

# The Flavonoid Quercetin Inhibits Proinflammatory Cytokine (Tumor Necrosis Factor Alpha) Gene Expression in Normal Peripheral Blood Mononuclear Cells via Modulation of the NF- $\kappa$ B System

Madhavan P. Nair,\* Supriya Mahajan, Jessica L. Reynolds, Ravikumar Aalinkeel, Harikrishnan Nair, Stanley A. Schwartz, and Chithan Kandaswami

*Department of Medicine and Microbiology, Kaleida Health System, Buffalo General Hospital, State University of New York at Buffalo, Buffalo, New York 14203*

Received 11 July 2005/Returned for modification 11 August 2005/Accepted 4 January 2006

**The flavonoids comprise a large class of low-molecular-weight plant metabolites ubiquitously distributed in food plants. These dietary antioxidants exert significant antitumor, antiallergic, and anti-inflammatory effects. The molecular mechanisms of their biological effects remain to be clearly understood. We investigated the anti-inflammatory potentials of a safe, common dietary flavonoid component, quercetin, for its ability to modulate the production and gene expression of the proinflammatory cytokine tumor necrosis factor alpha (TNF- $\alpha$ ) by human peripheral blood mononuclear cells (PBMC). Our results showed that quercetin significantly inhibited TNF- $\alpha$  production and gene expression in a dose-dependent manner. Our results provide direct evidence of the anti-inflammatory effects of quercetin by PBMC, which are mediated by the inhibition of the proinflammatory cytokine TNF- $\alpha$  via modulation of NF- $\kappa$ B1 and I $\kappa$ B.**

The natural antioxidant flavonoids constitute significant components of the diet and display a diverse array of biological effects (16, 19, 21, 26). Polyphenolic compounds, including a large class of flavonoids, are enriched in certain vegetables, fruits, seeds, and beverages (e.g., tea and wine) and are regarded as a class of semiessential nutrients for humans. Dietary intake rich in these compounds has been suggested to improve the health of individuals and decrease the risk of cardiovascular disease. The beneficial effects of flavonoids have been attributed to their antioxidant and anti-inflammatory properties (18, 19, 31). The effects of flavonoids, including quercetin, on a variety of inflammatory processes and immune functions have been extensively reviewed (4, 6, 11, 17, 25, 27, 28, 40). Tumor necrosis factor alpha (TNF- $\alpha$ ) is one of the major proinflammatory cytokines involved in the pathogenesis of chronic inflammatory diseases and is modulated by oxidative stress (5, 35). TNF- $\alpha$  is a multifunctional cytokine that regulates the growth, proliferation, differentiation, and viability of activated leukocytes. TNF- $\alpha$  also triggers the cellular release of other cytokines, chemokines, or inflammatory mediators and displays antiviral and antimicrobial effects (1, 2, 39).

Numerous signaling cascades have been elucidated in promotion of proinflammatory conditions by proinflammatory cytokines, such as TNF- $\alpha$ , which involves the activation of inducible transcriptional factors (1, 12, 13, 14, 29, 39). NF- $\kappa$ B is one of the principal inducible transcription factors whose modulation triggers a cascade of signaling events involving an integrated sequence of protein-regulated steps, some of which are potential key targets for intervention in treating inflammatory

conditions (3, 7, 20, 29, 33, 34). Previous studies have shown that quercetin inhibits lipopolysaccharide (LPS)-stimulated NF- $\kappa$ B activation in RAW 264.7 macrophage (8, 37) and also inhibits LPS-induced I $\kappa$ B phosphorylation in bone marrow-derived macrophage (11). Although quercetin exhibits several biological effects, the molecular mechanisms of its anti-inflammatory effects by peripheral blood mononuclear cells (PBMCs) have not been clearly elucidated. We hypothesize that flavonoids exert anti-inflammatory effects by PBMCs by inhibiting the endogenous production of the proinflammatory cytokine TNF- $\alpha$  and that these effects are mediated through the regulation of NF- $\kappa$ B and I $\kappa$ B. Therefore, the present study was undertaken to investigate the direct effect of quercetin on the gene expression and protein secretion of the proinflammatory cytokine TNF- $\alpha$ . We further investigated whether the transcription factor NF- $\kappa$ B was involved in the regulation of TNF- $\alpha$  by quercetin by normal PBMCs.

## MATERIALS AND METHODS

**Cell culture.** Total PBMCs were separated by Ficoll Hypaque centrifugation. PBMCs ( $3 \times 10^6$  cells/ml) were cultured in RPMI 1640 medium (Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (complete medium) with quercetin (Sigma-Aldrich, St. Louis, MO) at concentrations ranging between 1 to 50  $\mu$ M for 24, 48, 72, and 96 h at 37°C in a 5% CO<sub>2</sub> incubator. These concentrations selected for the in vitro studies are similar to levels found in plasma in human subjects that have ingested 150 mg or 300 mg of quercetin (15). For phosphorylation studies, PBMCs were treated with quercetin at 50  $\mu$ M for 30 and 60 min at 37°C in a 5% CO<sub>2</sub> incubator.

**Stimulated TNF- $\alpha$  production.** PBMCs ( $3 \times 10^6$  cells/ml) were treated with quercetin and additionally stimulated with phorbol myristate acetate (PMA) (5 ng/ml) and Ca<sup>2+</sup> ionophore (50 ng/ml) for 24 h.

**RNA extraction.** After cell stimulation, cytoplasmic RNA was extracted by an acid guanidinium thiocyanate-phenol chloroform method as described by Chomczynski and Sacchi (9). Cultured cells were pelleted by centrifugation and resuspended in a 4 M solution of guanidinium thiocyanate. Cells were lysed by repeat pipetting and phenol-chloroform extracted in the presence of sodium acetate. After centrifugation, RNA was precipitated from the aqueous layer by the addition of an equal volume of isopropanol. The mixture was kept at -20°C for

\* Corresponding author. Mailing address: Department of Medicine, Division of Allergy, Immunology, and Rheumatology, 310 Multi Research Bldg., Buffalo General Hospital, 100 High Street, Buffalo, NY 14203. Phone: (716) 859-2985. Fax: (716) 859-2999. E-mail: mnair@acsu.buffalo.edu.

1 h and then centrifuged to pellet the RNA. The RNA pellet was washed with 75% ethanol to remove any remaining traces of guanidium. The final pellet was dried and resuspended in diethyl pyrocarbonate water, and the concentration of RNA was determined using a spectrophotometer at 260 nm. DNA contamination in the RNA preparation was removed by treating the RNA preparation with DNase (1 IU/ $\mu$ g of RNA; Promega, Madison, WI) for 30 min at 37°C, followed by proteinase K digestion at 37°C for 15 min and subsequent extraction with phenol-chloroform-isoamyl alcohol and precipitation with ammonium acetate-ethanol. The isolated RNA was stored at -70°C until use. The DNA contamination in the RNA preparation was checked by including a control in which the reverse transcriptase enzyme was not included in the PCR amplification procedure. RNA preparation, which was devoid of any DNA contamination, was used in the subsequent experiments in semiquantitative real-time Q-PCR.

**Real-time Q-PCR.** TNF- $\alpha$  and NF- $\kappa$ B1 gene expressions were quantitated using real-time PCR. The relative abundance of each mRNA species was assessed using the SYBR green master mix from Stratagene (La Jolla, CA) to perform quantitative PCR (Q-PCR) using the ABI Prism 5700 instrument that detects and plots the increase in fluorescence versus PCR cycle number to produce a continuous measure of PCR amplification. To provide precise quantification of the initial target in each PCR, the amplification plot is examined at a point during the early log phase of product accumulation. This is accomplished by assigning a fluorescence threshold above the background and determining the time point at which each sample's amplification plot reaches the threshold (defined as the threshold cycle number, or  $C_T$ ). Differences in threshold cycle number are used to quantify the relative amount of PCR target contained within each tube (32). Relative mRNA species expression was quantitated and expressed as the transcript accumulation index ( $TAI = 2^{-\Delta\Delta CT}$ ), calculated using the comparative  $C_T$  method (22). All data were controlled for quantity of RNA input by performing measurements on an endogenous reference gene,  $\beta$ -actin. In addition, results on RNA from treated samples were normalized to results obtained on RNA from the control, untreated sample.

