The effect of crocin on phenotype switching of murine macrophages depends on their polarization state at the time of exposure to crocin

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Research Article

Keywords: crocin, Saffron, Arg-I, iNOS, macrophage, inflammation

Posted Date: July 22nd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1846416/v1

Abstract

Background

Macrophages have plastic phenotypes. M1 macrophages release inflammatory cytokines and have microbicidal activities. M2 cells release anti-inflammatory cytokines and repair tissue damages. The imbalance between M1 and M2 responses has an important effect on many pathological events. Crocin is an antioxidant and can eliminate free radicals, the properties involved in its anti-inflammatory capacities. We investigated the effects of crocin on macrophages phenotype switching in view of their primary activation states.

Methods

The effect of crocin different concentrations on macrophage viability was evaluated by MTT. TNF-α and IL-6 secretion as well as iNOS/Arg-I ratio were measured in cells treated with crocin and or LPS + INF-γ (M1 inducers). The cytokine secretion levels and the ratio were also determined in cells concurrently treated with crocin and LPS + INF-γ or cells pretreated with crocin before M1 induction.

Results

Crocin did not show any toxicity at a concentration of 500 μM and lower. When M0 uncommitted macrophages were exposed to crocin (25–100 μM), it raised some M1 activity indicators including iNOS/Arg-I ratio and TNF-α secretion, but not IL-6. Crocin in concurrent treatment with LPS + INF-γ could halt raising in M1 indicators, iNOS/Arg-I ratio and TNF-α secretion. Pretreatment of cells by crocin before the addition of LPS + INF-γ, could not converse the M1 induction in macrophages and caused a further increase in iNOS/Arg-I ratio and TNF-α secretion.

Conclusion

It seems that crocin modulatory effects on macrophage M1/M2 phenotype switching at least partly depends on the presence or absence of inflammatory mediators and accordingly the preliminary state of macrophage commitment.

Introduction

Macrophages are differentiated immune cells that are widely existing in the body and are generally used to maintain homeostasis and battle pathogen invasion [1]. They often act as immune control switches, balancing pro-inflammatory and anti-inflammatory activities. Primary macrophages (M0) present in different tissues are polarized according to alterations in their environment forming different subtypes, such as M1 and M2 macrophages [2]. M1/M2 ratio describes two opposite activities of macrophages.

They are naturally phagocytic and have specific phenotypic characteristics [3]. Polarized M1 cells are strong defenders against microbes and produce pro-inflammatory cytokines, including TNF-α and IL-6 and M1 activity stops cell proliferation. While polarized M2 cells secrete anti-inflammatory cytokines such as IL-10, and their activity promotes tissue repair and cell proliferation. The molecules that are mostly responsible for these activities are nitric oxide and ornithine named “fight” and “repair” mediators, respectively. They are both produced from arginine through enzymatic pathways. The favorite of macrophages to metabolize arginine by inducible nitric oxide synthase (iNOS) to citrulline and NO or by arginase to urea and ornithine describes them as M1 (iNOS) or M2 (Arginase- I) macrophages. Therefore, the M1/M2 classifying of macrophage can be summarized into these two differing procedures for the metabolism of arginine amino acid [4, 5]. as claimed by past studies, during the macrophages polarization procedure, there was an modification in the ratio of the pro-/anti-inflammatory marker including iNOS/Arg-I [6-9]. M2-activated macrophages increase the expression of Arg-I, which changes their iNOS/Arg-I ratio and results in their reduced ability to produce NO. M1 macrophage activation conversely increases this ratio [10]. It is worth mentioning that the definite polarization state of macrophages is more intricate than the simple dual M1/M2 classification. Macrophages are greatly flexible cells involving a range of activation states, with M1 and M2 representing the limits on each opposite end [3, 11]. The names M1 and M2 were chosen because macrophages M1 and M2 elicit Th1 and Th2 responses, respectively. Th sells are strong conductors of immune system orchestra and products of Th1 and Th2 responses (ie, IFN-γ and IL-4) also reduce M2 and M1 activity, respectively [12].

