Peer-reviewed version available at *Journal of Exploratory Research in Pharmacology* **2020**, *0*, 1-11; doi:10.14218/JERP.2020.00006

Quercetin exerts anti-inflammatory effects via meanwhile suppressing *TLR2* gene expression and STAT3 protein phosphorylation in activated inflammatory macrophages

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Running title: Anti-inflammatory signaling of Q in macrophages

Peer-reviewed version available at Journal of Exploratory Research in Pharmacology 2020, 0, 1-11; doi:10.14218/JERP.2020.00006

ABSTRACT

Our previous studies demonstrated that quercetin (Q) could be ingested and metabolized by macrophages and exerted prophylactic immuno-stimulatory activity and therapeutic anti-inflammatory effects on lipopolysaccharide (LPS)-treated macrophages *ex vivo*. To further clarify its possible anti-inflammatory mechanism, Q was selected to treat mouse peritoneal macrophages that obtained from female BALB/c mice exposed to LPS i.p. for 12 h. Relative gene expression of pro-/anti-inflammatory (*TNF-α*/*IL-10*) cytokines and components of inflammation-related intracellular signaling pathways (*TLR2, TLR4, NF-κB*, *JAK2* and *STAT3*) was analyzed using two-step reverse transcription (RT) and real-time quantitative polymerase chain reaction (qPCR). STAT3 protein phosphorylation was determined using an in-cell ELISA method. As a result, Q and its metabolite quercetin-3-O-β-D-glucuronide (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, supporting a substantial anti-inflammatory potential of Q and Q3G treatments. However, Q3G had lower effects than those of Q. Importantly, Q inhibited *TLR2* gene expression and phosphorylation of STAT3 protein in the inflamed cells. Our results are the first report to suggest that Q inhibits LPS-induced inflammation *ex vivo* through suppressing *TLR2* gene expression and STAT3 protein phosphorylation in activated inflammatory macrophages. Q has potential to further apply for treating inflammation-associated diseases.

Keywords: activated inflammatory macrophages; quercetin; pro-/anti-inflammatory cytokine genes; STAT3 protein phosphorylation; *TLR2*

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1. Introduction

Quercetin (Q) is a potent bioflavonoid and widely found in foods. Q exhibits extensive physiological and pharmacological benefits, including anti-inflammatory, anti-proliferative, and anti-atherosclerotic effects in humans [1-3]. Among immune cells, murine peritoneal macrophages are proven to ingest and metabolize Q *in vitro* [4]. After assimilation in macrophages, Q is metabolized to quercetin-3-O-β-D-glucuronide (Q3G) that may further serve as an antioxidant metabolite in plasma [5,6]. Activated inflammatory macrophages might be a potential target for Q metabolites within injured/inflamed arteries [7]. Moreover, we found that treatments with Q inhibited lipopolysaccharide (LPS)-induced inflammation in mouse peritoneal macrophages *ex vivo* [8]. Most recently, oral supplementation with Q and galangin, alone or in combination, are suggested to be promising therapeutic agents for atopic dermatitis using a 2,4-dinitrochlorobenzene-induced mouse model [9].

Macrophages are innate cells throughout the body and function to trigger immune responses and inflammation by mainly producing pro-inflammatory cytokine tumor necrosis factor (TNF)-α and anti-inflammatory cytokine interleukin (IL)-10 [10,11]. Pro-/anti-inflammatory cytokine expression profiles by activated inflammatory cells, particularly macrophages, may reflect inflammation status in the cells. Activated inflammatory macrophages have been used to investigate anti-inflammatory effects of active Q compounds [8,12,13].