**Fluorescence-activated cell sorter (FACS) analysis. (i) Detection of the surface markers CD4 and CD14 and intracellular TNF- $\alpha$ .** Immunofluorescent staining was used to identify and quantitate the number of cells that express the intracellular cytokine TNF- $\alpha$  as described previously (22, 28). To determine which cell population of PBMCs predominantly contributes to TNF- $\alpha$  production, cells were stained for the surface markers, CD4 (T cells) and CD14 (monocytes). Golgi stop (BD Pharmingen, San Diego, CA), an intracellular protein transport inhibitor, was used to enhance the ability to detect cytokine-producing cells. The CD4 (R&D Systems Minneapolis, MN) and CD14 (eBioscience, San Diego, CA) antibodies were fluorescein isothiocyanate-conjugated antibodies. The TNF- $\alpha$  monoclonal antibody (R&D Systems) was conjugated to phycoerythrin. After stimulation, cells were harvested, washed, suspended in staining buffer, and stained for TNF- $\alpha$  alone, CD4 alone, CD14 alone, TNF- $\alpha$  and CD4, and TNF- $\alpha$  and CD14. When staining for both the surface marker and the intracellular cytokine, cells were stained for surface markers prior to fixation. Cells were fixed with cytofix/cytoperm buffer (BD Pharmingen) and permeabilized by washing twice in 1 $\times$  Perm solution (BD Pharmingen). Cells were stained for intracellular TNF- $\alpha$  and resuspended in staining buffer prior to flow cytometric analysis. Stained cells were subjected to a light scatter analysis. A fixed population of cells were gated and represented as side scatter on the y axis and forward scatter on the x axis. Cells positive for TNF- $\alpha$  were expressed as a percentage of the total cells gated.

**(ii) Detection of the phosphorylated forms of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ .** After stimulation, cells were harvested, washed, and suspended in staining buffer. Cells were fixed with cytofix/cytoperm buffer (BD Pharmingen) and were permeabilized by washing twice in 1 $\times$  Perm solution (BD Pharmingen). Cells were stained with antibodies for I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , phospho-I $\kappa$ B $\alpha$ , and phospho-I $\kappa$ B $\beta$  (rabbit polyclonal antibodies; Cell Signaling), followed by detection using a goat anti-rabbit immunoglobulin G (IgG) fluorescein isothiocyanate-conjugated secondary antibody (Sigma-Aldrich). Cells were resuspended in staining buffer prior to flow cytometric analysis.

**Western blot analysis.** PBMCs were cultured with quercetin (1 to 50  $\mu$ M) for 48 h, and protein was extracted for Western blot analyses (10), using mammalian protein extraction reagent (Pierce, Rockford, IL). Protein concentrations were determined using Coomassie protein reagent (Bio-Rad, Hercules, CA). Thirty micrograms of total protein was loaded per lane and separated by 7.5% sodium dodecyl sulfate-Tris-glycine polyacrylamide gel electrophoresis (ISC Bioexpress, Kaysville, UT). Proteins were transferred to nitrocellulose membranes and blocked overnight in 1 $\times$  Tris-buffered saline (TBS) containing 0.1% Tween and 5% nonfat dry milk. Membranes were probed with the rabbit polyclonal antibody directed against TNF- $\alpha$  (Cell Signaling, Beverly, MA) and a goat polyclonal  $\beta$ -actin antibody (Santa Cruz Biotech, Santa Cruz, CA) per the manufacturer's

TABLE 1. Effect of quercetin on viability of PBMCs

Treatment <sup>a</sup>	% Viability after incubation period (h) (n) <sup>b</sup> :			
	24	48	72	96
Control	95.4 $\pm$ 2.5	94.14 $\pm$ 4.5	95.4 $\pm$ 4.5	93.1 $\pm$ 3.6
Quercetin, 1 $\mu$ M	96.9 $\pm$ 3.4	97.39 $\pm$ 2.4	96.1 $\pm$ 3.3	96.8 $\pm$ 2.1
Quercetin, 5 $\mu$ M	98.1 $\pm$ 1.2	97.9 $\pm$ 1.7	98.8 $\pm$ 5.2	97.6 $\pm$ 4.9
Quercetin, 10 $\mu$ M	97.8 $\pm$ 1.9	95.4 $\pm$ 2.8	96.4 $\pm$ 3.7	96.7 $\pm$ 7.8
Quercetin, 25 $\mu$ M	93.9 $\pm$ 3.3	93.4 $\pm$ 2.7	95.3 $\pm$ 7.3	92.7 $\pm$ 4.1
Quercetin, 50 $\mu$ M	92.4 $\pm$ 2.3	93.6 $\pm$ 4.9	94.1 $\pm$ 1.8	90.4 $\pm$ 3.8

<sup>a</sup> PBMCs ( $3 \times 10^6$  cells/ml) were cultured with and without quercetin at different concentrations for 24, 48, and 72 h. Viability was determined using the trypan blue dye exclusion technique.

<sup>b</sup> Values represent mean  $\pm$  SD of results from three separate experiments done in triplicate.

instructions. After an overnight incubation with primary antibodies, the membranes were washed three times in 1 $\times$  TBS with 0.5% Tween 20 prior to incubation with secondary antibodies. The membranes were incubated for 2 h at room temperature with secondary antibodies (biotin-conjugated goat anti-rabbit IgG, conjugated donkey anti-goat IgG; Santa Cruz Biotech) per the manufacturer's instructions. After secondary antibody incubations, the membranes were washed three times, for 10 min each, in 1 $\times$  TBS with 0.5% Tween 20 and then incubated for another 30 min with a streptavidin-alkaline phosphatase conjugate (Invitrogen) followed by colorimetric detection using nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate reagent (Roche, Indianapolis, IN). Densitometry analyses were done using a Syngene image analyzer with Gene Tools Analysis software, version 3.02.00 (Syngene, Frederick, MD). Data were normalized to levels of  $\beta$ -actin.

**ELISA.** After 96 h of stimulation with quercetin, supernatants were harvested and stored at -70°C until further analysis. TNF- $\alpha$  protein secretion in culture supernatants was quantitated using an enzyme-linked immunosorbent assay (ELISA) kit obtained from BioSource International (Camarillo, CA) and used as described by the manufacturer. The sensitivity of the TNF- $\alpha$  ELISA was 1.7 pg/ml.

## RESULTS

**Effect of quercetin on viability of PBMCs.** Data presented in Table 1 show the effect of quercetin on the viability of PBMCs at 24, 48, 72, and 96 h in culture. PBMCs ( $3 \times 10^6$  cells/ml) were cultured with and without quercetin at different concentrations for 24, 48, 72, and 96 h, and the viability was determined using the trypan blue dye exclusion technique. Results show that at 96 h of incubation, the viability of the PBMCs remained greater than 90% at the highest concentration of quercetin (i.e., 50  $\mu$ M). Additionally, the MTT assay, a cell toxicity assay, also shows comparable viability results (data not shown). Previous studies have also shown long-term viability (up to 120 h) of PBMCs in media supplemented with 10% fetal calf serum alone (41).

**Quercetin downregulates TNF- $\alpha$  gene expression.** Data presented in Fig. 1 show the effects of different concentrations of quercetin on TNF- $\alpha$  gene expression in PBMCs at 24, 48, and 72 h of incubation as quantitated by real-time Q-PCR. At 24 h, quercetin at 10 to 50  $\mu$ M concentrations produced a significant dose-dependent decrease in TNF- $\alpha$  gene expression, while at 48 and 72 h of incubation, quercetin at all concentrations, including the lowest concentration of 1  $\mu$ M, produced a significant dose-dependent decrease in TNF- $\alpha$  gene expression. Heat-inactivated quercetin had no effect on TNF- $\alpha$  gene expression (data not shown). These data suggest that quercetin significantly modulates TNF- $\alpha$  gene expression.