Changes in the balance between the responses of macrophages M1 and M2 play a key role in many disorders such as infections, cancer, autoimmune diseases and atherosclerosis [13]. For example, both M1 and M2 macrophages are present in atherosclerotic plugs, and a progressive switch from M2 to M1 has been reported during atherosclerotic lesions [14]. Therefore, modifying the direction of macrophage polarization to the desired phenotype in diseased tissues might be helpful in improving the condition toward a normal healthier state of the tissue.

Carotenoids, the bioactive compounds found in many herbal foods, appear to be able to modulate inflammation and immunological processes [15]. Among the approximately 700 carotenoids synthesized by photosynthetic algae, plants, and bacteria, about 50 items are used in the human regime, and some of them can be detected in human blood and tissue [16]. For many years, saffron, the dried stigma of *Crocus sativus* plant has been widely used as herbal dye and a spice and is well known in some countries' traditional medicines for its effectiveness in alleviating different diseases. One of the carotenoids in saffron is crocin (mono and diglycosyl esters of polyND carboxylic acid called crocetin) which has beneficial health features such as anti-inflammatory, antioxidant, memory booster, anti-tumor, and anti-depressant. Crocin has shown high effectiveness and low toxicity in laboratory animal models [17]. The potential for free radical scavenging and the antioxidant properties of crocin has been suggested to be implicated in its anti-inflammatory attributes [18].

To achieve an enhanced mechanistic intuition to the immunomodulatory effects of the crocin, we investigated the outcomes of crocin treatment on phenotype switching in murine macrophages with

uncommitted (M0) or M1 commitment states induced by LPS and INF-γ. We assessed IL-6 and TNF-α secretion and iNOS/Arg-1 ratio as well-known indicators of macrophage polarization status.

Materials And Methods

Cell culture and treatment

J774A.1 which is murine macrophage cell line, was purchased from the Pasteur Institute of Iran (Tehran, Iran). The cells were cultured in DMEM high glucose supplemented with 10% (v/v) FBS, 100,000 U/L penicillin, and 100 mg/L streptomycin, at 37 °C in a humidified incubator (5% CO_2, 95% air).

Macrophages were cultured at a starting density of 2.0- 4.0 × 10^5 cells/mL per well of 6 and 12-well plates (for cytokine secretion and mRNA Expression analysis, respectively) for 48 h. The cells then were treated for 12 h with LPS (100 ng/mL) + INF-γ (20 ng/mL) (for M1 activation), crocin (25, 50 and 100 μM) for 24 h, crocin (25, 50 or 100 μM) and LPS+INF-γ for 24 h (concurrent treatment), crocin (25, 50 or 100 μM) for 24 h and then LPS+INF-γ for additional 12 h (pretreatment with crocin).

Cell viability assay

5.0 × 10^3 Macrophage cells were cultured in each well of 96-well microplates for 48 h. Following 24 h treatment with different concentrations of crocin (10, 20, 50, 100, 250 and 500 μM) MTT assay was performed. MTT solution (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (5 mg/ml) was added to each well, and the plates were maintained (for 4 h) at 37°C and 5% CO_2. Formazan crystals were dissolved using DMSO (Dimethyl sulfoxide) solution, and the optical density (OD) was measured at 570 nm.

Cytokine secretion measurements

The levels of cell-released TNF-α and IL-6 were measured in the collected supernatants of untreated and treated cultured cells, using enzyme-linked immunosorbent assay (ELISA) (Invitrogen, USA) following the manufacturer's instructions. Optical density was evaluated by the Epoch™ Microplate Spectrophotometer (BIO TEK, Instruments, USA).

Arg-I and iNOS mRNA expression analysis

RNeasy®Mini Kit (Qiagen, USA) was used for RNA extraction and One-step RT-qPCR was performed using One-step SYBR®PrimeScript™ RT-PCR Kit II (TAKARA, China) according to the manufacturer's protocol in a Real-Time PCR machine (StepOne Applied Biosystem, USA). Each Ct (threshold cycle) value of the target genes was normalized to β-Actin Cts as a reference gene. The efficiency values of reference and target gene amplification reactions were between 90 and 110%. Consequently, fold changes of Arg-I and iNOS mRNA were calculated using the $2^{-\Delta\Delta ct}$ method. Primer sequences were declared in Table 1.