Inflammation is a complicated biological response that can be triggered by extracelluar or/and intracellular factors such as lipopolysaccharides (LPS), pro-inflammatory cytokines, and growth factors etc. [14]. Many adaptor proteins and transcription factors in inflammatory cells continue inflammatory signal transductions [14]. One

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of particular transcription factors, signal transducer and activator of transcription 3 (STAT3), is a member of the STAT protein family in response to LPS, cytokines, growth factors etc. [14]. After stimulated by a ligand through its specific receptor (e.g. LPS versus Toll like receptors (TLRs)), STAT3 located in cytoplasm is phosphorylated by an adaptor protein, receptor-associated Janus kinases (JAK), and forms homo- or heterodimers [14]. Activated STAT3 finally translocate to the compartment of the cell nucleus where they serve as transcription activators and mediate a variety of gene expressions in response to different cell stimuli [15]. Particularly, STAT3 is activated until tyrosine 705 is phosphorylated in response to ligands such as interferons, epidermal growth factor, IL-5, IL-6 etc. [14]. In contrast to tyrosine 705 phosphorylation, STAT3 may also be activated via serine 727 phosphorylation by mitogen-activated protein kinases [16]. Undoubtedly, STAT3 plays an essential role in cellular processes in response to inflammation, cell growth and apoptosis [15]. Targeted inhibition of JAK-STAT pathways, particularly STAT3, has been a potential treatment strategy for obesity [17], atherosclerosis [18], prostate cancer, breast cancer and hepatoma [19].

Recently, Q administration was found to reduce GP130, JAK1, and STAT3 activation via IL-6 in glioblastoma cells, providing new insight into the role of Q as a blocker of the STAT3 activation pathway stimulated by IL-6 [20]. The role of JAK-STAT signaling in the anti-proliferative effects of dietary flavonoids in prostate cancer cells has been found [21]. Q was found to actively accumulated in nuclear structures and trigger specific gene expression in epithelial cells [22]. In addition, Q dose-dependently inhibited TNF-α production and gene expression in peripheral blood mononuclear cells by modulating *nuclear factor (NF)-κβ1* and *Iκβ* [23]. Despite of anti-inflammatory effects of Q have been demonstrated in both *in vitro* and *in vivo*

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studies [8,24], its anti-inflammatory mechanism via intracellular signaling pathways in activated inflammatory macrophages were not fully understood.

To unravel the possible anti-inflammatory mechanism of Q, activated inflammatory macrophages isolated from the peritoneal cavity of mice injected intraperitoneally (i.p.) with LPS for 12 h were treated with Q for 3 h *in vitro*. For comparison with the effect of Q, Q3G, a major metabolite of Q, was also selected to perform. Changes in gene expression amounts of pro-/anti-inflammatory cytokines, such as *TNF-α*/*IL-10*, and components of inflammation-related intracellular signaling pathways, including *TLR2*, *TLR4*, *NF-κB*, *JAK2* and *STAT3*, in the activated inflammatory macrophages were measured using two-step 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 immuno-sorbent assay (ELISA) method.

2. Materials and methods

2.1. Sample preparation

Quercetin (Q) (Sigma-Aldrich Co., Steinheim, Switzerland) and its metabolite quercetin-3-O-β-D-glucuronide (Q3G) (Carbosynth Limited, Berkshire, UK) were purchased at the highest available purity (>98%, HPLC) and prepared as described previously [24].

2.2. Experimental animals

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Female BALB/cByJNarl mice (7-week-old) were provided by the National Laboratory Animal Center, National Applied Research Laboratories, Ministry of Science and Technology in Taipei, Taiwan, ROC, and maintained in the Department of Food Science and Biotechnology at National Chung Hsing University, Taiwan, ROC. The mice were housed in an animal room with a 12-h-light and 12-h-dark cycle, constant temperature (23 ± 2 ℃) and relative humidity (50-75%). The experimental mice were fed a laboratory standard diet (Diet MF 18, Oriental Yeast Co., Ltd., Osaka, Japan) and free access to water ad libitum. After acclimatization for 1 week, the experimental mice (8 weeks old) were randomly divided into two groups, including normal mice and LPS-treated mice. The animal experiments used in the present study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), National Chung Hsing University, Taiwan, ROC (IACUC Approval No: 98-101).