**Quercetin modulates TNF- $\alpha$ -positive phenotypes.** Data presented in Fig. 2 show the effect of quercetin (48 h) on the

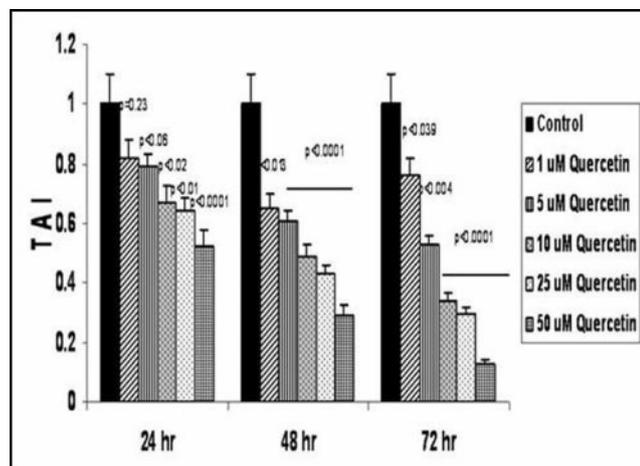


FIG. 1. Effect of quercetin on TNF- $\alpha$  gene expression as quantitated by real-time Q-PCR. PMBCs were cultured for 24, 48, and 72 h with quercetin at 1, 5, 10, 25, and 50  $\mu$ M, and TNF- $\alpha$  gene expression was quantitated. Data represented are means  $\pm$  SD of results from three separate experiments. Statistical significance was determined by Student's *t* test. TAL, transcript accumulation index.

intracellular marker TNF- $\alpha$  as determined by FACS analysis. Data shown in Fig. 2a to f are representative histograms showing TNF- $\alpha$ -positive cells. The percentage of TNF- $\alpha$ -positive cells at 1, 5, 10, and 50  $\mu$ M quercetin were 3.4 (Fig. 2c), 2.9 (Fig. 2d), 1.2 (Fig. 2e), and 0.7 (Fig. 2f), respectively, with the control value being 5.9% (Fig. 2b). Figure 2g shows the mean percentage  $\pm$  standard deviation (SD) of TNF- $\alpha$ -positive cells, from three separate experiments compared to control cultures. PMBCs treated with 5  $\mu$ M (2.4%,  $P = 0.007$ ), 10  $\mu$ M (1.03%,  $P = 0.001$ ), and 50  $\mu$ M (0.66%,  $P = 0.0001$ ) quercetin significantly decrease the percentage of TNF- $\alpha$ -positive cells compared to the untreated control (5.2%). Quercetin at 1  $\mu$ M (3.2%,  $P = 0.06$ ) had no effect of the percentage of TNF- $\alpha$ -positive cells compared to the control. These results confirm gene expression data as analyzed by real-time Q-PCR for TNF- $\alpha$  (Fig. 1).

**CD14<sup>+</sup> monocytes and CD4<sup>+</sup> T cells release TNF- $\alpha$ .** Since PMBCs are a heterogeneous population of cells, we sought to determine which cell populations produce TNF- $\alpha$ . Data presented in Fig. 3a to e are representative dot plots showing that both CD4<sup>+</sup> and CD14<sup>+</sup> cell populations produce TNF- $\alpha$ . Data shown in Fig. 3b demonstrate that 89.7% of CD4<sup>+</sup> T cells express TNF- $\alpha$ , which is decreased to 62.9% (Fig. 3c) following 48 h of 50  $\mu$ M quercetin treatment. Figure 3d demonstrates that 44.3% of CD14<sup>+</sup> monocytes express TNF- $\alpha$ , which is decreased to 23.1% (Fig. 3e.) following 48 h of 50  $\mu$ M quercetin treatment. Data shown in Fig. 3f show the mean percentage  $\pm$  SD of positive cells from three separate experiments compared to control cultures. These data suggest that CD4<sup>+</sup> and CD14<sup>+</sup> cells are the major cell populations in PMBCs in which quercetin modulates TNF- $\alpha$  expression.

**Quercetin modulates stimulated TNF- $\alpha$  production.** Data presented in Fig. 4 show the effect of quercetin on stimulated (PMA/Ca<sup>2+</sup> ionophore) TNF- $\alpha$  production as determined by FACS analysis. Data shown in Fig. 4a to g are representative histograms showing TNF- $\alpha$ -positive cells. The percentage of

TNF- $\alpha$ -positive cells in the unstimulated and stimulated controls and in the presence of stimulation (1, 5, 10, and 50  $\mu$ M quercetin) were 2.8 (Fig. 4b), 15.9 (Fig. 4c), 11.5 (Fig. 4d), 5.8 (Fig. 4e), 3.1 (Fig. 4f), and 1.8 (Fig. 4g), respectively. Figure 4g shows the mean percentage  $\pm$  SD of TNF- $\alpha$ -positive cells from three separate experiments compared to respective control cultures. The percentage of TNF- $\alpha$ -positive cells was significantly increased in PMA/Ca<sup>2+</sup> ionophore-stimulated PMBCs (17.6%,  $P = 0.0001$ ) compared to the unstimulated control (4.5%). Quercetin at 1  $\mu$ M (11.8%,  $P = 0.001$ ), 5  $\mu$ M (5.2%,  $P = 0.001$ ), 10  $\mu$ M (2.7%,  $P = 0.001$ ), and 50  $\mu$ M (1.4%,  $P = 0.0001$ ) significantly decreased the percentage of TNF- $\alpha$ -positive cells induced by PMA/Ca<sup>2+</sup> ionophore stimulation compared to stimulation alone (17.6%).

**Quercetin downregulates TNF- $\alpha$  production.** Data presented in Fig. 5 show the effects of quercetin (48 h) on TNF- $\alpha$  protein expression by PMBCs as detected by Western blot analysis. Figure 5a shows the  $\beta$ -actin loading control with no change in protein expression by 1, 5, 10, or 50  $\mu$ M quercetin treatment compared to the untreated control. Data demonstrate (Fig. 5b) that quercetin at 5, 10, and 50  $\mu$ M downregulated TNF- $\alpha$  protein (26 kDa) expression by PMBCs. Figure 5c shows the densitometric analysis (% change in optical density units) of TNF- $\alpha$  protein  $\pm$  SD from four separate experiments compared to control cultures. PMBCs treated with 5  $\mu$ M (21.3% inhibition,  $P = 0.05$ ), 10  $\mu$ M (26.33% inhibition,  $P = 0.044$ ), and 50  $\mu$ M (39.30% inhibition,  $P = 0.001$ ) quercetin showed a significant suppression in TNF- $\alpha$  protein expression. These results confirm gene expression data as analyzed by real-time Q-PCR for TNF- $\alpha$  (Fig. 1) as well as FACS analysis data (Fig. 2).

**ELISA.** Data presented in Fig. 6 show the effects of quercetin on the endogenous production of TNF- $\alpha$  by PMBCs as quantitated by ELISA. Levels of TNF- $\alpha$  were measured in the culture supernatants at 96 h of treatment with various concentrations of quercetin. Quercetin (5 to 50  $\mu$ M) significantly downregulated TNF- $\alpha$  production by PMBCs. Thus, the quantitation of TNF- $\alpha$  through ELISA is consistent with the gene expression data as analyzed by real-time Q-PCR (Fig. 1) and TNF- $\alpha$ -positive phenotypic analysis by FACS (Fig. 2).

**Quercetin modulates NF- $\kappa$ B gene expression.** The transcription factor NF- $\kappa$ B is a significant mediator of various immune and inflammatory responses and is negatively modulated by I $\kappa$ B. Cellular activation induces the phosphorylation of I $\kappa$ B proteins (I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ ). Phosphorylation of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  targets them for ubiquitination and degradation. Degradation of I $\kappa$ B and I $\kappa$ B results in the translocation of NF- $\kappa$ B to the nucleus where it binds to specific promoter regions of various genes encoding for inflammatory cytokines (3, 7, 14, 20, 29, 33, 34). Since the NF- $\kappa$ B complex regulates inflammatory cytokine production, we examined the effects of quercetin on NF- $\kappa$ B1 gene modulation. Data presented in Fig. 7 show the effect of quercetin on NF- $\kappa$ B1 gene expression by real-time PCR by PMBCs. As shown at 24 and 48 h of incubation, quercetin significantly downregulated NF- $\kappa$ B1 gene expression at concentrations of 5 to 50  $\mu$ M. At 72 h, quercetin significantly downregulated NF- $\kappa$ B1 gene expression at 10 to 50  $\mu$ M concentrations. These data suggest that the inhibitory effects of quercetin on TNF- $\alpha$  production may be mediated by downregulation of NF- $\kappa$ B1.