Statistical Analysis

All data were reported as mean ± standard error of mean (SEM) of 3 independent experiments (n=3). Statistical analysis was made using one-way ANOVA followed by Tukey–Kramer post-hoc test and Dunnett's multiple comparisons test for MTT assay, by Prism 7 software. Brown-Forsythe and Shapiro-Wilk tests were used for variance equality and verifying the normality of the data, respectively. *p*-value < 0.05 was considered to be statistically significant.

Results

Effect of crocin on macrophage viability

The results of the MTT assay showed that 24 h treatment of macrophage cells with crocin at concentrations range of 10-500 μM had no toxic effect on macrophages (Figure 1, a one-way ANOVA: F 6, 21 = 3.568, $p < 0.0135$).

Proinflammatory cytokines secretion analysis

LPS+IFN-γ treatment

Treatment of cells with LPS (100 ng/mL) + INF-γ (20 ng/mL) for 12 h caused significant increased secretion of TNF-α and IL-6 proteins (about 1.8 and 202 mean fold changes, respectively) in comparison to untreated M0 cells (Figure 2a and 2b, a one-way ANOVA, F 4, 10 = 2881, $p < 0.0001$ and F 4, 10 = 26.65, $p < 0.0001$, respectively).

Crocin treatment

TNF-α secretion by macrophages treated with different concentrations of crocin (25-100 μM) for 24 h was significantly higher (1.6 to 1.8 fold) than untreated M0 cells. Crocin was as potent as LPS+IFN-γ in induction of TNF-α secretion (Figure 2a, a one-way ANOVA, F 4, 10 = 26.65, $p < 0.0001$).

Crocin didn't cause any changes in the secretion of IL-6 compared to untreated M0 cells. (Figure 2b, a one-way ANOVA, F 4, 10 = 2881, $p < 0.0001$).

Crocin pretreatment

Pretreatment of macrophages with crocin (25-100 μM) for 24h before LPS+INF-γ exposure, significantly increased TNF-α secretion compared to untreated M0 cells (about 19-22 mean fold change) and even compared to M1 activated cells (1.4-1.6 fold) (Figure 3a, a one-way ANOVA, F 4, 10 = 121.6, $p < 0.0001$).

Crocin pretreated cells (25-100 μM) showed higher IL-6 secretion levels (about 43 fold) compared to M0 cell ($p < 0.0001$). Crocin exposure before LPS+INF-γ treatment couldn't subside the IL-6 increased expression brought by M1 induction (Figure 3b, a one-way ANOVA, F 4, 10 = 3826, $p < 0.0001$).

Concurrent treatment with crocin and LPS+IFN-γ

In cells treated with crocin and LPS+IFN-γ simultaneously, TNF-α secretion was significantly lower (about 0.4-0.5 fold) than M1 activated cells and remained at the same level with untreated ground state M0 cells (Figure 4a, a one-way ANOVA, F 4, 10 = 29.20 $p < 0.0001$). The presence of crocin in concurrent treatment couldn't prevent IL-6 escalation in the supernatant of the cells induced by LPS+INF-γ. Cotreated cells showed higher IL-6 levels in their supernatants (163-165 fold) compared to the M0 cells ($p < 0.0001$) (Figure 4b, a one-way ANOVA, F 4, 10 = 91.96, $p < 0.0001$).

The effect of crocin on Arg-I and iNOS mRNA expression and their ratio:

iNOS: M1 activated macrophage cells showed a significant increase (130.3± 0.3 fold change) in iNOS mRNA expression level ($p < 0.0001$). Crocin concurrent treatment with M1 inducers could diminish this iNOS-expression enhancement to about one-tenth (10.1 ± 0.4 fold change, $p < 0.0001$). In cells pretreated with crocin before exposure to LPS+INF-γ, iNOS expression increased remarkably compared to untreated cells (73.8 ± 0.06 fold change, $p = 0.0001$), although this increase was significantly lower than M1 activated cells (about 0.6 folds lower, $p < 0.0001$). No substantial change in iNOS was detected in crocin-treated cells (Figure 5a, a one-way ANOVA, F 4, 10 = 2099, $p < 0.0001$).

