Original Article

Quercetin Modulates Cytokine Expression and Inhibits TLR2 Expression and STAT3 Activation in Mouse Activated Inflammatory Macrophages

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Abstract

Background and objective: Our previous studies evidenced that quercetin (Q) could be ingested and metabolized by macrophages, and exerted both prophylactic immuno-stimulatory activity and therapeutic anti-inflammatory effects on lipopolysaccharide-treated macrophages *ex vivo*. To ascertain potential mechanism of anti-inflammatory action by Q, the present study evaluated changes of pro-/anti-inflammatory cytokines and components of inflammation-related intracellular signaling pathways in activated macrophages.

Methods: In this *ex vivo* study, BALB/c mice were first administered intraperitoneally injected with lipopolysaccharide for 12 h; then, mouse peritoneal macrophages were isolated and treated with Q for 3 h *in vitro*. Quercetin 3-glucuronide (a major metabolite of Q) and dexamethasone (a glucocorticoid) were selected to perform comparative analysis. Relative gene expression amounts of pro-/anti-inflammatory cytokines (*TNF-α*/*IL-10*) and components involved in inflammation-related intracellular signaling pathways in macrophages (*TLR2*, *TLR4*, *NF-κB*, *JAK2*, and *STAT3*) were measured using two-step reverse transcription and real-time quantitative polymerase chain reaction. STAT3 protein phosphorylation was determined using an in-cell enzyme-linked immunosorbent assay method.

Results: Q decreased *TNF-α* gene expression amounts and ratios of pro-/anti-inflammatory (*TNF-α*/*IL-10*) cytokine gene expressions but increased *IL-10* gene expression amounts in activated inflammatory macrophages, supporting a substantial anti-inflammatory potential of Q treatments. Importantly, Q inhibited *TLR2* gene expression and phosphorylation of STAT3 protein in the activated inflammatory macrophages.

Conclusions: Our results are the first to suggest that Q inhibits lipopolysaccharide-induced inflammation *ex vivo* through suppression of *TLR2* gene expression and STAT3 protein phosphorylation in activated inflammatory macrophages. Q has potential further application for treating inflammation-associated diseases.

Keywords: Activated inflammatory macrophages; Quercetin; Pro-/Anti-inflammatory cytokines; STAT3 phosphorylation; *TLR2*.

Abbreviations: ANOVA, one-way analysis of variance; DEX, dexamethasone; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; i.p., injected intraperitoneally; JAK, Janus kinase; LPS, lipopolysaccharide; NF, nuclear factor; Q, quercetin; Q3G, quercetin-3-O-β-D-glucuronide; PBS, phosphate-buffered saline; qPCR, quantitative polymerase chain reaction; RT, reverse transcription; STAT3, signal transducer and activator of transcription 3; TCM, tissue culture medium; TLR, toll-like receptor; TNF, tumor necrosis factor.

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Introduction

Quercetin (Q) is a potent bioflavonoid and widely found in health foods. It exhibits extensive physiological and pharmacological benefits, including anti-inflammatory, anti-proliferative, and anti-atherosclerotic effects in humans.[1–3] Recently, Q was found to have strong potential for decreasing lipopolysaccharide (LPS)-stimulated inflammation in murine peritoneal macrophages, in a therapeutic anti-inflammatory manner *ex vivo*.[4] We have evidenced that Q could be ingested and metabolized by murine peritoneal macrophages.[5] After assimilation in macrophages, Q is metabolized to quercetin-3-O-β-D-glucuronide (Q3G).[6] Both Q and Q3G may provide particular anti-oxidative protection in rat plasma.[6,7] However, the anti-inflammatory mechanism of Q in activated inflammatory macrophages remains unclear.

Macrophages are recognized as inflammatory cells in the innate immune system and function to trigger immune responses and inflammation by producing pro- (interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6) or anti-inflammatory (IL-10) cytokines.[8,9] Inflammation status *in vitro* or *in vivo* may be described according to pro-/anti-inflammatory cytokine secretion or expression profiles of activated cells, particularly macrophages. It has been suggested that activated macrophages might be a potential target for Q metabolites within injured/inflamed arteries.[10] Activated inflammatory macrophages have been investigated to determine the anti-inflammatory effects of active Q compounds.[4,11,12]

Recently, Q administration was found to reduce GP130, Janus kinase (JAK)1, and signal transducer and activator of transcription 3 (STAT3) activation via IL-6 in glioblastoma cells. This finding provided new insight into the role of Q as a blocker of the STAT3 activation pathway stimulated by IL-6.[13] The role of JAK-STAT signaling in the anti-proliferative effects of dietary flavonoids in prostate cancer cells has also been studied.[14] Q was found to actively accumulate in nuclear structures and trigger specific gene expression in epithelial cells by regulating mechanisms related to gene transcription.[15] Moreover, Q administration was found to dose-dependently inhibit TNF-α production and gene expression in peripheral blood mononuclear cells by modulating *nuclear factor* (*NF*)-*κβ1* and *Iκβ*.[16] Most recently, the anti-inflammatory effects of Q have been demonstrated in *in vitro* and *in vivo* studies[4,17]; however, the role of Q in inflammatory and intracellular signaling pathways remains unclear.

The aim of the present study was to unravel the role of Q in inflammatory and intracellular signaling pathways. To determine this mechanism, *in vivo* activated macrophages were isolated from the peritoneal cavity of mice injected intraperitoneally (i.p.) with LPS for 12 h and treated with Q for 3 h *in vitro*. Q3G, a major metabolite of Q, and dexamethasone (DEX), a glucocorticoid, were selected for comparison. Changes in the gene expression levels of pro-/anti-inflammatory cytokines, including *TNF-α* and *IL-10*, and components of inflammation-related intracellular signaling pathways, including *toll-like receptor* (*TLR*)2, *TLR4*, *NF-κB*, *JAK2* and *STAT3*, in the activated inflammatory macrophages were measured using reverse transcription (RT) and real-time quantitative polymerase chain reaction (qPCR). Phosphorylation of the STAT3 protein in the activated inflammatory macrophages was determined using an in-cell enzyme-linked immunosorbent assay (ELISA) method.

Materials and methods

Sample preparation

Q and Q3G (purity >98%) were purchased and prepared to be a stock solution for use as described previously.[17]

Experimental animals

BALB/c mice (females, 7 weeks-old) were furnished for experiments and fed a laboratory standard diet (Diet MF 18; Oriental Yeast Co., Ltd., Osaka, Japan).[17] The animal room maintained a 12-h light and 12-h dark cycle, with constant temperature (23 ± 2 °C) and relative humidity (50–75%). After acclimatization for 1 week, the experimental mice (8 weeks-old) were grouped for different experiments. The use of experimental mice in this study was performed according to the Guideline for the Care and Use of Laboratory Animals of the National Institutes of Health. The study was approved by the Institutional Animal Care and Use Committee (IACUC Approval No: 98-101) of National Chung Hsing University.