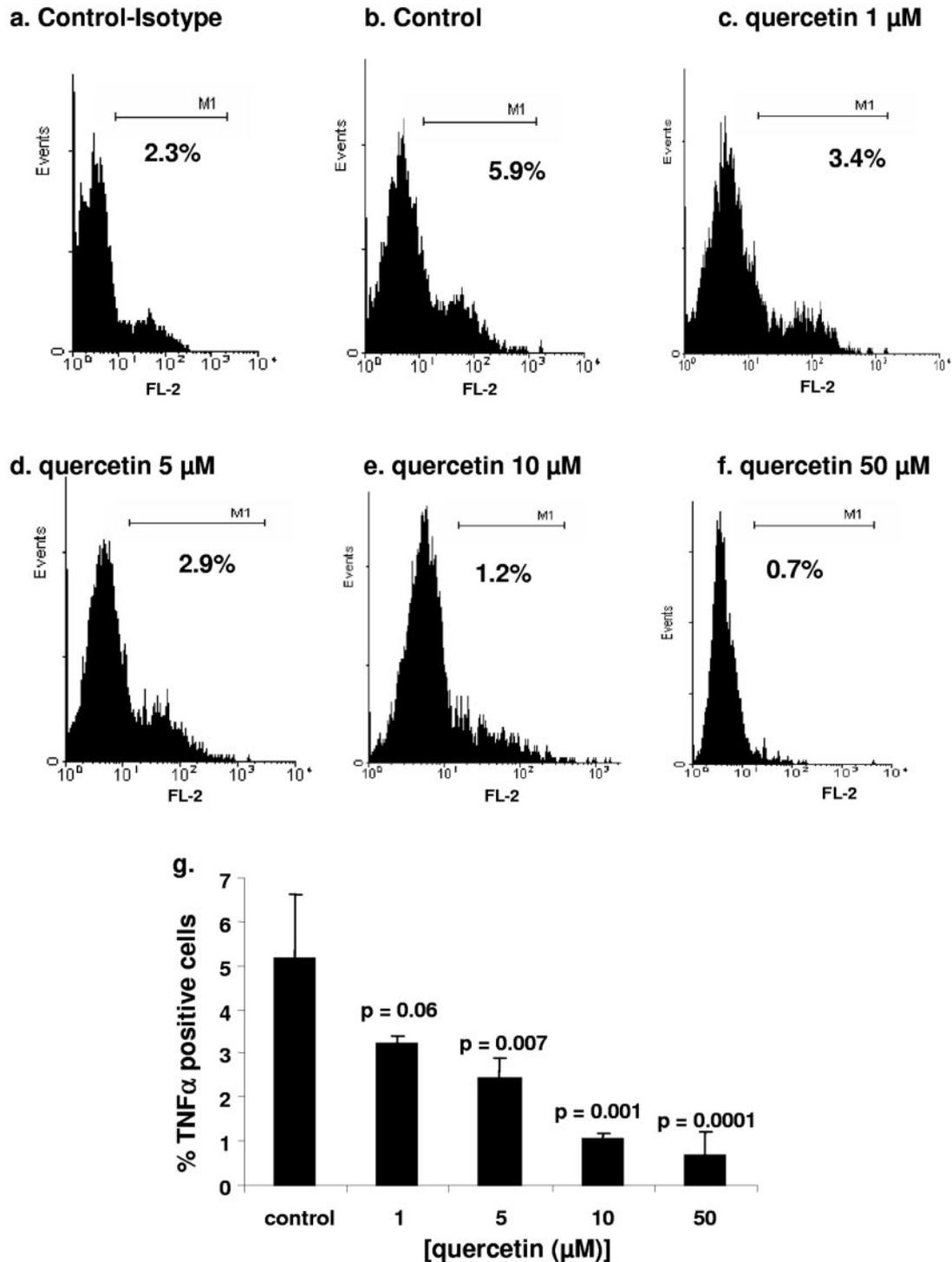


FIG. 2. Effect of quercetin on the phenotypic expression of intracellular TNF- $\alpha$  by PBMCs. PBMCs were cultured with 1, 5, 10, and 50  $\mu$ M quercetin for 48 h and subjected to FACS analysis. (a) Isotype control (mouse IgG<sub>1</sub>); (b) control; (c) 1  $\mu$ M quercetin; (d) 5  $\mu$ M quercetin; (e) 10  $\mu$ M quercetin; (f) 50  $\mu$ M quercetin; (g) graph showing the mean percentage  $\pm$  SD of TNF- $\alpha$ -positive cells. Statistical significance was determined by analysis of variance followed by Tukey's test ( $n = 3$ ).

**Quercetin modulates phosphorylation of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ .** Phosphorylation of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  targets these proteins for ubiquitination and degradation, which results in activation of NF- $\kappa$ B; therefore, it was of interest to investigate the effects of quercetin

on phosphorylation of both I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ . Data shown in Fig. 8 and 9 demonstrate the effects of quercetin (50  $\mu$ M) on the phosphorylation state of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  as determined by FACS analyses. Data shown in Fig. 8a to d are representative histograms

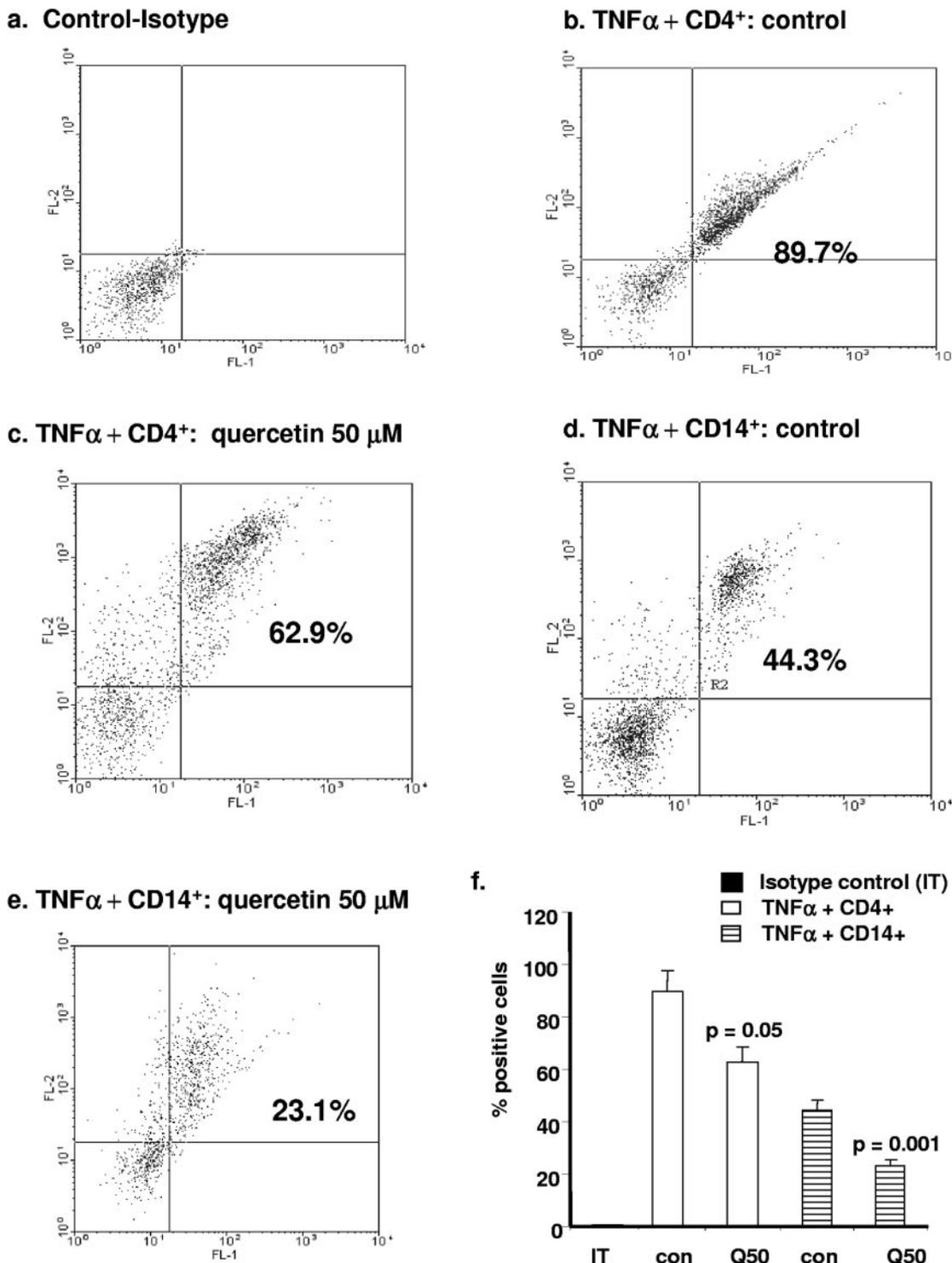


FIG. 3. FACS analyses to determine the cell population in PBMCs producing TNF- $\alpha$ . (a) Isotype control; (b) TNF- $\alpha$  and CD4<sup>+</sup> cells, control; (c) TNF- $\alpha$  and CD4<sup>+</sup>, quercetin (50  $\mu$ M, 48 h); (d) TNF- $\alpha$  and CD14<sup>+</sup> cells, control; (e) TNF- $\alpha$  and CD14<sup>+</sup> cells, quercetin (50  $\mu$ M, 48 h); (f) graph showing the mean percentage  $\pm$  SD of positive cells. Statistical significance was determined by Student's *t* test (*n* = 3). con, control.