Arg-I: Treatment of macrophages with LPS+IFN-γ increased Arg-I expression compared to untreated cells (2.433 ± 0.06 fold change, $p = 0.0489$). Cells treated with crocin showed a significant reduction in Arg-I mRNA expression level compared to M0 activated cells (about 0.04 fold, $p < 0.0019$). Arg-I relative expression was also increased in the cells treated with crocin and LPS+INF-γ simultaneously compared to both untreated M0 cells (4.0 ± 0.1 fold change, $p = 0.0003$) and LPS+INF-γ treated ones (about 1.65 fold change, $p = 0.0280$). In crocin pretreated cells no significant change of Arg-I was observed compared to both M0 cells and M1 activated cells (Figure 5b, a one-way ANOVA, F 4, 10 = 24.75, $p < 0.0001$).

iNOS/Arg-I: in comparison to the uncommitted M0 cells (ratio=1), M1 activation by LPS+INF-γ significantly increased the ratio to 55.2 ± 1.2, $p < 0.00010$. Pretreatment of the cells with crocin could not reverse this increase (57.5± 0.1, $p < 0.0001$). Crocin (50 μM) itself elevated the ratio to 31.7± 0.7, $p = 0.0004$. Concurrent treatment restored the raised ratio by LPS and IFN-γ to the levels close to untreated cells (2.5 ± 0.07, $p = 0.0001$) (Figure 5c, a one-way ANOVA, F 4, 10 = 72.13, $p < 0.0001$).

Discussion

For a long time crocin has been known for its anti-inflammatory effects [19]. In this study, for the first time, we examined whether the primary commitment status of macrophages plays role in the ultimate impact of crocin on phenotype switching of them.

We used a combination of LPS and INF-γ to simulate the inflammatory conditions and induce the M1 phenotype in macrophages [20]. This activation significantly increased IL-6 and TNF-α secretion and iNOS/Arg-I ratio. Simultaneous treatment of macrophages with crocin and LPS/INF-γ did not allow any

significant change in TNF-α secretion and iNOS/Arg-I ratio. It has been previously described that downstream of LPS signaling pathway reactive oxygen species (ROS) are produced which mediate production of pro-inflammatory cytokines leading to the M1 phenotype induction rather than M2 [21]. Therefore, it is expectable that antioxidant substances such as crocin are able to modify macrophage responses to LPS probably by scavenging produced ROS and discontinuing the transduction of the signal to the downstream mediators of the inflammatory response. The ability of crocin to reduce TNF-α secretion and iNOS/Arg-I ratio in macrophages in the presence of LPS+INF-γ may explain partly why crocin shows therapeutic benefits when it is used in inflammatory diseases, by changing the activity of macrophages from overriding M1 inflammatory state in the diseases to dominant M2 anti-inflammatory phenotype [22]. In cultured rat brain microglial cells, crocin and crocetin (other ingredient of saffron and an important active metabolite of crocin in the body) were effective in inhibiting LPS-induced NO release. These compounds reduced the production of TNF-α, IL-β, and reactive oxygen species following LPS stimulation in th cells [18] The anti-inflammatory potential of crocin has also been reported in inflammatory neurological diseases such as multiple sclerosis [23]. Crocin also subsided the enhanced ratio of Th1/Th2 induced by Concanavalin A [24].

In concurrent treatment with crocin and LPS+INF-γ, crocin couldn't modify IL-6 secretion in the murine macrophages. LPS and INF-γ bind to TLR4 and INF-γ receptors on the macrophage, respectively [21]. It has been reported that following the ligation of LPS to TLR4 receptors on BV-2 microglial cells, downstream signaling pathways leading to the production of inflammatory cytokines TNF-α (e.g. ERK1/2 and NF-κB) and IL-6 (e.g. JNK, p38, and c-Jun) were different from each other and a specific compound might only modify one pathway without any effects on the other one [21, 25]. Accordingly, it seems that in our study crocin was just able to modify the signaling pathway responsible for the regulation of TNF-α expression, but not IL-6. In addition to concurrent treatment with LPS+INF-γ, in all other treatment conditions examined in this study crocin couldn't modify IL-6 secretion in the murine macrophages regardless to their primary commitment state.