Isolation of mouse macrophages

In our previous study, a mouse systemic inflammation model was established using single injection i.p. with LPS at 8 mg/kg body weight through 12 h.[4] Based on the established animal model, activated inflammatory macrophages from the mouse peritoneal cavity were isolated for *in vitro* study. Experimental mice (8 weeks-old) were challenged with phosphate-buffered saline (PBS; pH 7.4, 0.22 μm filtered) consisting of NaCl at 137 mM, KCl at 2.7 mM, Na_2HPO_4 at 8.1 mM, and KH_2PO_4 at 1.5 mM or *Escherichia coli* LPS (O127:B8, L-3129; Sigma-Aldrich Co., St. Louis, MO, USA) at a dose of 8 mg/kg body weight dissolved in aliquots of 100 μL sterilized PBS. After PBS or LPS i.p. injection through 12 h, mice were performed to isolate normal or activated peritoneal macrophages.[12,18] The mice were anaesthetized with isoflurane (4900-1605; Panion & BF Biotech Inc., Taipei, Taiwan) using a vaporizer (CAS-01; Northern Vaporiser Limited, Cheshire, England, UK), bled by retro-orbital venous plexus puncture to collect blood, and then immediately sacrificed with CO_2 to isolate primary peritoneal macrophages. Peritoneal macrophages were isolated from mouse peritoneal cavity, as described previously.[17] The obtained normal (PBS-treated) and activated (LPS-treated) macrophages from individual corresponding mice were respectively modulated to a density of 2×10^6 cells/mL in tissue culture medium (TCM) and counted using a hemocytometer with the trypan blue dye exclusion method for following experiments.[19]

Determination of an optimal incubation time for activated inflammatory macrophages to express target cytokine genes

To determine the optimal incubation time for expressing target cytokine genes in activated inflammatory macrophages, isolated macrophages (2 mL/well) were cultured with TCM medium (2 mL/well) in 6-well plates to achieve a final cell density of 1×10^6 cells/mL. The plates were incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for 0, 3, 6 or 12 h. After incubation, the plate was centrifuged at 400×*g* for 10 m. The supernatant was discarded and the cell pellet was washed with 1 mL sterile PBS/well. The cell pellet in the wells was collected to extract total RNA to analyze the gene expression of pro-inflammatory cytokine (*TNF-α*) and anti-inflammatory cytokine (*IL-10*) using RT and real-time qPCR assay. Changes in pro-/anti-inflammatory cytokine gene expression profiles were selected as biomarkers for evaluating inflammation status in the activated inflammatory macrophages. Based on the target cytokine gene expression profiles, incubation of normal or activated macrophages with samples for 3 h *in vitro* was selected as an optimal incubation time for following studies.

Effect of Q and Q3G on gene expression of target cytokines and components of the intracellular inflammation-related signaling pathway

In our previous study, we found that either Q or Q3G treatments at 50 μM did not produce any cytotoxicity on mouse peritoneal macrophages *in vitro*.[17] Thus, Q and Q3G, at the indicated concentrations of 20 and 50 μM, were selected to treat isolated normal or activated macrophages to verify anti-inflammatory potential and

determine a possible mechanism of action. Isolated normal or activated macrophages (2 mL/well) were cultured with Q or Q3G (2 mL/well) at the indicated final concentrations of 0, 20 and 50 μM in 6-well plates. In addition, DEX at 1 μM was performed as a treatment control *in vitro* for comparison in the present study, because DEX, which is a glucocorticoid, was shown to effectively decrease LPS-stimulated inflammation at 0.1 to 10 μM in J774 macrophages.[20] The plates were incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for 3 h, and then centrifuged at 400×*g* for 10 m. The supernatant was discarded and the cell pellet washed with 1 mL sterile PBS. The cell pellet in the wells was used to extract total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The isolated RNA samples from the treated cells were stored at −80 °C for future RT and real-time qPCR assay. Changes in the gene expression levels of both pro-inflammatory cytokine (*TNF-α*) and anti-inflammatory cytokine (*IL-10*) were selected as indicators for evaluating anti-inflammatory potential of Q or Q3G. Changes in the gene expression levels of intracellular inflammation-related signaling pathway components, including *TLR2*, *TLR4*, *MyD88*, *TRIF*, *TRAF6*, *NF-κB*, *JAK2*, and *STAT3*, were measured to determine the possible anti-inflammatory mechanisms of Q or Q3G.

Targeted gene expression assays

Extraction of total RNA from treated cells

The extraction method was performed as described previously.[21,22] To evaluate the RNA quality, an aliquot of 2 μL of isolated RNA solution was pipetted into a clean tube and then diluted 50 times with 10 mM Trizma hydrochloride (Tris-HCl/DEPC; Sigma-Aldrich) buffer. The absorbance (A) at 260 and 280 nm was measured using a spectrophotometer (U2900 UV-vis spectrophotometer; Hitachi, Tokyo, Japan). The ratios of A260/A280 ranged from 1.5 to 2.0, which indicates high quality RNA and low protein concentration in the obtained RNA sample. To obtain a fixed quantity for assay, the RNA concentration in the solution was approximately calculated using the equation: 1 unit of A260 = 40 μg RNA/mL. Finally, the obtained high-quality RNA samples were stored at −80 °C for subsequent RT and real-time qPCR assay.

Synthesis of the first-strand cDNA using RT

An aliquot of total RNA (2 μg) isolated from the treated cells was pipetted into a clean tube. To avoid DNA contamination, DNA was removed from the RNA sample using a commercial kit of RQ1 RNase-Free DNase (Promega, Madison, WI, USA). The first-strand cDNA was synthesized from mRNA using a commercial kit of M-MLV Reverse Transcriptase (Promega) containing reaction buffer (Promega), 10 mM dNTP Mix (Promega), and Oligo dT (Invitrogen) in a total volume of 25 μL. The RT reaction was performed for one cycle in a PCR thermal cycler (Genesis 96; Pebio Scientific Company, Taipei, Taiwan) with the following program: 25 °C for 5 m, 42 °C for 60 m, and 70 °C for 15 m, followed by cooling to 4 °C. After the first single-strand cDNA was synthesized, it was diluted 10-fold (v/v) in nuclease-free water and stored at −80 °C until use.