showing that the percentage of phospho-I $\kappa$ B $\alpha$ -positive cells were 31.7 (Fig. 8c) and 18.7 (Fig. 8d) following 30 and 60 min of exposure to quercetin (50  $\mu$ M), respectively, compared to the control (44.9%, Fig. 8b). Figure 8e shows the mean percentage  $\pm$

SD of phospho-I $\kappa$ B $\alpha$ -positive cells from two separate experiments compared to control cultures. PBMCs treated with 50  $\mu$ M quercetin for 60 min showed a significant decrease in the percentage of phospho-I $\kappa$ B $\alpha$ -positive cells (18.55%, *P* = 0.05), while a 30-min

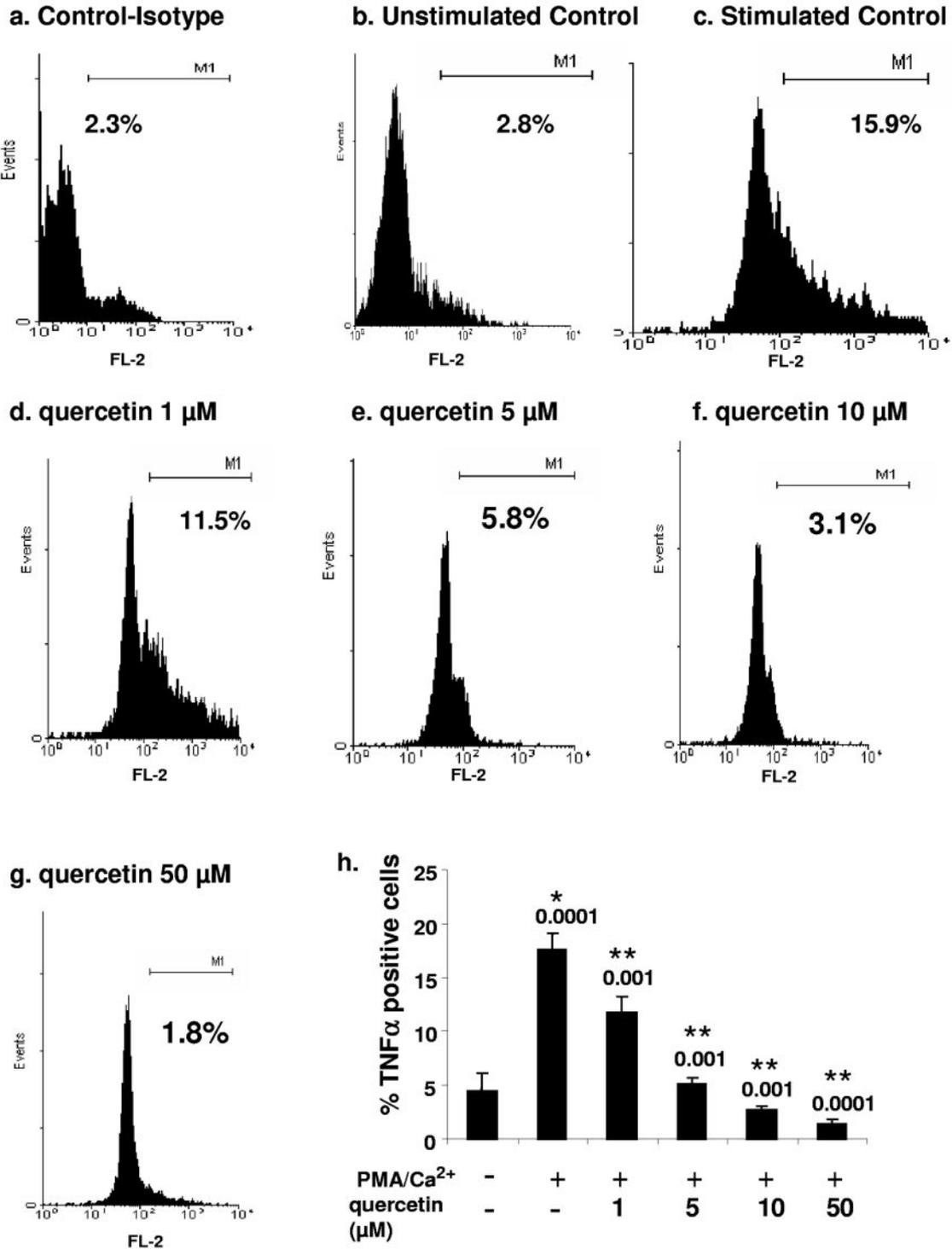


FIG. 4. Effect of quercetin on stimulated intracellular TNF- $\alpha$  production by PBMCs. PBMCs were cultured with 1, 5, 10, and 50  $\mu$ M quercetin for 24 h, stimulated with PMA/Ca<sup>2+</sup> ionophore for an additional 24 h, and then subjected to FACS analysis. (a) Isotype control (mouse IgG<sub>1</sub>); (b) unstimulated control; (c) PMA/Ca<sup>2+</sup> ionophore alone (stimulated control); (d) 1  $\mu$ M quercetin plus PMA/Ca<sup>2+</sup> ionophore; (e) 5  $\mu$ M quercetin plus PMA/Ca<sup>2+</sup> ionophore; (f) 10  $\mu$ M quercetin plus PMA/Ca<sup>2+</sup> ionophore; (g) 50  $\mu$ M quercetin plus PMA/Ca<sup>2+</sup> ionophore; (h) graph showing the mean percentage  $\pm$  SD of TNF- $\alpha$ -positive cells. Statistical significance was determined by analysis of variance followed by Tukey's test ( $n = 3$ ); \*, compared to unstimulated control; \*\*, compared to stimulated control.

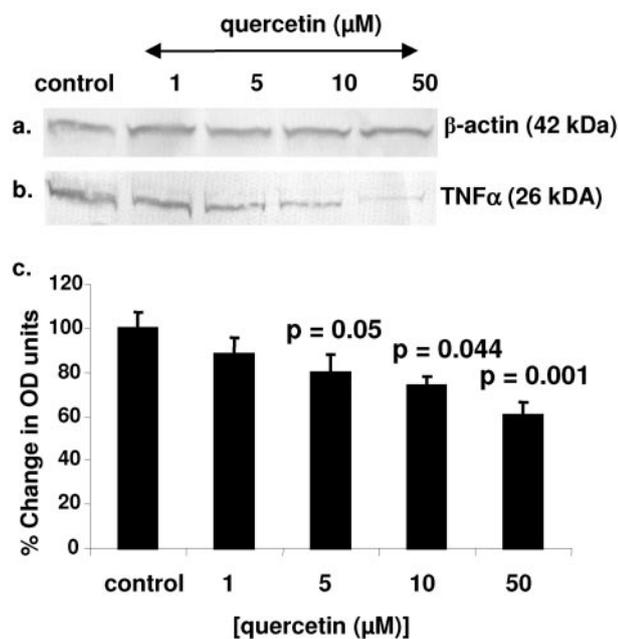


FIG. 5. Effect of quercetin (48 h) on TNF- $\alpha$  protein expression as quantitated by Western blot analysis. (a)  $\beta$ -Actin loading control in the untreated control and 1, 5, 10 and 50  $\mu$ M quercetin-treated samples; (b) TNF- $\alpha$  expression in the untreated control and 1, 5, 10, and 50  $\mu$ M quercetin-treated samples; (c) densitometric analysis (% change in optical density [OD] units) of TNF- $\alpha$  protein in the quercetin treated samples compared to control cultures. Data represented are means  $\pm$  SD of results from four separate experiments and are normalized to levels of  $\beta$ -actin. Statistical significance was determined by Student's *t* test (*n* = 4).

exposure to quercetin had no effect on the percentage of phospho-I $\kappa$ B $\alpha$ -positive cells (32.55%, *P* = 0.171) compared to 42.1% in the untreated population. Quercetin (50  $\mu$ M, 30 and 60 min) had no effect on total I $\kappa$ B $\alpha$ -positive cells (data not shown).