In our study, when cells were pretreated with crocin and then exposed to LPS/INF-γ, iNOS mRNA and TNF-α secretion increased and Arg-I mRNA decreased compared to untreated cells and therefore, crocin could not stop M1 induction by LPS+INF-γ. In accordance with our results, when Zhang et al. pre-treated monocytes with the antioxidant butylated hydroxyanisole (BHA) prior to LPS and IFN-γ exposure, BHA could not inhibit M1 state induction [28].

In untreated M0 macrophages, crocin treatment didn't cause any significant changes in iNOS mRNA level, while decreased Arg-I mRNA expression and increased TNF-α secretion, which were indicative of induction of a M1-like phenotype. In an *in vivo* study when crocin (40 mg/kg) was examined in mice to evaluate its protective effects against d-galactose-mediated oxidative stress and inflammatory response, in healthy control animals, it significantly augmented TNF-α level in serum, but not IL-6 level [26].In an earlier study designed to evaluate the protective effects of crocin against rat hepatic damage induced by D-galactose, crocin by itself (30 mg/kg/day, intraperitoneally) did not change iNOS protein levels in hepatic tissue of healthy control rats. However, when administered concurrently with D-galactose, crocin

was able to inhibit D-galactose-mediated iNOS induction [27]. Shen et al. demonstrated that crocin can decrease the differentiation of RAW264.7 cells into M2-like macrophages, and increased secretion of the cytokines associated to M1-like phenotype [28]. The same results have been reported for some other well-known antioxidants. The antioxidant Berberine isolated from *Coptis chinensis* suppressed intestinal tumorigenesis in the mice model. This antitumor effect of berberine was shown to be mediated by a decrease in M2 macrophage polarization [29]. Accumulating data revealed that ginsenosides as an antioxidant and the main active constituent of ginseng, had a potential to efficiently alter TAM to the M1 subset of macrophages [30, 31]. Since crocin has been used as a supplement in a number of clinical trial studies [32-36], these findings highlight that crocin may possibly be able to modify the immune system of the recipient even in the absence of any inflammatory condition and also put emphasis on reassessing crocin administration as a preventive approach toward the diseases with a well-known etiology of M1 macrophage dominancy.

In conclusion, it seems that crocin modulatory effects on macrophage M1/M2 phenotype switching is at least partly dependent on the primary commitment state of the cells when exposed to crocin. Crocin in concurrent treatment with M1 inducing mediators, LPS/INF-γ, can suppress M1 induction. However, when crocin is solely applied to the macrophages, it acts as an M1 inducer by itself. Pretreatment of cells by crocin before the addition of LPS/INF-γ, cannot converse the M1 induction in macrophages. As crocin is widely used as an active component of some supplements in different countries, more detailed studies, especially in vivo investigations are required to clarify the differential immunomodulatory capabilities of crocin and related involved mechanisms in clinical health and disease states.

Abbreviations

Arg-1 Arginase-I

Ct threshold cycle

DMSO Dimethyl sulfoxide

IFN-γ Interferon-γ

IL Interleukin

iNOS Inducible nitric oxide synthase

NO Nitric oxide

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Th T helper

TNF-α Tumor necrosis factor-α

Declarations

Funding statement: The authors gratefully acknowledge the Iran National Science Foundation and the Vice-Chancellor of Research in Mashhad University of Medical Sciences, Mashhad, Iran (grant number 940740) for approval and financial support of this research.

Conflict of interest statement: The authors declare that there is no conflict of interest.