Assay and data calculations of real-time qPCR

Briefly, 5 μL diluted cDNA (cDNA template) was pipetted into a reaction tube, which is used for each real-time PCR, containing a mixture consisting of 4 μL nuclease-free water, 10 μL Smart Quant Green Master Mix with dUTP low ROX (Protech), 0.5 μL target gene-specific forward PCR primer (10 μM), and 0.5 μL target gene-specific reverse PCR primer (10 μM) in a final volume of 20 μL. Primer sequences for detection of expression of mouse cytokines and inflammation-related component genes using qPCR assays were devised according to their corresponding cDNA sequences using online software, and are shown in Table 1. Reactions and the qPCR detection were carried out in a real-time rotary analyzer (Rotor-Gene 6000; Corbett Life Science, Sydney, Australia) with the following program: hot-start activation for 15 m at 95 °C, followed by 40–50 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 60 °C, and extension for 30 s at 72 °C. The Ct (threshold cycle number) value of the target gene expression was achieved from fluorescence intensity measured using the real-time rotary analyzer. Each biological determination was performed in triplicate. Relative expression levels of mRNA species were quantified using the comparative Ct method.[14] Mouse β-actin, a stably expressed "housekeeping" gene, was selected as a reference gene. The following pro-/anti-inflammatory cytokine genes and intracellular inflammation-related signaling genes expressed in mouse normal or activated peritoneal macrophages administered with either Q or Q3G were selected: pro-inflammatory cytokines *TNF-α* and *IL-6*; anti-inflammatory cytokine *IL-10*; and components of intracellular inflammation-related signaling, including *TLR2*, *TLR4*, *NF-κB*, *JAK2* and *STAT3*. Relative mRNA expression levels in differently-treated cells are presented as the fold-change value. The expression ratio (R) of individual mRNA level at treated versus control condition in the cells was calculated using the following equation: $R = 2^{-\Delta\Delta Ct}$.[23,24] The comparative Ct value, which is the threshold cycle number of the target mRNA, was calculated from the fluorescence intensity measured using the real-time rotary analyzer, and indicated that a lower Ct value corresponds to a higher mRNA expression level. The equations $\Delta Ct = Ct_{target\ gene} - Ct_{reference\ gene}$ and $\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{calibration}$ or $\Delta\Delta Ct = (Ct_{target\ gene} - Ct_{\beta\text{-actin gene}})_{time\ x} - (Ct_{target\ gene} - Ct_{\beta\text{-actin gene}})_{time\ 0}$ were used to calculate each target gene expression (*e.g.*, cytokines or inflammation-related signaling) with respect to its control situation.[25]

Determination of STAT3 phosphorylation at tyrosine 705 using in-cell ELISA method

Phosphorylation of STAT3 generates an active form of the transcription factor STAT3 protein. After phosphorylation in cytoplasm, the phosphorylated STAT3 protein is able to move into the cell nucleus to mediate targeted gene transcription. To evaluate changes of activated STAT3 transcription factor amounts in target cells, STAT3 phosphorylation at tyrosine 705 were measured using an in-cell ELISA method. STAT3 phosphorylation in normal or activated inflammatory macrophages treated without or with Q were further measured to clarify the role of STAT3 phosphorylation at tyrosine 705 in inflammation. Briefly, normal or activated inflammatory macrophages (50 μL/well) were cultured in the absence or presence of Q (50 μL/well) at the indicated final concentrations of 0, 20, and 50 μM in 96-well plates and incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for 3 h. After incubation, the plate was centrifuged at 25 °C, 400×*g* for 10 min to remove the supernatant. The cell pellet was collected to measure STAT3 phosphorylation at tyrosine 705 using a STAT3 Colorimetric In-Cell ELISA Kit (Pierce Biotechnology, Rockford, IL, USA). Data were calculated with the average A450 value for each experimental condition (*e.g.*, with and without treatment) for each target.

Table 1. Primer sequences for detection of expressions of mouse cytokines and inflammation-related component genes using real-time qPCR assays

Cytokine genes		Primer sequences[a]	Length[b], bp
TNF-α	FW	AGCCCCCAGTCTGTATCCTT	212
	RV	CTCCCTTTGCAGAACTCAGG	
IL-10	FW	CATGGGTCTTGGGAAGAGAA	194
	RV	CATTCCCAGAGGAATTGCAT	

Inflammation-related component genes	Primer sequences		Length, bp
TLR2	FW	TGCTTTCCTGCTGGAGATTT	197
	RV	TGTAACGCAACAGCTTCAGG	
TLR4	FW	GGCAGCAGGTGGAATTGTAT	198
	RV	AGGCCCCAGAGTTTTGTTCT	
NF-κB	FW	TTCCTGGCGAGAGAAGCAC	202
	RV	AAGCTATGGATACTGCGGTCT	
JAK2	FW	GTCCACCCGTGGAATTTATG	198
	RV	GAAGGGAAAGGTCCCTGAAG	
STAT3	FW	GAGGAGCTGCAGCAGAAAGT	190
	RV	TCGTGGT AAA CTG GACACCA	

Housekeeping gene	Primer sequences		Length, bp
β-actin	FW	GCTACAGCTTCACCACCACA	208
	RV	AAGGAAGGCTGGAAAAGAGC	

RV, reverse primer. [a]FW, forward primer; [b]Amplicon length in base pair.

For assessing STAT protein modification with treatment, the fold-change as a ratio of A450 values from the treated and non-treated modified protein were calculated.

Statistical analysis

Data are represented as mean ± standard error of the mean (n = 3–6 biological determinations). Results were analyzed with one-way analysis of variance (ANOVA), followed by post hoc tests, including Duncan's New Multiple Range test and unpaired Student's t-test. A p-value less than 0.05 was considered significantly different among treatments. Statistical analyses were assayed with SPSS version 12.0 (SPSS, Inc., Chicago, IL, USA).

Results

Determination of incubation time for activated inflammatory macrophages in vitro for mRNA expression assays

To determine the optimal incubation time of mouse primary activated inflammatory macrophages for mRNA expression assays, the target cytokine mRNA expression in the cells was analyzed. The results showed that relative expression of target cytokines in mouse primary activated inflammatory macrophages, including TNF-α and IL-10, changed in a time-dependent manner (Table 2). The relative expression level of TNF-α was significantly different at all incubation times ($p < 0.05$). The expression of the pro-inflammatory cytokine TNF-α was dominant at the early stage (*e.g.*, incubation for 3 h), while that of the anti-inflammatory cytokine IL-10 was dominant at the late stage (*e.g.*, incubation for 12 h), which indicates inhibition of the synthesis of pro-inflammatory cytokines during the inflammatory process. Thus, the highest fold-change in the ratio of pro-/anti-inflammatory cytokine gene expression (*TNF-α*/*IL-10*) in mouse primary activated inflammatory macrophages was 6.89 ± 1.88 at 3 h-incubation. Based on the most significant ($p < 0.05$) difference of cytokine gene expression profile (Table 2), the 3-h incubation time was selected as optimal incubation time for following studies.