2.3. Isolation of normal and activated inflammatory macrophages from experimental mice

A mild mouse systemic inflammation model using an intraperitoneal injection of LPS at a concentration of 8 mg/kg body weight (BW) for 12 h was established in our laboratory [8]. To isolate normal or activated inflammatory macrophages, experimental mice (8 weeks old) were challenged with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4, 0.22 μm filtered) or *Escherichia coli* LPS (O127:B8, Sigma-Aldrich Co., L-3129, St. Louis, MO) at a dose of 8 mg/kg BW in a volume of 100 μl of LPS dissolved in sterilized PBS using aliquots from a single lot of PBS or LPS. After PBS or LPS injection for 12 h, the experimental mice sacrificed to isolate normal or activated inflammatory

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macrophages, respectively [13,25]. The mice were anaesthetized with isoflurane (cat. no., 4900-1605, Panion & BF Biotech Inc., Taipei, Taiwan) using a vaporizer (CAS-01, Northern Vaporiser Limited, Cheshire, England, UK) and bled by retro-orbital venous plexus puncture to collect blood. Then, the mice immediately sacrificed with CO_2 to isolate primary peritoneal macrophages. Each mouse peritoneal cavity was lavaged through peritoneum with 2 aliquots of 5 ml sterile Hank's balanced salts solution (HBSS). The lavage fluid was collected and centrifuged at 4 ℃, 400 ×*g* for 10 min to harvest cell pellets. The cell pellets that are peritoneal macrophages (>90%) were collected and re-suspended in tissue culture medium (TCM, a serum replacement; Celox Laboratories Inc., Lake Zurich, IL). The peritoneal macrophages isolated from each animal were adjusted to a density of 2×10^6 cells/ml in TCM medium with a hemocytometer using the trypan blue dye exclusion method for following experiments.

2.4. 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 activated inflammatory 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 ℃ in a humidified incubator with 5% CO_2 and 95% air for 0, 3, 6, or 12 h, respectively. Throughout the incubation, the plate was then centrifuged at 25℃, 400 × g for 10 min. The supernatant was discarded and the cell pellet 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 (T*NF-α*) and anti-inflammatory cytokine (*IL-10*) using

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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 inflammatory macrophages with samples for 3 h *in vitro* was selected as an optimal incubation time for the following studies.

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

In our previous study, Q or Q3G treatments lower than 50 μM could not result in any cytotoxicity on mouse peritoneal macrophages *in vitro* [24]. Therefore, Q or Q3G at 20 and 50 μM were selected to treat isolated normal or activated inflammatory macrophages to re-verify anti-inflammatory potential and further determine a possible anti-inflammatory mechanism. Isolated normal or activated inflammatory 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. Since glucocorticoid dexamethasone (Dex) at 0.1 to 10 μM have been found to inhibit LPS-induced inflammation in J774 macrophages *in vitro* [26], we chosen Dex at 1 μM as a positive control for comparison. The plates were incubated at 37 ℃ in a humidified incubator with 5% CO_2 and 95% air for 3 h, and then centrifuged at 25℃, 400 × g for 10 min. The supernatant was discarded and the cell pellet washed with 4 ml sterile PBS. The cell pellet in the wells was used to extract total RNA using TRIzol reagent (Invitrogen, CA, USA). The isolated RNA samples from the treated cells were stored at -80℃ for future RT and real-time qPCR assay. Changes in the gene expression amounts of pro-

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inflammatory cytokine (*TNF-α*) and anti-inflammatory cytokine (*IL-10*) were selected as indicators for evaluating the anti-inflammatory potential of Q or Q3G. Changes in the gene expression amounts of intracellular inflammation-related signaling components, including *TLR2* and *STAT3*, were measured to determine possible anti-inflammatory mechanisms of Q or Q3G.

2.6. Targeted gene expression assays

2.6.1. Extraction and quality evaluation of total RNA from treated cells

The extraction method was performed as described previously [27,28]. To evaluate the quality of extracted total RNA, an aliquot of 2 μl of RNA solution was pipetted into a clean tube and then diluted 50 times with 10 mM Trizma hydrochloride (Tris-HCl/DEPC, Sigma, MO, USA) buffer. Using a spectrophotometer (Hitachi-U2900 UV-vis spectrophotometer, Tokyo, Japan), both absorbance (A) at 260 and 280 nm of each individual extracted RNA sample were measured. Based on the ratio of A260/A280, values ranged from 1.5 to 2.0, indicating a high quality RNA and low protein concentration in the extracted RNA sample. To load a fixed quantity for RNA assay, the RNA concentration in the sample solution was roughly calculated using the equation: 1 unit of A260 = 40 μg RNA/ml. At last, the extracted RNA samples were stored at -80°C for subsequent two-step RT and real-time qPCR assay.

