Actb	0.257	Ppia	1.088
9	B2m	0.266	Ywhaz	1.165	Hmbs	0.283	Hprt1	1.147
10	Rplp0	0.335	Ppia	1.268	B2m	0.322	Alsasl	1.241
11	Hmbs	0.46	Hprt1	1.403	Alsasl	0.388	Rplp0	1.401
12	Alsasl	0.714	Gusb	1.529	Rplp0	0.7	Gusb	1.542

the selection of suitable RGs for the accurate quantitation and normalization in different macrophage cell types such as in rat osteoblasts [14], PMA-induced THP-1 macrophages [18], J774A1 macrophage cell line [16], LPS-stimulated murine macrophages [15], and differentiating osteoblasts, osteoclasts and macrophages under different biological conditions [17]. To our knowledge, selection and validation of stable RGs in polarized macrophages have not been performed. So, in this study, we aimed to validate reference genes in a well-established primary macrophage model, BMDMs (M0) and their polarized forms (M1 and M2 cells).

Reference genes refer to the genes whose expression level does not change under specific experimental conditions which are selected from housekeeping genes with different functions. The large-scale gene expression data such as microarray can be used to identify condition-stable RGs [40, 41]. First, we compared 14 reference genes based on published microarray data of M0, M1, and M2 cells and found out that the expression levels of Tbp and Tfrc were too low to be used as suitable RGs in these three cell lines. The expression levels of Alas1 and Hprt1 in M0, M1, and M2 were different, suggesting that they do not qualify as RGs in polarized macrophages. But the stability of these two genes needs further RT-qPCR verification to confirm, so we designed 12 reference gene primers except Tbp and Tfrc to evaluate their gene stability in M0, M1, and M2 cells. Gapdh and Actb are the two most widely used reference genes [42], but it has been proven that they are not the most stable RGs, even under certain experimental conditions they appeared to be the most unstable genes [16]. In this study,