Effect of Q or Q3G in vitro on the cytokine gene expression profile in normal and activated inflammatory macrophages

The results showed that treatments of normal macrophages (from mice treated i.p. with PBS for 12 h) with Q at 20 μM significantly ($p < 0.05$) increased mRNA expression amounts of both TNF-α and IL-10, but did not significantly ($p > 0.05$) change the ratio of pro-/anti-inflammatory (*TNF-α*/*IL-10*) cytokine gene expressions (Fig. 1). Our results suggest that Q administration *in vitro* at the indicated appropriate concentration of 20 μM might activate primary normal macrophages by increasing the mRNA expressions of both pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokines but overall slightly decreased inflam-

Table 2. Effects of different incubation time with TCM medium *in vitro* on cytokine gene expressions in activated inflammatory macrophages from female BALB/c mice intraperitoneally injected with lipopolysaccharide at 8 mg/kg BW through 12 h

Cytokine gene name	Incubation time in h			
	0	3	6	12
	Relative expression amount, fold			
TNF-α	1.00 ± 0.00^{B}	2.25 ± 0.04^{A}	1.96 ± 0.16^{A}	0.38 ± 0.08^{C}
IL-10	1.00 ± 0.00^{AB}	0.37 ± 0.09^{B}	0.57 ± 0.23^{AB}	1.22 ± 0.32^{A}
TNF-α/IL-10	1.00 ± 0.00^{B}	6.89 ± 1.88^{A}	4.48 ± 1.48^{AB}	0.69 ± 0.23^{B}

Values are mean ± standard error of mean (n = 3 biological determinations), analyzed using one-way analysis of variance, followed by Duncan's new multiple range test. Values within the same row not sharing a common superscript capital letter are significantly different ($p < 0.05$) from each other. TCM, tissue culture medium.

mation status by decreasing the ratio of pro-/anti-inflammatory (*TNF-α*/*IL-10*) cytokine gene expression. Importantly, treatment of activated inflammatory macrophages (from mice treated i.p. with LPS for 12 h) with Q at either 20 or 50 μM significantly decreased ($p < 0.05$) the mRNA expressions of TNF-α but obviously increased those of IL-10 (Fig. 1). Q administration at either 20 or 50 μM overall and significantly ($p < 0.05$) inhibited the ratio of pro-/anti-inflammatory (*TNF-α*/*IL-10*) cytokine gene expressions in activated inflammatory macrophages (Fig. 1). Our results evidence that Q exerts substantive anti-inflammatory effects on activated inflammatory macrophages by decreasing TNF-α mRNA expression amounts and ratios of pro-/anti-inflammatory (*TNF-α*/*IL-10*) cytokine gene expressions but increasing IL-10 mRNA expression amounts. Interestingly, we found that DEX treatment effects on normal and activated inflammatory macrophages *in vitro* were similar to those of Q, indicating that either DEX or Q treatments had a therapeutic effect against inflammation. Corticosteroid-like DEX is already used in clinical treatments for anti-inflammatory medications, even though it may cause adverse side effects. Q administration for inflammation treatment may be an alternative choice to replace or reduce the clinical use of DEX in the future.

After Q is assimilated by macrophages, it may be further metabolized into Q3G. To compare the anti-inflammatory potential of Q and Q3G, Q3G was also selected to treat normal and activated inflammatory macrophages for 3 h. The results showed that Q3G administration to normal macrophages *in vitro* significantly increased *TNF-α* but just slightly increased *IL-10* gene expressions (Fig. 2). Moreover, Q3G significantly ($p < 0.05$) increased the ratio of pro-/anti-inflammatory (*TNF-α*/*IL-10*) cytokine gene expressions, suggesting that Q3G overall slightly increased inflammation status in normal macrophages. Importantly, treatment of activated inflammatory macrophages (from mice treated i.p. with LPS for 12 h) with Q3G at either 20 or 50 μM significantly decreased ($p < 0.05$) the mRNA expressions of TNF-α but obviously increased those of IL-10 (Fig. 2). Q3G administration at either 20 or 50 μM overall and significantly ($p < 0.05$) inhibited the ratio of pro-/anti-inflammatory (*TNF-α*/*IL-10*) cytokine gene expressions in activated inflammatory macrophages (Fig. 2). Our results evidence that Q3G also exerts substantive anti-inflammatory effects on activated inflammatory macrophages, but not normal macrophages, by decreasing TNF-α mRNA expression amounts and ratios of pro-/anti-inflammatory (*TNF-α*/*IL-10*) cytokine gene expressions but increasing IL-10 mRNA expression amounts.

Our results suggest that DEX exerts therapeutic (curative) effects in activated inflammatory diseases by regulating cytokine secretion profiles in inflammatory cells. Similar to DEX administration effects, Q and Q3G *in vitro* administrations overall decreased inflammation status in activated inflammatory macrophages (Figs. 1 and 2). Obviously, Q had the better effect against inflammation than that of Q3G in both and normal and activated inflammatory macrophages. To more accurately describe the anti-inflammatory mechanism of Q, it was further applied to normal and activated inflammatory macrophages for analysis of inflammation-related intracellular signaling pathways.

Effect of Q administration in vitro on relative gene expression levels of components of inflammation-related intracellular signaling pathway in normal or activated inflammatory macrophages

Table 3 shows the *in vitro* effects of Q on relative gene expression amounts of components of inflammation-related intracellular signaling pathway, including *TLR2*, *TLR4*, *NF-κB*, *JAK2*, and *STAT3*, in normal or activated inflammatory macrophages. The results showed that Q administration more or less increased *TLR2*, *TLR4*, *NF-κB*, *JAK2*, and *STAT3* gene expression amounts compared to those of controls in normal macrophages (Table 3). In general, cultured primary macrophages that were isolated from the body may result in slight spontaneous inflammation due to the change of oxygen content in the environment. However, our results suggest that Q administration at 50 μM might inhibit spontaneous inflammation in normal macrophages via inhibition of the TLR2 signaling pathway. The physiological significance of increased *NF-κB* and *STAT3* gene expression amounts induced by Q might result from the immune-stimulatory property of Q and remains to be further studied. In addition, we found that *TLR2* and *NF-κB* gene expression amounts significantly ($p < 0.05$) increased, but *JAK2* and *STAT3* gene expression amounts significantly decreased in activated inflammatory macrophages as compared to those in normal macrophages (Table 3). Our results suggest that mice treated with LPS i.p. may develop systemic inflammation and activate macrophage inflammation through the TLR2-to-NF-κB intracellular signaling pathway in the activated inflammatory macrophages. However, LPS treatment i.p. for 12 h may inhibit *JAK2* and *STAT3* gene expressions in the activated inflammatory macrophages. Importantly, Q administration *in vitro* significantly ($p < 0.05$) rectified the inflammation injury in the activated inflammatory macrophages, via decreasing *TLR2* gene expression dose-dependently, and improved inflammation damage to activated inflammatory macrophages by increasing *JAK2* and *STAT3* gene expressions that were hindered in the activated inflammatory macrophages (Table 3). The physiological significance of increased *NF-κB* and *JAK2* gene expression in the activated inflammatory macrophages by Q administration remains to be further investigated.