2.6.2. Synthesis of the first-strand cDNA using RT

An aliquot of 2 μg of total RNA isolated from the treated cells was pipetted into a clean tube. To prevent DNA contamination, DNA in the RNA sample was digested using a commercial kit of RQ1 RNase-Free DNase (Promega, Madison, WI, USA). Then,

the first strand cDNA was produced from mRNA using a commercial kit of M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) which contains reaction buffer (Promega, Madison, WI, USA), dNTP Mix 10 mM (Promega, Madison, WI, USA), and Oligo dT (Invitrogen, CA, USA) in a total volume of 25 µl. The reaction of reverse transcription was performed for one cycle with the following program using a PCR thermal cycler machine (Genesis 96; Pebio Scientific Company, Taipei, Taiwan): 25°C for 5 min, 42°C for 60 min, 70 °C for 15 min, and followed by cooling to 4°C. After the first cDNA was completely synthesized, the single strand cDNA sample was diluted 10-fold (v/v) in nuclease-free water and then stored at -80 °C for use.

2.6.3. Assay and data calculations of real-time qPCR

An aliquot of 5 µl of diluted cDNA (cDNA template) was pipetted into a reaction tube, which contained a mixture consisting of 4 µl nuclease-free water, 10 µl Smart Quant Green Master Mix with dUTP low ROX (Protech, Taipei, Taiwan), 0.5 µl target gene-specific forward PCR primer (10 µM), and 0.5 µl target gene-specific reverse PCR primer (10 µM), to a final volume of 20 µl. Primer sequences for detection of expression of mouse cytokines and inflammation-related component genes using real-time qPCR assays are shown in Table 1. Real-time qPCR reaction and detection were performed in a real-time rotary analyzer (Rotor-Gene 6000; Corbett Life Science, Sydney, Australia) using the following program: hot-start activation at 95 °C for 15 min, followed by 40–50 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The Ct (threshold cycle number) value of the target gene expression was achieved according to fluorescence intensity measured using the real-time rotary analyzer. Each biological determination was carried out in triplicate. The comparative Ct method was used to quantify relative expression amounts of

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targeted mRNA species, indicating that a lower Ct value corresponds to a higher mRNA expression amount [21]. The stably expressed mouse *β-actin*, a housekeeping gene, was selected as a reference for calibration. Relative gene expressions of pro-inflammatory *TNF-α* and anti-inflammatory *IL-10* cytokines, as well as components of intracellular inflammation-related signaling, including *TLR2* and *STAT3* were measured. Relative mRNA expression amounts in differently treated cells are expressed as the fold change value. The expression ratio (R) of individual mRNA amount at treated vs. control condition in the cells was determined using the equation: $R = 2^{-\Delta\Delta Ct}$ [29,30]. The following equations were used to calculate each target gene expression (e.g., cytokines or inflammation-related signaling) with respect to its control situation [31]: $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$ and $\Delta\Delta Ct = \Delta Ct_{\text{treatment}} - \Delta Ct_{\text{control}}$ or $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\beta\text{-actin gene}})_{\text{time x}} - (Ct_{\text{target gene}} - Ct_{\beta\text{-actin gene}})_{\text{time 0}}$.

2.7. Assay of STAT3 phosphorylation at tyrosine 705 using in-cell ELISA method

Phosphorylation of STAT3 is an active form of transcription factor STAT3 protein. After phosphorylation in cytoplasm, phosphorylated STAT3 protein allows to move into the cell nucleus for 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

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and 95% air for 3 h. After incubation, the plate was centrifuged at 25 ℃, 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. 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.

2.8. Statistical analysis

Values are expressed as means ± SEM and analyzed using one-way ANOVA, followed by either Duncan's New Multiple Range test or unpaired Student's t-test. Differences among treatments were considered statistically significant if $P < 0.05$. Statistical tests were performed using SPSS version 12.0 (SPSS, Inc., Chicago, IL, USA).