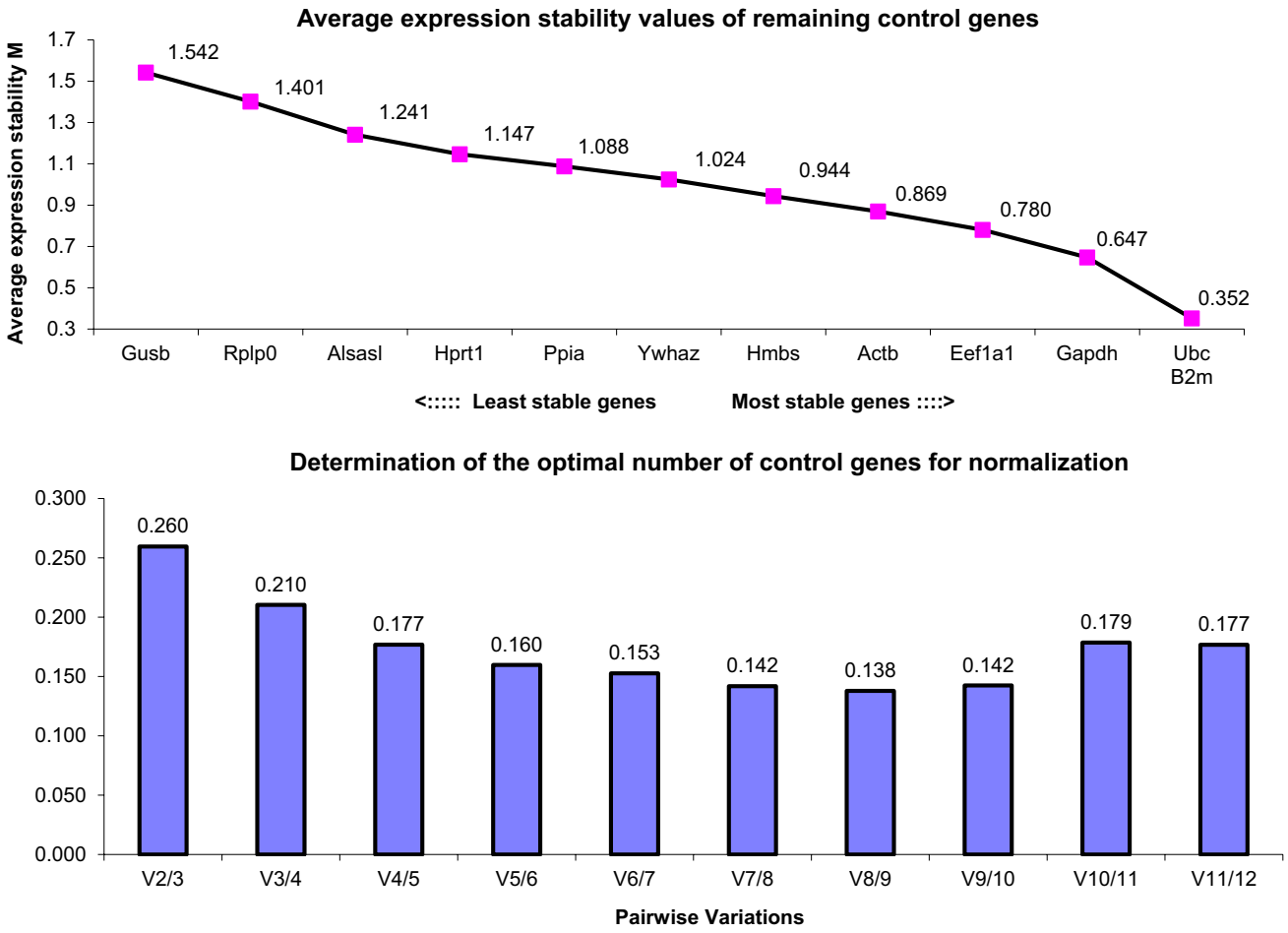

Fig. 3 Graphical presentation of stability value by GeNorm. **a** Showing the ranking of the12 reference by geNorm software, with the most stable toward the right and least toward the left. **b** Determination of the minimal number of reference genes by pairwise variation (Vn/n + 1). It was shown the determination of the optimal number of housekeeping genes by pairwise variation. A cut-off value of 0.15 was set. The V value defined the pair-wise variation between two sequential normalization factors. The two most stably expressed reference genes may be accurate for qRT-PCR normalization. More reference genes for qRT-PCR normalization will not increase the stability of reference genes

Table 6 Overall ranking of candidate genes in all samples by NormFinder analysis

Ranking	M0		M1		M2		Combination	
	Gene name	Stability value	Gene name	Stability value	Gene name	Stability value	Gene name	Stability value
1	Ubc	0.076	Ubc	0.504	Gapdh	0.084	Eef1a1	0.422
2	Gapdh	0.076	B2m	0.652	Eef1a1	0.089	Ubc	0.627
3	B2m	0.12	Eef1a1	0.795	Gusb	0.154	Ywhaz	0.653
4	Eef1a1	0.128	Hmbs	0.821	Ubc	0.16	Actb	0.766
5	Actb	0.196	Actb	0.922	Ppia	0.17	Hmbs	0.776
6	Ywhaz	0.322	Gapdh	1.049	Hprt1	0.209	Ppia	0.836
7	Hprt1	0.378	Ppia	1.079	Ywhaz	0.222	B2m	0.896
8	Ppia	0.419	Ywhaz	1.083	Actb	0.313	Gapdh	1.085
9	Gusb	0.545	Alsasl	1.112	Hmbs	0.387	Hprt1	1.113
10	Rplp0	0.594	Hprt1	1.7	B2m	0.561	Alsasl	1.443
11	Hmbs	0.793	Rplp0	1.821	Alsasl	0.577	Rplp0	2.033
12	Alsasl	1.97	Gusb	2.053	Rplp0	2.243	Gusb	2.096