Similar to the administration effects of Q, DEX (positive con-

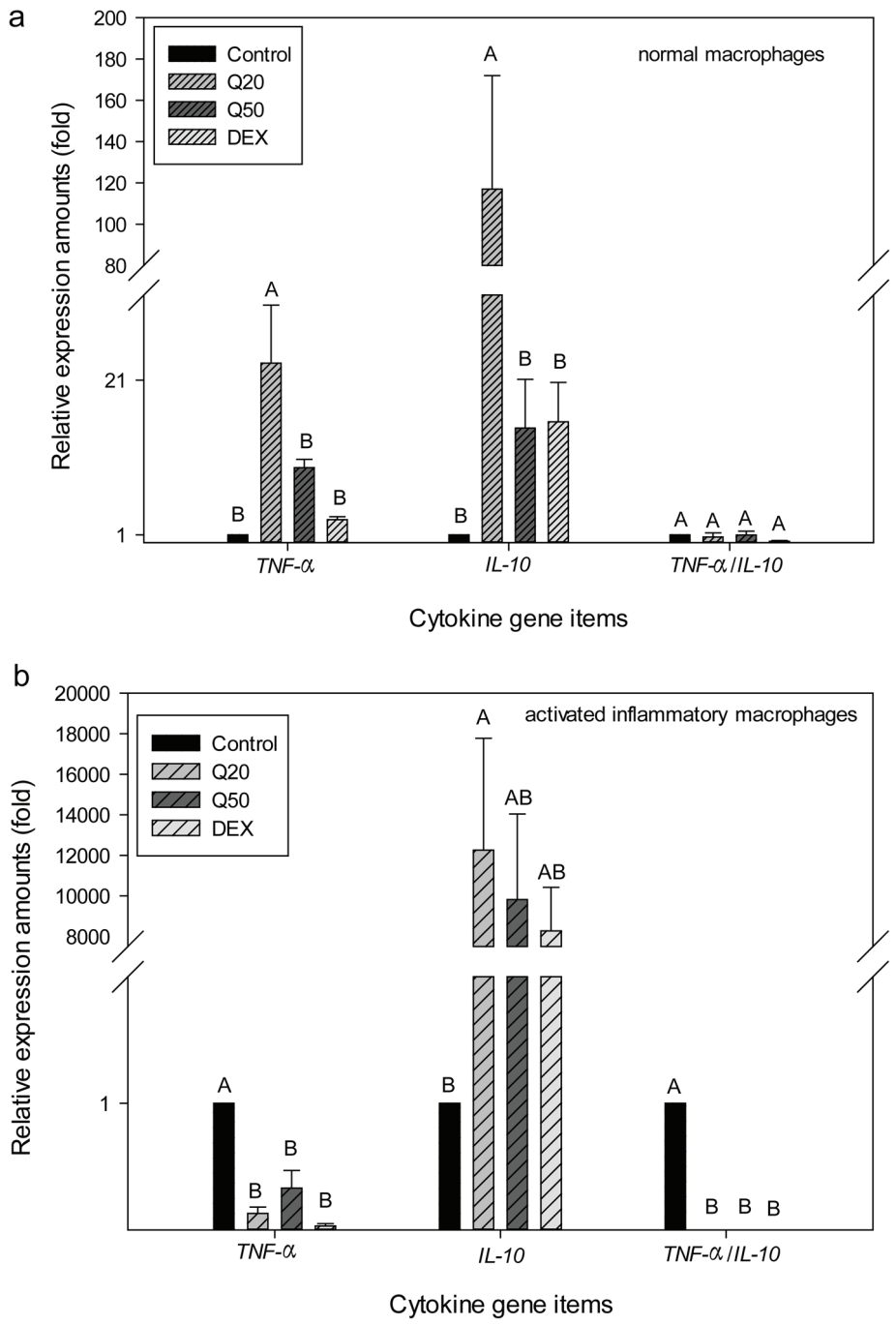

Fig. 1. Effects of quercetin (Q) administration on cytokine gene expression in normal (a) and activated inflammatory macrophages (b) from female BALB/c mice intraperitoneally injected with phosphate-buffered saline or lipopolysaccharide at 8 mg/kg BW through 12 h. Values are mean ± standard error of the mean (n = 4 biological determinations), analyzed using one-way analysis of variance, followed by Duncan's new multiple range test. Bars within same items not sharing a common capital letter are significantly different ($p < 0.05$) from each other. Q20, Q treatment at 20 μM; Q50, Q treatment at 50 μM; dexamethasone (DEX) treatment at 1 μM (a positive control).

trol) at 1 μM *in vitro* significantly improved the inflammation-induced injury in the activated inflammatory macrophages ($p < 0.05$), by decreasing *TLR2* gene expression. However, the gene expressions of *NF-κB* and *JAK2* in the activated inflammatory macrophages were significantly ($p < 0.05$) increased by DEX administration (Table 3). Moreover, our results showed that *STAT3* gene expression in both normal and activated inflammatory macrophages were significantly increased by Q administration at appropriate concentrations *in vitro*, as compared to those of the controls ($p < 0.05$). Undoubtedly, *STAT3* gene expression and activation influenced by Q plays an important role in inflammation. Thus, the possible mechanism of STAT3 activation through phosphorylation