3. Results and discussion

To unravel a possible anti-inflammatory mechanism of Q, normal and activated inflammatory macrophages were isolated from mouse peritoneal cavities and cultured with Q or its major metabolite Q3G *in vitro*. Changes of targeted cytokines gene expression and components of inflammation-related intracellular signaling pathway in the cells were determined using RT qPCR.

3.1. Optimal incubation time for activated inflammatory macrophages in vitro for mRNA expression assays

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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 significance ($P < 0.05$) of cytokine gene expression profile (Table 2), the 3 h-incubation time was selected as optimal incubation time for the following studies.

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

To re-confirm the anti-inflammatory potential of Q based on pro- and anti-inflammatory cytokine gene expression profile, the normal or activated inflammatory macrophages were treated with Q or Q3G at the indicated non-toxic optimal concentrations (20 and 50 μM) for 3 h [24]. 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

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mRNA expression amounts of both TNF-α and IL-10, but could not significantly ($P > 0.05$) change the ratio of pro-/anti-inflammatory (*TNF-α/IL-10*) cytokine gene expressions (Table 3). 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 inflammation 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 (Table 3). 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 (Table 3). 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. Pharmacokinetic areas under the plasma concentration-time curves of daily oral supplementation with Q (50- and 150-mg dosages, respectively) ranged from 76.1 μM·min to 305.8 μM·min in volunteers with no apparent toxicity, suggesting Q

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administration safety *in vivo* [32].

After Q is assimilated by macrophages, it may be further metabolized into Q3G. To compare anti-inflammatory potential of Q and Q3G, Q3G were 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 (Table 4). 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 (Table 4). 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 (Table 4). 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.

Dex is a glucocorticoid that has been widely used to treat many inflammatory and autoimmune diseases including rheumatoid arthritis, bronchospasm, and idiopathic thrombocytopenic purpura [33]. In the present study, we show that Dex has 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 (Tables 3 and 4). Q has been found to have diverse

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physiological effects, including antioxidant and anti-inflammatory effects in different tissues [34,35]. Q3G is the major quercetin metabolite that is reported to have antioxidant functions *in vitro*, and regulate coronary venous barrier function by improving blood-borne inflammatory mediators in a novel microvascular wall model [36]. In the present study, our results further suggest that Q and Q3G treatments *in vitro* might have an immuno-stimulatory effect on normal macrophages, but inhibit inflammation status in activated inflammatory macrophages by regulating cytokine gene expressions (Tables 3 and 4). 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 [37]. The uptake and metabolism of Q in mouse primary macrophages have been previously described in our report [4]. Obviously, Q has 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 analyzing inflammation-related intracellular signaling pathways.

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

Table 5 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.

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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 5). 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 inhibiting 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 5). Our results suggest that mice treated with LPS i.p. may result in systemic inflammation and activate macrophage inflammation through 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 5). The physiological significance of increased *NF-κB* and *JAK2* gene expression in the activated inflammatory macrophages by Q administration remains to be further investigated. It was found that purified active lotus plumule (*Nelumbo nucifera* Gaertn) polysaccharides inhibited

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inflammation in mouse primary splenocytes by decreasing *TLR2* and *TLR4* gene expression [38]. Our results are identical to the published literature [38].

Similar to the administration effects of Q, Dex (positive control) 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, *NF-κB* and *JAK2* gene expression in the activated inflammatory macrophages were significantly ($P < 0.05$) increased by Dex administration (Table 5). 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 of STAT3 protein at tyrosine 705 was further measured in the present study.

3.4. Effect of Q in vitro 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 1 shows Q *in vitro* administration effects on STAT3 phosphorylation at tyrosine 705 in normal or activated inflammatory macrophages. The results showed that STAT3 protein phosphorylation at tyrosine 705 in activated inflammatory macrophages significantly ($P < 0.05$) increased compared to that of normal control (Fig. 1), indicating

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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* could not significantly ($P > 0.05$) change phosphorylation levels of STAT3 protein at tyrosine 705 in both normal and activated inflammatory macrophages ($P > 0.05$).