As shown in the representative histograms (Fig. 9a to d), the percentages of phospho-I $\kappa$ B $\beta$ -positive cells were 22.9% (30 min of quercetin) (Fig. 9c) and 18.2% (60 min of quercetin) (Fig. 9d) compared to the control (34.1%) (Fig. 9b). Figure 9e shows the mean percentage  $\pm$  SD of phospho-I $\kappa$ B $\beta$ -positive cells from two separate experiments compared to control cultures. PMBCs treated with 50  $\mu$ M quercetin for 30 and 60 min showed a significant decrease in phospho-I $\kappa$ B $\beta$ -positive cells, 24.3% (*P* = 0.022) and 17.86% (*P* = 0.023), respectively, compared to 48.7% in the untreated population. Data are represented as the mean percentages of positive cells from two separate experiments. Quercetin (50  $\mu$ M, 30 and 60 min) had no effect on total I $\kappa$ B $\beta$ -positive cells (data not shown).

### DISCUSSION

Many polyphenolic compounds from plants, including a large class of bioflavonoids, are known to offer health benefits to humans. Flavonoids represent a group of phytochemicals exhibiting a wide range of biological activities arising mainly from their antioxidant properties and ability to modulate several enzymes or cell receptors (16, 19, 21, 26). Flavonoids have been recognized to exert antibacterial and antiviral activity,

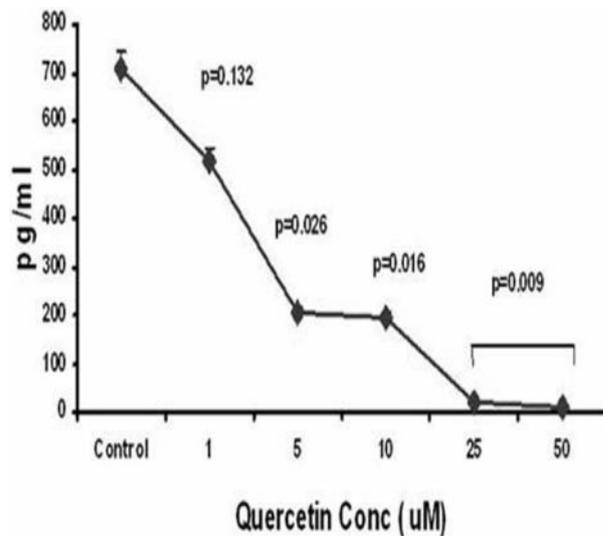


FIG. 6. Effect of quercetin (96 h) on TNF- $\alpha$  protein expression as quantitated by ELISA (BioSource International). The sensitivity of the TNF- $\alpha$  ELISA was 1.7 pg/ml. Data represented are means  $\pm$  SD of results from three separate experiments. Statistical significance was determined by Student's *t* test (*n* = 3). Conc, concentration.

anti-inflammatory, antiangiogenic and antiallergic effects, analgesic, hepatoprotective, cytostatic, apoptotic, estrogenic, and antiestrogenic properties (4, 6, 11, 16, 19, 21, 25–28, 40). The beneficial effects have been attributed to their antioxidant and anti-inflammatory properties. The current study shows that the anti-inflammatory effects of quercetin may be mediated by down-regulating endogenous and PMA/Ca<sup>2+</sup>-stimulated TNF- $\alpha$  production by PMBCs. The findings from this study further support data demonstrating that quercetin inhibits LPS-induced

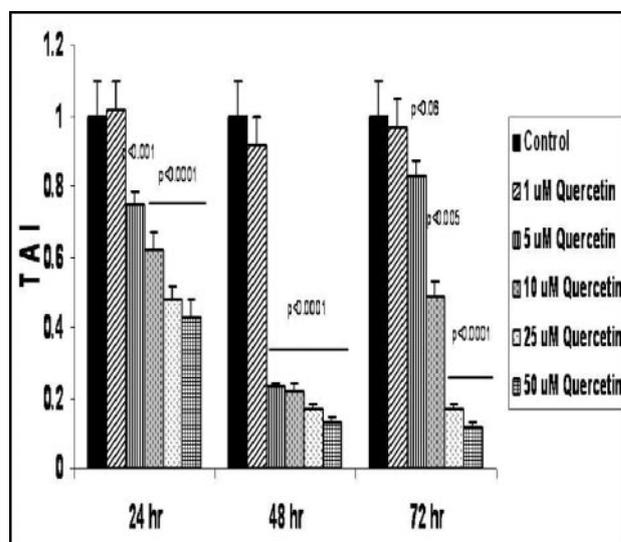


FIG. 7. Effect of quercetin on NF- $\kappa$ B1 gene expression as quantitated by real-time PCR. PMBCs were cultured for 24, 48, and 72 h with quercetin at (1 to 50  $\mu$ M); NF- $\kappa$ B1 gene expression was quantitated. Data represented are means  $\pm$  SD of results from three separate experiments. Statistical significance was determined by Student's *t* test (*n* = 3). TAI, transcript accumulation index.