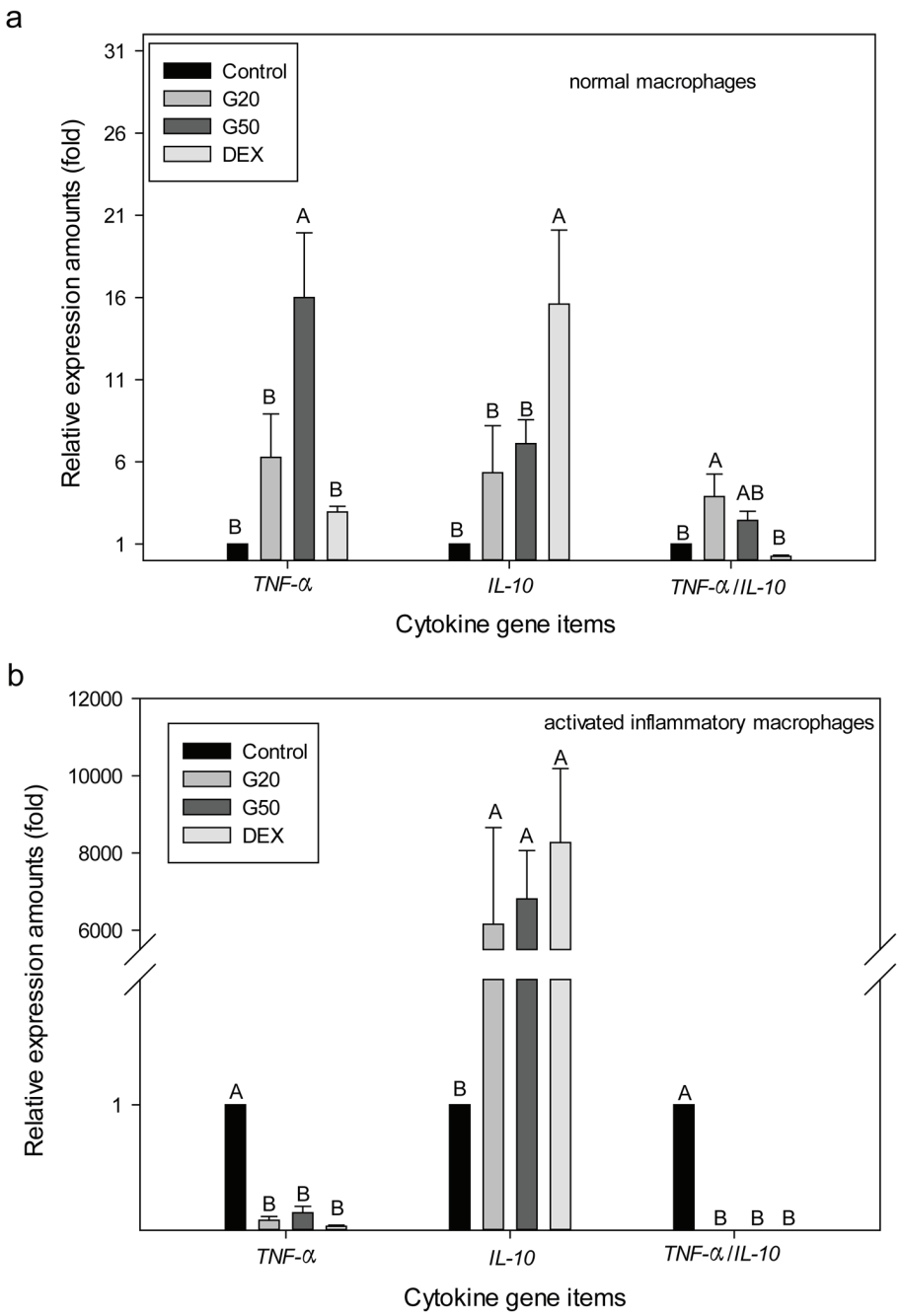

Fig. 2. Effects of quercetin-3-glucuronide (Q3G) administration on cytokine gene expression in normal (a) and activated inflammatory macrophages (b) from female BALB/c mice intraperitoneally injected with phosphate-buffered saline or lipopolysaccharide at 8 mg/kg BW through 12 h. Values are mean ± standard error of the mean (n = 5 biological determinations), analyzed using one-way analysis of variance, followed by Duncan's new multiple range test. Bars within same items not sharing a common capital letter are significantly different (p < 0.05) from each other. G20, Q3G treatment at 20 μM; G50, Q3G treatment at 50 μM; dexamethasone (DEX) treatment at 1 μM (a positive control).

of STAT3 protein at tyrosine 705 was further measured.

Effect of in vitro Q administration on phosphorylation of STAT3 at tyrosine 705 in normal or activated inflammatory macrophages

Phosphorylated STAT3 protein is an active form of this transcription factor. To clarify whether Q administration activated JAK-STAT3 signaling through phosphorylation of STAT3 protein in normal or activated inflammatory macrophages, levels of STAT3 phosphorylation at tyrosine 705 were measured using an in-cell ELISA method. Figure 3 shows Q *in vitro* administration effects on STAT3 phosphorylation at tyrosine 705 in normal or activated inflammatory macrophages. The results showed that STAT3 pro-

Table 3. Effects of quercetin administrations on relative gene expression folds of components in the inflammation-related signaling pathway in normal and activated inflammatory macrophages from female BALB/c mice intraperitoneally injected with phosphate-buffered saline or lipopolysaccharide at 8 mg/kg BW through 12 h

Gene	Macrophages	Quercetin, μM			DEX, 1 μM
		0	20	50	
		Relative expression amount, fold			
TLR2	normal	1.00 ± 0.00^{A}	1.20 ± 0.10^{A}	0.18 ± 0.07^{C}	0.60 ± 0.04^{B}
	inflammatory	2.76 ± 0.31^{A*}	0.83 ± 0.15^{B}	0.49 ± 0.24^{B}	0.18 ± 0.06^{B}
TLR4	normal	1.00 ± 0.00	11.8 ± 8.29	14.1 ± 12.1	1.42 ± 0.16
	inflammatory	0.78 ± 0.22^{C}	3.11 ± 0.63^{B}	6.95 ± 0.66^{A}	0.33 ± 0.03^{C}
NF-κB	normal	1.00 ± 0.00^{AB}	2.60 ± 1.75^{A}	2.39 ± 0.39^{A}	0.60 ± 0.17^{B}
	inflammatory	2.19 ± 1.20^{B*}	3.90 ± 1.65^{AB}	3.90 ± 1.65^{AB}	6.68 ± 2.83^{A}
JAK2	normal	1.00 ± 0.00	1.61 ± 0.71	1.60 ± 0.84	0.54 ± 0.22
	inflammatory	0.54 ± 0.11^{B*}	1.01 ± 0.23^{AB}	1.74 ± 0.33^{A}	1.74 ± 0.35^{A}
STAT3	normal	1.00 ± 0.00^{B}	6.00 ± 1.50^{A}	1.81 ± 0.86^{B}	0.67 ± 0.17^{B}
	inflammatory	0.59 ± 0.17^{B*}	1.17 ± 0.21^{A}	1.14 ± 0.19^{A}	0.40 ± 0.12^{B}