The present study indicates that Q administration inhibits 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* (Table 3). In addition, Q administration *in vitro* ameliorated the inflammation-induced injury in the activated inflammatory macrophages by decreasing *TLR2* gene expression in a dose-dependent manner and by increasing that of *JAK2* and *STAT3* genes, which had been suppressed in the activated inflammatory macrophages (Table 5). 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 (Fig. 1). It was found that Q or its metabolites could enter macrophages to exert their anti-inflammatory functions [5]. In the present study, we further determined the effects of Q administration *in vitro* on components of inflammation-related signaling pathway (TLR2 and TLR4) in activated inflammatory macrophages. In addition, we infer that increased *NF-κB* expression might inhibit *STAT3* expression

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in the activated inflammatory macrophages in the absence of Q (Table 5). Both NF-κB and STAT3 are transcription factor in cells. Interestingly, Q administration seemed to simultaneously increase both *NF-κB* and *STAT3* gene expression in normal and activated inflammatory macrophages (Table 5). The relationship between *STAT3* and *NF-κB* gene expression influenced by Q remains to be further clarified.

Some achievements have been obtained in the present study, and Q may be further applied for anti-inflammatory clinical use including tumor therapy [9,14,18,39,40]. However, there are limitations in the present study. Firstly, this is still an *ex vivo* study; therefore confirmation of the key findings *in vivo* using peritoneal challenge model should be performed in the future. The findings with the murine cells may not be recapitulated in the 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 are the first report to suggest that Q inhibits LPS-induced inflammation *ex vivo* through suppressing *TLR2* gene expression and STAT3 phosphorylation in activated inflammatory macrophages.

4. Conclusion

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 inflamed macrophages. The present study supports that Q exerts anti-

inflammatory effects via meanwhile suppressing *TLR2* gene expression and STAT3 protein phosphorylation in activated inflammatory macrophages. Q has potential to further apply for treating inflammation-associated diseases.

Conflict of interest: The authors declared no conflict of interest.

Acknowledgment: This study was kindly supported by research grant NSC99-2628-B-005-004-MY3 from the Ministry of Science and Technology, Taipei, Taiwan, ROC.

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Table 1

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

Cytokine genes[a]		**Primer sequences**[b]	**Length (bp)**[c]
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	
House-keeping gene		Primer sequences	Length (bp)
β-actin	FW	GCTACAGCTTCACCACCACA	208
	RV	AAGGAAGGCTGGAAAAGAGC	

[a] IL, interleukin; TNF, tumor necrosis factor; TLR, toll-like receptor; ; NF-κB, nuclear factor-kappaB; JAK2, Janus kinase 2; STAT3, signal transducers and activators of transcription 3. [b] FW, forward primer; RV, reverse primer. [c] Amplicon length in base pair.

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Table 2

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

Cytokines gene name	Incubation time (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}

[a] Values are means ± SEM (n = 3 biological determinations), analyzed using one-way ANOVA, followed by Duncan's new multiple range test. [b] Values within the same row not sharing a common superscript capital letter are significantly different ($P < 0.05$) from each other.

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Table 3

Effects of quercetin administration on cytokine gene expression in activated peritoneal macrophages from female BALB/c mice intraperitoneally injected with phosphate-buffered saline or lipopolysaccharide at 8 mg/kg BW through 12 h.[a, b, c].

			Quercetin (μM)		Dex
	Macrophages	control	20	50	(1 μM)
Gene		Relative expression amount (fold)			
TNF-α	normal	1.00 ± 0.00^{B}	23.2 ± 7.5^{A}	9.69 ± 1.06^{B}	2.95 ± 0.39^{B}
	inflammatory	1.00 ± 0.00^{A}	0.13 ± 0.05^{B}	0.33 ± 0.14^{B}	0.03 ± 0.02^{B}
IL-10	normal	1.00 ± 0.00^{B}	117 ± 55^{A}	14.8 ± 6.3^{B}	15.6 ± 5.1^{B}
	inflammatory	1.00 ± 0.00^{B}	12258 ± 5518^{A}	9823 ± 4212^{AB}	8271 ± 2148^{AB}
TNF-α/	normal	1.00 ± 0.00	0.71 ± 0.54	0.96 ± 0.51	0.16 ± 0.09
IL-10	inflammatory	1.00 ± 0.00^{A}	0.00 ± 0.00^{B}	0.00 ± 0.00^{B}	0.00 ± 0.00^{B}