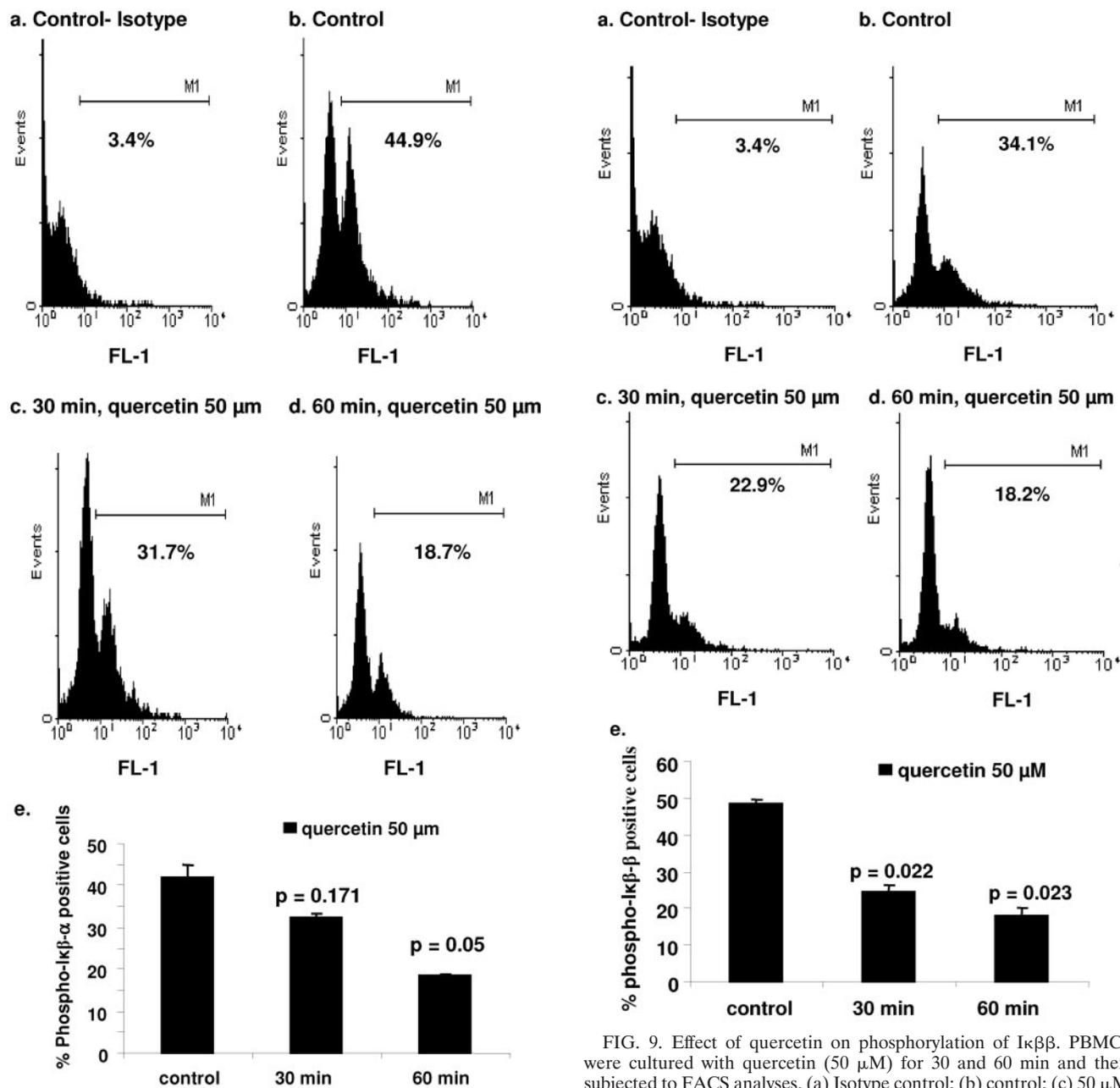


FIG. 8. Effect of quercetin on phosphorylation of  $I\kappa\beta\alpha$ . PBMCs were cultured with quercetin ( $50\ \mu\text{M}$ ) for 30 and 60 min and then subjected to FACS analyses. (a) Isotype control; (b) control; (c)  $50\ \mu\text{M}$  quercetin, 30 min; (d)  $50\ \mu\text{M}$  quercetin, 60 min; (e) graph showing the mean percentages  $\pm$  SD of phospho- $I\kappa\beta\alpha$ -positive cells. Statistical significance was determined by Student's  $t$  test ( $n = 3$ ).

TNF- $\alpha$  production (11, 17, 23, 24, 30, 36–38, 40). Previous studies from this laboratory demonstrate that quercetin inhibits interleukin 4 expression and increases gamma interferon production in PBMCs (28). These data and the findings from the present study suggest that quercetin has the ability to modulate the immune response.

An impressive variety of stimuli (TNF- $\alpha$ , interleukin-1, T-cell activation signals, bacterial endotoxins, viral transforming

FIG. 9. Effect of quercetin on phosphorylation of  $I\kappa\beta\beta$ . PBMCs were cultured with quercetin ( $50\ \mu\text{M}$ ) for 30 and 60 min and then subjected to FACS analyses. (a) Isotype control; (b) control; (c)  $50\ \mu\text{M}$  quercetin, 30 min; (d)  $50\ \mu\text{M}$  quercetin, 60 min; (e) graph showing the mean percentages  $\pm$  SD of phospho- $I\kappa\beta\beta$ -positive cells. Statistical significance was determined by Student's  $t$  test ( $n = 3$ ).

proteins, certain growth factors, and reactive oxygen intermediates) lead to the rapid nuclear accumulation of the transcription factor NF- $\kappa\beta$  induced by phosphorylation and degradation of  $I\kappa\beta$ . NF- $\kappa\beta$  is widely recognized as a critical mediator of immune and inflammatory responses (3, 7, 14, 20, 29, 33, 34). In most cell types, NF- $\kappa\beta$  is found in the cytoplasm, where it is associated with an inhibitory protein known as  $I\kappa\beta$  ( $I\kappa\beta\alpha$  and  $I\kappa\beta\beta$ ).  $I\kappa\beta$  negatively modulates NF- $\kappa\beta$  by preventing its translocation to the nucleus. Upon cellular activation,  $I\kappa\beta\alpha$  and  $I\kappa\beta\beta$  are phosphorylated by the cellular kinase complex IKK. This complex is composed of two kinases:  $IKK\alpha$  and

IKK $\beta$ . Phosphorylation of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  results in their ubiquitination and degradation, resulting in the translocation of NF- $\kappa$ B to the nucleus, where it binds to specific promoter regions of various genes encoding for inflammatory cytokines (3, 7, 14, 20, 29, 33, 34). Previous studies demonstrate that quercetin inhibits LPS-stimulated NF- $\kappa$ B activation in RAW 264.7 macrophage (8, 37) and also inhibits LPS-induced I $\kappa$ B $\alpha$  phosphorylation in bone marrow-derived macrophage (11). Our studies show that a possible mechanism of quercetin-mediated suppression of TNF- $\alpha$  gene and protein expression is mediated by downregulating gene expression for NF- $\kappa$ B1. Furthermore, our FACS analyses show that quercetin decreases the phosphorylation of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ , suggesting that quercetin decreases the activation of NF- $\kappa$ B. This decrease in phosphorylation of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  may be a direct mechanism by which quercetin inhibits the activity of NF- $\kappa$ B, thereby decreasing endogenous TNF- $\alpha$  expression. Further studies are necessary to elucidate the intricate roles that NF- $\kappa$ B and I $\kappa$ B play in the inhibition of TNF- $\alpha$  expression.

These findings suggest that the cytokine TNF- $\alpha$  can be inhibited by quercetin, which may be of clinical significance in host defense mechanisms against various infections. Our data suggest that the major population of cells in which quercetin modulates TNF- $\alpha$  expression are predominantly CD4<sup>+</sup> T cells and CD14<sup>+</sup> monocytes. A decrease in endogenous TNF- $\alpha$  production in the presence of quercetin indicates that flavonoids have the capacity to modulate the immune response and have potential anti-inflammatory activity. In addition to its well-known proinflammatory role, TNF- $\alpha$  has complex effects on the growth, differentiation, and death of immune cells. TNF- $\alpha$  inhibition is a validated approach to treating several inflammatory diseases (5). Although the results of this study are preliminary, we believe that quercetin-induced suppression of TNF- $\alpha$  can result in the stimulation of anti-inflammatory cytokines via inhibiting the activation of NF- $\kappa$ B, and therefore, we anticipate that quercetin can be widely used as an anti-TNF- $\alpha$  therapy. Evaluation of the molecular mechanisms of quercetin-induced anti-inflammatory effects may be a promising area for the development of new flavonoid-based neutrophilic agents for the treatment of various inflammatory diseases.

#### ACKNOWLEDGMENTS

This work was supported in part by NIH grants NIDA, RO1-DA15628, RO1-DA12366, and RO-DA14218; the Margaret Duffy and Robert Cameron Troup Memorial Fund for Cancer Research of the Kaleida Health System; Buffalo General Foundation; and the State University of New York.