Values are mean ± standard error of the mean (n = 4 biological determinations), analyzed using one-way analysis of variance, followed by Duncan's new multiple range test. Values within the same row not sharing a common superscript capital letter are significantly different ($p < 0.05$) from each other. Asterisk (*) within same gene item means significantly different ($p < 0.05$) between normal and activated inflammatory macrophages in the absence of quercetin, analyzed using one-way analysis of variance, followed by unpaired Student's t-test.

tein phosphorylation at tyrosine 705 in activated inflammatory macrophages significantly ($p < 0.05$) increased compared to that of normal control (Fig. 3), indicating that LPS administration i.p. induced STAT3 phosphorylation in the activated inflammatory macrophages. Most importantly, Q *in vitro* administration at 20 μM significantly ($p < 0.05$) inhibited STAT3 phosphorylation at tyrosine 705 in the activated inflammatory macrophages but did not significantly ($p > 0.05$) influence normal macrophages. Our results suggest that Q administration might inhibit inflammation status in the activated inflammatory macrophages by inhibiting the signaling pathway involved in phosphorylation of STAT3 at tyrosine 705. However, DEX treatment *in vitro* did not significantly ($p > 0.05$) change phosphorylation levels of STAT3 protein at tyrosine 705 in either normal or activated inflammatory macrophages ($p > 0.05$).

Discussion

The present study indicates that Q administration inhibited the inflammation status in activated inflammatory macrophages via regulation of cytokine gene expression. This effect is mediated by decreased gene expressions of pro-inflammatory cytokine *TNF-α* but increased anti-inflammatory cytokine *IL-10*. We further determined the effects of Q administration *in vitro* on components of inflammation-related signaling pathway (*TLR2* and *TLR4*) in activated inflammatory macrophages. Q administration *in vitro* ameliorated the inflammation-induced injury in the activated inflammatory macrophages by decreasing *TLR2* gene expression in a dose-dependent manner. It was found that purified active lotus plumule (*Nelumbo nucifera* Gaertn) polysaccharides inhibited inflammation in mouse primary splenocytes by decreasing *TLR2* and *TLR4* gene expression.[26] Our results are identical to the published literature.[26] Contrary to our prediction, Q treatment increased expression amounts of *JAK2* and *STAT3* genes, which had been suppressed in the activated inflammatory macrophages. Although *STAT3* gene expression increased with Q administration, STAT3 phosphorylation at tyrosine 705 in activated inflammatory macrophages (which was increased by LPS treatment i.p.) was inhibited. Both NF-κB and STAT3 are transcription factors in cells. We infer that increased *NF-κB* expression might inhibit *STAT3* expression in the activated inflammatory macrophages in the absence of Q. Interestingly, Q administration seemed to simultaneously increase both *NF-κB* and *STAT3* gene expression in normal and activated inflammatory macrophages. The relationship between *STAT3* and *NF-κB* gene expression influenced by Q remains to be further clarified.

STAT3 is a molecular hub concerning immunosuppression, and STAT3 signaling pathways within malignant cells are generally over-activated.[27] IL-6 that is one of cancer-related inflammatory cytokines that activates STAT3 in glioblastoma cells; Q potentially serves as a blocker of the STAT3 activation pathway stimulated by IL-6.[13] In addition, Q decreases pro-inflammatory TNF-α production in peripheral blood mononuclear cells by regulating *NF-κβ1* and *Iκβ*.[16] Most importantly, we found that Q administration significantly increased *IL-10* gene expression amount. IL-10 is a potent anti-inflammatory cytokine; known as a cytokine synthesis inhibitor, it is produced in the late stage of inflammation to inhibit other pro-inflammatory cytokines, such as IL-6 and TNF-α. Our results suggest that Q administration might inhibit pro-inflammatory IL-6 or TNF-α through increasing IL-10, and consequently inhibiting IL-6- or TNF-α-induced STAT3 signaling pathway in activated inflammatory macrophages via decreasing STAT3 protein phosphorylation in the cells. However, more data should be accumulated in order to clarify the effect of Q on STAT3 signaling. Based on the present study, proposed effects of Q on the cell signaling in inflamed macrophages are illuminated in Figure 4.

Daily oral supplementation with Q (50- and 150-mg dosages, respectively) in volunteers led to this drug being recognized as safe; the pharmacokinetic areas under the plasma concentration-time curves ranged from 76.1 μmol·min·L^{-1} to 305.8 μmol·min·L^{-1}.[28] It was also found in another study that Q or its metabolites could

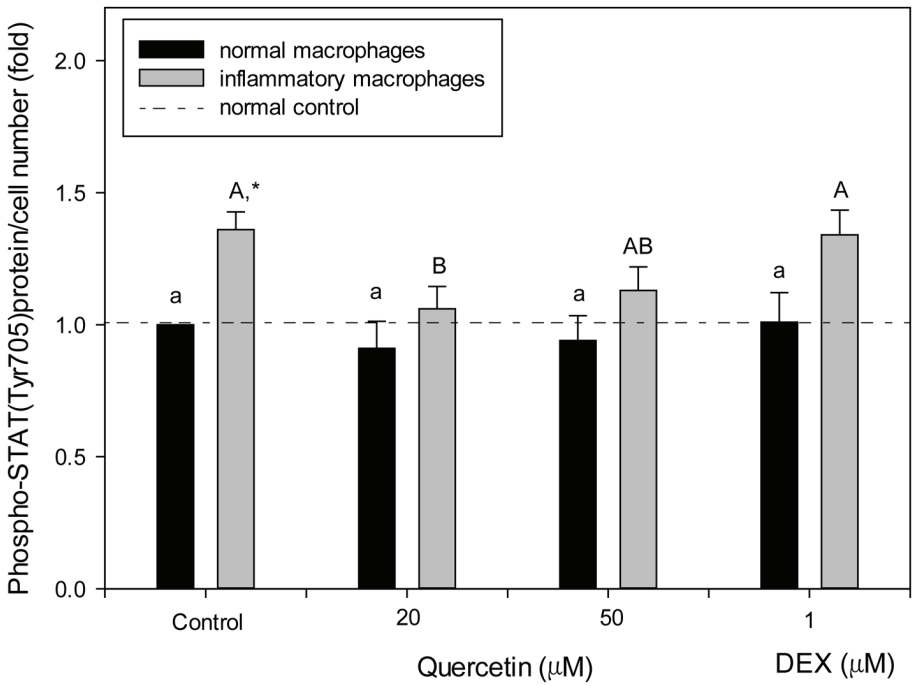

Fig. 3. Effect of quercetin (Q) administration on phospho-STAT3 (Tyr705) protein levels in normal and activated inflammatory macrophages from female BALB/c mice. Values are mean ± standard error of the means (n = 5 biological determinations) analyzed using one-way analysis of variance, followed by Duncan's new multiple range test. Bar under the same condition not sharing a common letter are significantly different ($p < 0.05$) from each other. Asterisk (*) means significantly different ($p < 0.05$) between normal and inflammatory cells in the absence of sample, analyzed using unpaired Student's t-test. Dexamethasone (DEX) at 1 μM (a positive control).