[a] Values are means ± SEM (n = 4 biological determinations), analyzed using one-way ANOVA, followed by Duncan's new multiple range test. [b] Values within same row not sharing a common superscript capital letter are significantly different ($P < 0.05$) from each other. [c] The collected peritoneal macrophages were cultured with quercetin or dexamethasone (Dex, a positive control) for 3 h *in vitro*.

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Table 4

Effects of quercetin-3-glucuronide administration on cytokine gene expression in activated peritoneal macrophages from female BALB/c mice intraperitoneally injected with phosphate-buffered saline or lipopolysaccharide at 8 mg/kg BW through 12 h.[a, b, c].

Gene	Macrophages	Quercetin-3-glucuronide (μM) 0	20	50	Dex (1 μM)
		Relative expression amount (fold)			
TNF-α	normal	1.00 ± 0.00^{B}	6.27 ± 2.64^{B}	16.0 ± 3.94^{A}	2.95 ± 0.34^{B}
	inflammatory	1.00 ± 0.00^{A}	0.08 ± 0.03^{B}	0.14 ± 0.05^{B}	0.03 ± 0.01^{B}
IL-10	normal	1.00 ± 0.00^{B}	5.33 ± 2.87^{B}	7.10 ± 1.46^{B}	15.6 ± 4.5^{A}
	inflammatory	1.00 ± 0.00^{B}	6155 ± 2502^{A}	6813 ± 1251^{A}	8271 ± 1917^{A}
TNF-α/ IL-10	normal	1.00 ± 0.00^{B}	3.89 ± 1.36^{A}	2.43 ± 0.56^{AB}	0.25 ± 0.07^{B}
	inflammatory	1.00 ± 0.00^{A}	0.00 ± 0.00^{BC}	0.00 ± 0.00^{B}	0.00 ± 0.00^{C}

[a] Values are means ± SEM (n = 5 biological determinations), analyzed using one-way ANOVA, followed by Duncan's new multiple range test. [b] Values within same row not sharing a common superscript capital letter are significantly different ($P < 0.05$) from each other. [c] The collected peritoneal macrophages were cultured with quercetin-3-glucuronide or dexamethasone (Dex, a positive control) for 3 h *in vitro*.

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Table 5

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

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	$2.76 \pm 0.31^{A,*}$	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	$2.19 \pm 1.20^{B,*}$	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	$0.54 \pm 0.11^{B,*}$	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	$0.59 \pm 0.17^{B,*}$	1.17 ± 0.21^{A}	1.14 ± 0.19^{A}	0.40 ± 0.12^{B}

[a] Values are means ± SEM (n = 4 biological determinations), analyzed using one-way ANOVA, followed by Duncan's new multiple range test. [b] Values within same row not sharing a common superscript capital letter are significantly different ($P < 0.05$) from each other. [c] Asterisk (*) within same gene item means significantly different ($P < 0.05$) between normal and inflammatory macrophages in the absence of quercetin, analyzed using one-way ANOVA, followed by unpaired Student's *t*-test. [d] The peritoneal macrophages were cultured with quercetin or dexamethasone (Dex, a positive control) for 3 h *in vitro*.

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Figure legends

Fig. 1. Effect of quercetin administration on phospho-STAT3 (Tyr705) protein levels in normal and inflammatory macrophages from female BALB/c mice.

[a] Values are mean ± SEM (n = 5 biological determinations) analyzed using one-way ANOVA, followed by Duncan's new multiple range test. [b] Bar under the same condition not sharing a common letter are significantly different ($P < 0.05$) from each other. [c] Asterisk (*) means significantly different ($P < 0.05$) between normal and inflammatory cells in the absence of sample, analyzed using unpaired Student's *t*-test. [d] The peritoneal macrophages were cultured with quercetin or dexamethasone (Dex, a positive control) for 3 h *in vitro*.

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Fig. 1