#### REFERENCES

- Aggarwal, B. B. 2000. Tumour necrosis factor receptor associated signaling molecules and their role in activation of apoptosis. JNK and NF-kappa B. *Ann. Rheum. Dis.* **59**:6–16.
- Aggarwal, B. B., A. Samanta, and M. Feldmann. 2001. TNF- $\alpha$ , p. 413. *In J. J. Oppenheim, M. Feldman, S. K. Durum, T. Hirano, J. Vilcek, and N. A. Nicola (ed.)*, Cytokine reference, vol. I. Academic Press, San Diego, Calif.
- Bremner, P., and M. Heinrich. 2002. Natural products as targeted modulators of the nuclear factor-kappaB pathway. *J. Pharm. Pharmacol.* **54**:453–472.
- Busse, W. W., D. E. Kopp, and E. Middleton, Jr. 1984. Flavonoid modulation of human neutrophil function. *J. Allergy Clin. Immunol.* **73**:801–809.
- Calamia, K. T. 2003. Current and future use of anti-TNF agents in the treatment of autoimmune, inflammatory disorders. *Adv. Exp. Med. Biol.* **528**:545–549.
- Camuesco, D., M. Comalada, M. E. Rodriguez-Cabeza, A. Nieto, M. D. Lorente, A. Concha, A. Zarzuelo, and J. Galvez. 2004. The intestinal anti-inflammatory effect of quercitrin is associated with an inhibition in iNOS expression. *Br. J. Pharmacol.* **143**:908–918.
- Celec, P. 2004. Nuclear factor kappa B-molecular biomedicine: the next generation. *Biomed. Pharmacother.* **58**:365–371.
- Cho, S. Y., S. J. Park, M. J. Kwon, T. S. Jeong, S. H. Bok, W. Y. Choi, W. I. Jeong, S. Y. Ryu, S. H. Do, C. S. Lee, J. C. Song, and K. S. Jeong. 2003. Quercetin suppresses proinflammatory cytokines production through MAP kinases and NF-kappaB pathway in lipopolysaccharide-stimulated macrophage. *Mol. Cell. Biochem.* **243**:153–160.
- Chomczynski, P., and N. Sacchi. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159.
- Coligan, J. E., A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober (ed.). 1991. *Current protocols in immunology*, p. 145–186. Wiley, New York, N.Y.
- Comalada, M., D. Camuesco, S. Sierra, I. Ballester, J. Xaus, J. Galvez, and A. Zarzuelo. 2005. In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-kappaB pathway. *Eur. J. Immunol.* **35**:584–592.
- Dempsey, P. W., S. E. Doyle, J. Q. He, and G. Cheng. 2003. The signaling adaptors and pathways activated by TNF superfamily. *Cytokine Growth Factor Rev.* **14**:193–209.
- Duh, E. J., W. J. Maury, T. M. Folks, A. S. Fauci, and A. B. Rabson. 1989. Tumor necrosis factor alpha activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF-kB sites in the long terminal repeat. *Proc. Natl. Acad. Sci. USA* **86**:5974–5978.
- Hsu, H., J. Xiong, and D. V. Goeddel. 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-kB activation. *Cell* **81**:495–504.
- Hubbard, G. P., S. Wolfram, J. A. Lovegrove, and J. M. Gibbins. 2004. Ingestion of quercetin inhibits platelet aggregation and essential components of the collagen-stimulated platelet activation pathway in humans. *J. Thromb. Haemost.* **2**:2138–2145.
- Kandaswami, C., and E. Middleton, Jr. 1994. Free radical scavenging and antioxidant activity of plant flavonoids. *Adv. Exp. Med. Biol.* **366**:351–376.
- Kawada, N., S. Seki, M. Inoue, and T. Kuroki. 1998. Effect of antioxidants, resveratrol, quercetin, and N-acetylcysteine, on the functions of cultured rat hepatic stellate cells and Kupffer cells. *Hepatology* **27**:1265–1274.
- Kim, H. P., K. H. Son, H. W. Chang, and S. S. Kang. 2004. Anti-inflammatory plant flavonoids and cellular action mechanisms. *J. Pharmacol. Sci.* **96**:229–245.
- Korkina, L. G., and I. B. Afanas'ev. 1997. Antioxidant and chelating properties of flavonoids. *Adv. Pharmacol.* **38**:151–163.
- Krakauer, T. 2004. Molecular therapeutic targets in inflammation: cyclooxygenase and NF-kappaB. *Curr. Drug Targets Inflamm. Allergy* **3**:317–324.
- Li, B. Q., T. Fu, Y. Dongyan, J. A. Mikovits, F. W. Ruscetti, and J. M. Wang. 2000. Flavonoid baicalin inhibits HIV-1 infection at the level of viral entry. *Biochem. Biophys. Res. Commun.* **276**:534–538.
- Mahajan, S. D., S. A. Schwartz, and M. P. Nair. 2003. Immunological assays for chemokine detection in in-vitro culture of CNS cells. *Biol. Proced. Online* **5**:90–102.
- Mamani-Matsuda, M., J. Rambert, D. Malvy, H. Lejoly-Boisseau, S. Daulouede, D. Thiolat, S. Coves, P. Courtois, P. Vincendeau, and M. D. Mossalayi. 2004. Quercetin induces apoptosis of *Trypanosoma brucei gambiense* and decreases the proinflammatory response of human macrophages. *Antimicrob. Agents Chemother.* **48**:924–929.
- Manjeet, K. R., and B. Ghosh. 1999. Quercetin inhibits LPS-induced nitric oxide and tumor necrosis factor-alpha production in murine macrophages. *Int. J. Immunopharmacol.* **21**:435–443.
- Middleton, E., Jr. 1998. Effect of plant flavonoids on immune and inflammatory cell function. *Adv. Exp. Med. Biol.* **439**:175–182.
- Middleton, E., Jr., C. Kandaswami, and T. C. Theoharides. 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* **52**:673–751.
- Nair, H. K., K. V. Rao, R. Aalinkel, S. Mahajan, R. Chawda, and S. A. Schwartz. 2004. Inhibition of prostate cancer cell colony formation by the flavonoid quercetin correlates with modulation of specific regulatory genes. *Clin. Diagn. Lab. Immunol.* **11**:63–69.
- Nair, M. P., C. Kandaswami, S. Mahajan, K. C. Chadha, R. Chawda, H. Nair, N. Kumar, R. E. Nair, and S. A. Schwartz. 2002. The flavonoid, quercetin, differentially regulates Th-1 (IFN $\gamma$ ) and Th-2 (IL4) cytokine gene expression by normal peripheral blood mononuclear cells. *Biochim. Biophys. Acta* **1593**:29–36.
- Osborn, L., S. Kunkel, and G. J. Nabel. 1989. Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proc. Natl. Acad. Sci. USA* **86**:2336–2340.
- Rangan, G. K., Y. Wang, Y. C. Tay, and D. C. Harris. 1999. Inhibition of NFkappa B activation with antioxidants is correlated with reduced cytokine transcription in PTC. *Am. J. Physiol.* **277**:F779–789.

31. **Scalbert, A., I. T. Johnson, and M. Saltmarsh.** 2005. Polyphenols: antioxidants and beyond. *Am. J. Clin. Nutr.* **81**:215S–217S.
32. **Shively, L., L. Chang, J. M. LeBon, Q. Liu, A. D. Riggs, and J. Singer-Sam.** 2003. Real-time PCR assay for quantitative mismatch detection. *Bio-Techniques* **34**:498–502.
33. **Singh, S., and B. B. Aggarwal.** 1995. Activation of transcription factor NF-kappa B is suppressed by curcumin (diferulolylmethane). *J. Biol. Chem.* **270**:24995–25000.
34. **Suzuki, Y. J., and L. Packer.** 1993. Inhibition of NF-kappa B activation by vitamin E derivatives. *Biochem. Biophys. Res. Commun.* **193**:277–283.
35. **Taylor, P. C., R. O. Williams, and M. Feldmann.** 2004. Tumour necrosis factor alpha as a therapeutic target for immune-mediated inflammatory diseases. *Curr. Opin. Biotechnol.* **15**:557–563.
36. **Ueda, H., C. Yamazaki, and M. Yamazaki.** 2004. A hydroxyl group of flavonoids affects oral anti-inflammatory activity and inhibition of systemic tumor necrosis factor-alpha production. *Biosci. Biotechnol. Biochem.* **68**: 119–125.
37. **Wadsworth, T. L., and D. R. Koop.** 1999. Effects of the wine polyphenolics quercetin and resveratrol on pro-inflammatory cytokine expression in RAW 264.7 macrophages. *Biochem. Pharmacol.* **15**:941–949.
38. **Wadsworth, T. L., T. L. McDonald, and D. R. Koop.** 2001. Effects of Ginkgo biloba extract (EGb 761) and quercetin on lipopolysaccharide-induced signaling pathways involved in the release of tumor necrosis factor-alpha. *Biochem. Pharmacol.* **62**:963–974.
39. **Wajant, H., F. Henkler, and P. Scheurich.** 2001. The TNF-receptor-associated factor family: scaffold molecules for cytokine receptors, kinases and their regulators. *Cell. Signal.* **13**:389–400.
40. **Wang, J., and G. Mazza.** 2002. Effects of anthocyanins and other phenolic compounds on the production of tumor necrosis factor alpha in LPS/IFN-gamma-activated RAW 264.7 macrophages. *J. Agric. Food Chem.* **50**:4183–4189.
41. **Wong, E. C., V. E. Maher, K. Hines, J. Lee, C. S. Carter, T. Goletz, W. Kopp, C. L. Mackall, J. Berzofsky, and E. J. Read.** 2001. Development of a clinical-scale method for generation of dendritic cells from PBMC for use in cancer immunotherapy. *Cytotherapy* **3**:19–29.