Table 7 The stability expression results detected by the comparative CT method (ΔCT method)

Ranking	M0		M1		M2		Combination	
	Genes	Average of STDEV	Genes	Average of STDEV	Genes	Average of STDEV	Genes	Average of STDEV
1	Gapdh	0.48	Ubc	1.18	Ppia	0.47	Eef1a1	1.22
2	Actb	0.49	B2m	1.18	Gapdh	0.47	Ubc	1.24
3	Ubc	0.5	Actb	1.29	Ubc	0.48	Ywhaz	1.29
4	Eef1a1	0.5	Eef1a1	1.29	Hprt1	0.49	Ppia	1.35
5	Ywhaz	0.51	Gapdh	1.37	Ywhaz	0.49	Hmbs	1.37
6	Hprt1	0.53	Hmbs	1.38	Eef1a1	0.5	B2m	1.37
7	Ppia	0.55	Ywhaz	1.51	Gusb	0.55	Actb	1.37
8	B2m	0.57	Ppia	1.51	Actb	0.58	Gapdh	1.5
9	Gusb	0.64	Alsasl	1.62	Hmbs	0.59	Hprt1	1.51
10	Rplp0	0.79	Hprt1	1.88	B2m	0.71	Alsasl	1.79
11	Hmbs	1.03	Rplp0	1.97	Alsasl	0.81	Rplp0	2.24
12	Alsasl	1.98	Gusb	2.16	Rplp0	2.26	Gusb	2.25

Table 8 Overall ranking of candidate genes in all samples by RefFinder- comprehensive ranking methods

Ranking	M0		M1		M2		Combination	
	Genes	Geomean of ranking values	Genes	Geomean of ranking values	Genes	Geomean of ranking values	Genes	Geomean of ranking values
1	Gapdh	2.51	Ubc	1.57	Gapdh	2.11	Ubc	1.68
2	Hprt1	3.03	B2m	2	Ppia	2.51	Eef1a1	1.86
3	Ywhaz	3.08	Gapdh	3.31	Ubc	3.03	B2m	2.55
4	Ubc	3.81	Eef1a1	3.66	Eef1a1	3.83	Ywhaz	4.41
5	Actb	4.23	Actb	3.87	Ywhaz	4.79	Hmbs	5.23
6	Eef1a1	5.42	Hmbs	4.12	Hprt1	5.42	Gapdh	5.26
7	Rplp0	5.62	Ppia	8.43	Gusb	5.45	Actb	5.79
8	Ppia	5.63	Ywhaz	8.43	Alsasl	6.04	Ppia	6.45
9	B2m	5.73	Alsasl	8.49	Hmbs	7.35	Alsasl	9.15
10	Gusb	8.21	Rplp0	8.77	Actb	8.66	Hprt1	9.24
11	Hmbs	11	Hprt1	10.49	B2m	10	Rplp0	11
12	Alsasl	12	Gusb	12	Rplp0	12	Gusb	12

these two genes were also used and our results revealed that the expression stability of Actb in M0, M1 and M2 cells is strikingly similar to Gapdh even though they are not the most stable ones. We have realized that this result corroborates another previous study [15]. These findings also suggested the necessity of evaluating reference genes used for qPCR evaluation, even if they are traditionally used reference genes.

To our knowledge, a lot of software such as RefFinder, NormqPCR, RefGenes, OLIVER, GeNorm, BestKeeper, and NormFinder had been developed for stable reference gene selection. The grading of RG stability may vary due to the discrepancies of the above methods. There is no one computational program or statistical tool universally accepted to analyze gene expressions for specific experiments. A lot of studies including our previous study [20], usually employ several applets to determine gene expression stability. NormFinder [21], GeNorm [22], BestKeeper software [23] along with the delta CT [24] and RefFinder method [25, 26] are the most commonly used method to rank the stability of reference gene expression. These five methods were explored in our study to determine the most stable reference genes in M0, M1 and M2 cells. After over ranking of these methods, our results revealed that Ubc followed by Eef1a1 and B2m respectively were the three most stable RGs. This result is peculiar compared to other studies which selected reference genes in a different type of macrophages [14–17]. In rat osteoblasts [14], the researcher compared 31 reference genes in bone marrow osteoblasts, calvarial osteoblasts, and UMR-106 osteoblasts evaluated byΔCt method, DataAssist™, NormFinder and BestKeeper methods. They revealed that Eif2b1was the most stable reference gene, and