enter macrophages to exert their anti-inflammatory functions.[6] Furthermore, Q and its major quercetin metabolite Q3G have diverse physiological effects, including antioxidant and anti-inflammatory effects in different tissues.[29–31] In the present study, our results further suggest that Q and Q3G treatments *in vitro* might have an immunostimulatory effect on normal macrophages but inhibit

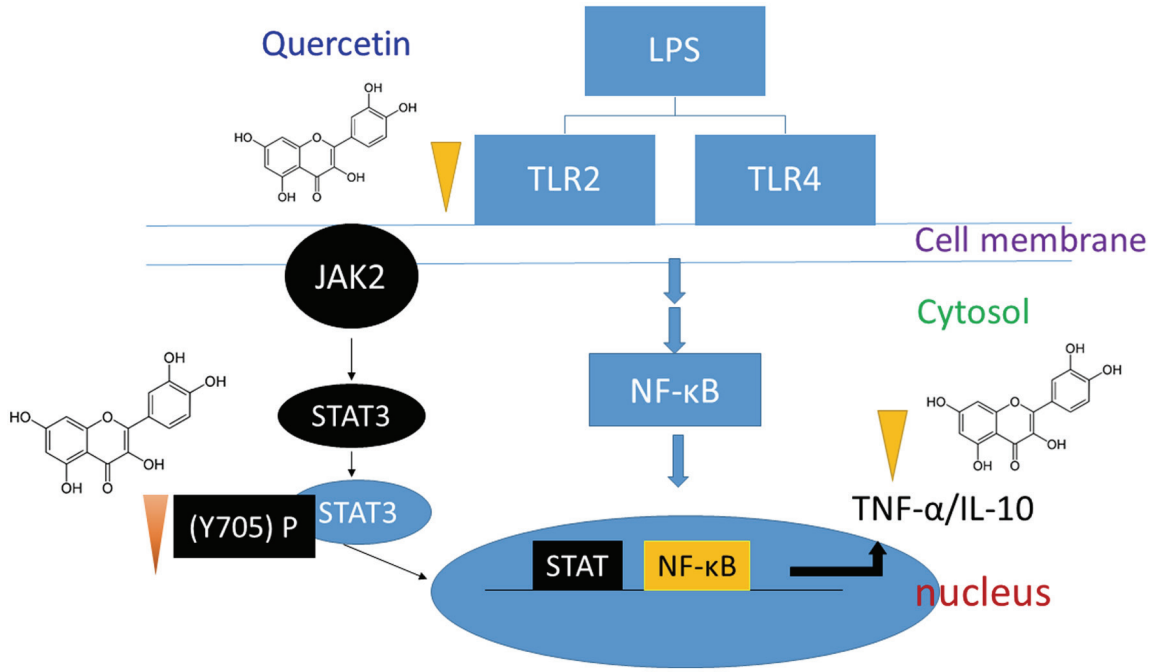

Fig. 4. Proposed effects of quercetin on the cell signaling in inflamed macrophages.

inflammation status in activated inflammatory macrophages by regulating cytokine gene expressions. However, Q3G was found to have a lower anti-inflammatory effect on normal macrophages than Q in this experimental model. We hypothesize that the glycoside moiety in Q3G improves its water solubility but decreases its uptake by macrophages.[32]

Interestingly, we found that DEX treatment effects on normal and activated inflammatory macrophages *in vitro* were more or less similar to those of Q, indicating that either DEX or Q treatments had a therapeutic effect against inflammation. Corticosteroid-like DEX is already used in clinical treatments for anti-inflammatory medications, even though it may cause adverse side effects. Q administration for inflammation treatment may be an alternative choice to replace or reduce the clinical use of DEX in the future.

Some achievements have been obtained in the present study, and Q may be further applied for anti-inflammatory clinical use, including tumor therapy.[33–37] However, there are limitations in the present study. First, this was an *ex vivo* study; therefore, confirmation of the key findings *in vivo* using a peritoneal challenge model should be performed in the future. The findings with murine cells may not be recapitulated in human cells. Unfortunately, changes of TLR and NF-κB protein levels in the cells were not determined, so that the findings' impact at the protein level could not be confirmed. It remains unclear why Q increased the gene expression of *STAT3* but inhibited its phosphorylation. However, our results provide the first indication that Q inhibits LPS-induced inflammation *ex vivo* through suppressing *TLR2* gene expression and STAT3 phosphorylation in activated inflammatory macrophages.

Future directions

Quercetin (Q) may act as a natural anti-inflammatory agent to inhibit LPS-induced inflammation via suppressing *TLR2* gene expression and STAT3 phosphorylation in activated inflammatory macrophages *ex vivo* (Fig. 4). Q has a beneficial and vital role in modulating chronic inflammation-related diseases. The daily supplementation of Q into the body of humans may greatly decrease the inflammation status *in vivo* via inhibition of inflammatory signaling pathways in macrophages. As the population starts to daily consume an appropriate dosage of Q, then it is postulated that the incidence of inflammation-related diseases will be markedly decreased. Since modulating the spontaneous or mild LPS-induced inflammation would be an important therapeutic target for improving inflammation-derived degenerative diseases, Q can be recommended as dietary and drug interventions for reducing inflammation-derived diseases. Future studies should focus on the intervention dosage and route of Q that will indeed alleviate inflammation status *in vivo*.

Conclusions

This study evidenced that Q and its metabolite Q3G decreased *TNF-α* gene expression amounts and ratios of pro-/anti-inflammatory (*TNF-α*/*IL-10*) cytokine gene expressions but increased *IL-10* gene expression amounts in activated inflammatory macrophages. However, Q3G has similar, but lower, effects on activated inflammatory macrophages. Importantly, Q inhibited *TLR2* gene expression and phosphorylation of STAT3 protein in the activated inflammatory macrophages. The present study supports that Q can modulate cytokine expression and inhibit *TLR2* expression and STAT3 activation in mouse activated inflammatory macrophages. Q has potential to further apply for treating inflammation-associated diseases.

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Data sharing statement

No additional data are available.

Conflict of interest

The authors declare having no conflicts of interest.

Author contributions

Conceptualization, methodology, validation, writing-reviewing and editing, funding acquisition (JYL); data curation, formal analysis, investigation, writing of the original draft (YRL).

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