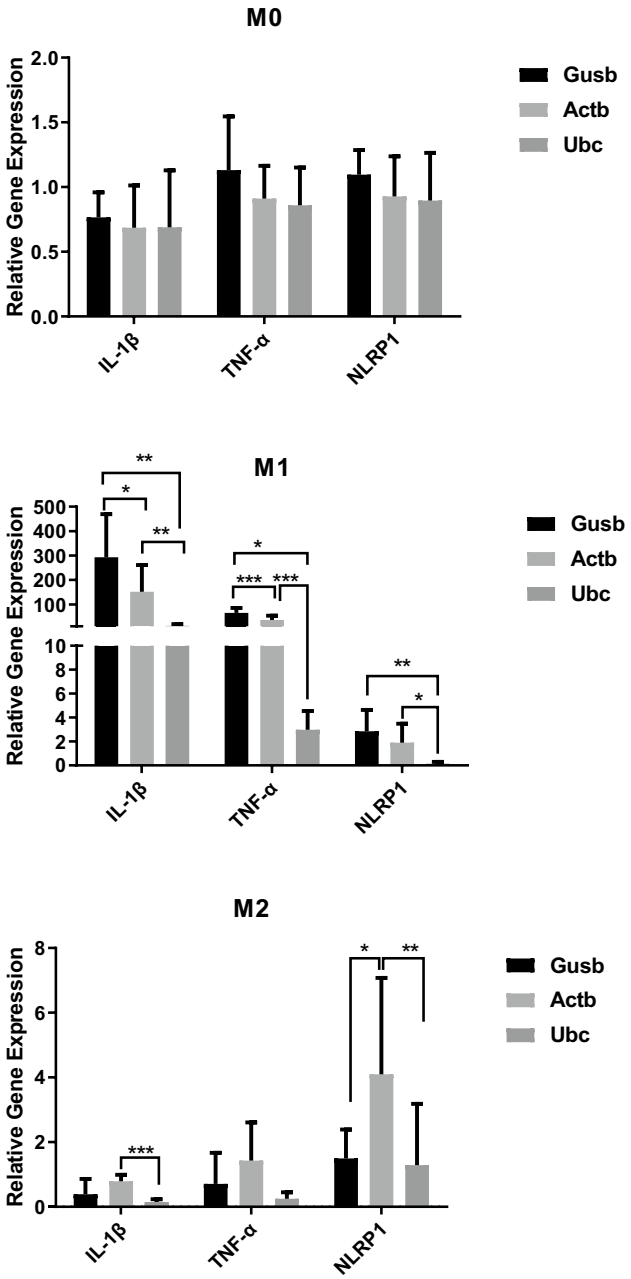

Fig. 4 Relative gene expression levels of IL-1β, TNF-αand NLRP-1gene using reference genes with different stability based on the $2^{-\Delta\Delta Ct}$ method. For normalization, the most (Ubc) and least consistently expressed reference genes (Gusb) and the traditionally used reference gene Actb which ranked in the midway were used for the calculation of ΔCt. Bars represent the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001

Rps17 was the least stable gene. In PMA-induced THP-1 macrophages [18], using GeNorm, NormFinder and BestKeeper, the author found that β-actin and RPL37A were the two most stable reference genes But in J774A1 macrophage cell line [16] indifferent culture condition, the most stability gene was β-actin and B2M. Alexandre S Stephens showed that the most stable gene in bone marrow macrophage were Hprt1/B2m by GeNorm which is similar to our result [17]. In our result we showed that in M0 cells the most stable gene was Hprt1/Ywhaz. However in LPS stimulated BMDMs, the Hnrnpab and Stx5a were better [15]. In LPS treated RAW 264.7 cells and peritoneal macrophages [15], the stability of reference genes were various due to the software selected, the cell type, the candidate reference gene selected and the culture conditions. And there was no comparable to our data. And all of the results suggested, the most stable RG is different in various macrophage cell types. The reasons were including peculiarities of cells, the analytical method, the candidate reference gene selected and different treatment conditions. These indicate that reference gene selection in macrophages of the different experimental conditions is necessary with over-ranking methods and enough candidate reference gene selected. This requires us to carry out routine internal reference gene screening on macrophages in different experimental conditions, to ensure the accuracy and reliability of qPCR results.

As far as we know, this is the first attempt to select and validate suitable RGs for RT-qPCR analysis in M0, M1 and M2 murine macrophages. Since distinct experimental settings in macrophages require individual validation of internal control genes and more and more methods were used for stable genes selection, we believe that another promising candidate reference gene stably expressed in macrophage and polarized macrophages may exist. Most importantly, reference genes selected from a large number of housekeeping genes by high-throughput technologies was needed and this alternative approach can be robust. Several studies had already shown that the NCBI GEO database is available for the discovery of novel reference genes [15, 40, 41] and RNA-seq has been more widely used for selecting stable reference genes from a large number of reference genes [43–45]. But reference genes selected by the above high-throughput technologies in M0, M1 and M2 murine macrophages were still needed to be validated.

Here we performed RT-qPCR using $2^{-\Delta\Delta Ct}$ methods to determine mRNA level of three target genes NLRP1, IL-1β and TNF-α based on three reference genes with different levels of stability. Interestingly, the gene expression levels in M1 and M2 were significantly different when RGs with different stability were used, which means that it is really necessary to select and validate suitable RGs before RT-qPCR analysis. But unfortunately, only a few researchers provided evidence for RG selection. Hence forth, our results will be very helpful in guiding investigators to select the appropriate RGs to accurately quantify mRNA in studies involving M0, M1 and M2 murine macrophages. Lastly, what we want to achieve more is to encourage researchers to ascertain RGs stability before gene expression analysis for every experimental setting.

Conclusion

Our study has clearly shown that Ubc followed by Eef1a1 were recognized as the two ideal RGs for mRNA level analysis in M0, M1, and M2 cells. Our results showed strong variation in fold changes of NLRP1, IL-1β, and TNF-α when reference genes with different stability were used in M1 and M2 cells. Our study indicates that it is necessary to carefully select RGs for gene expression normalization by RT-qPCR study.

Author contributions Conceptualization, AOS; Data curation, WJ and AOS; Formal analysis, KQ; Funding acquisition, WJ; Investigation, WJ, TS, WL, KQ and LZ; Methodology, TS, WL, YB, SYA and KQ; Project administration, WL; Resources, JQ and LZ; Software, TS, WL, YB and SYA; Supervision, LZ; Validation, WJ, KX, JQ and LZ; Visualization, JQ and LZ; Writing—original draft, WJ; Writing—review & editing, AOS, SYA, KX, JQ and LZ.

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Data availability The data and materials used to support the findings in this study are included in the article.

Compliance with ethical standards

Conflict of interest All authors have no dispute of interest to disclose.

Consent to publish All authors approved the submission of the manuscript.

Ethics approval and consent to participate All animal care and procedures were per the ethical standards, approved by Jiangsu Society for Animal Welfare, China (Acceptance number: XZMC20130226) and Science and Technology Department of Jiangsu Province, China (Acceptance number: SYXK(SU)-2015-0030 and SCXK(SU)-2015-0009).

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