Author contributions: Hossein Hosseinzadeh conceived the original idea, obtained the funding, and approved the final version of the manuscript. **Fatemeh Mosaffa** designed the research proposal and interpreted data, participated in revising the article, and supervised the project. **Hakimeh Abdi** carried out the experiments, was involved in the analysis and interpretation of results and wrote the manuscript with support from **Fatemeh Mosaffa**. **Marjan Roshanravan** carried out the experiments and was involved in the analysis and interpretation of results.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

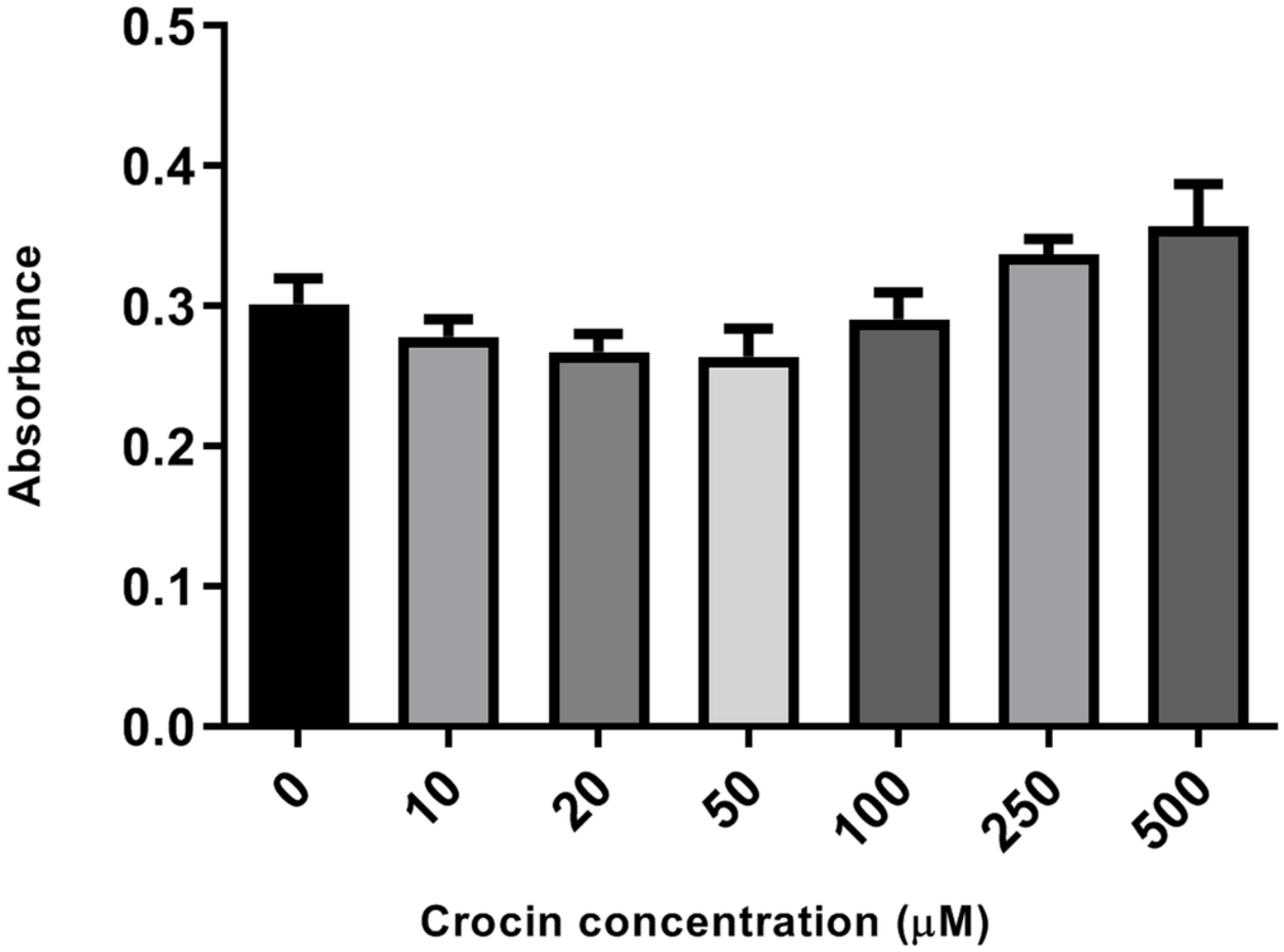

Figure 1

The effects of crocin on macrophage viability. After exposure to crocin (1-500 μM) for 24 h, cell viability was evaluated using MTT assay. All data were reported as mean ± SEM of four independent experiments (n= 4). (Dunnett's multiple comparisons test after One-way ANOVA)

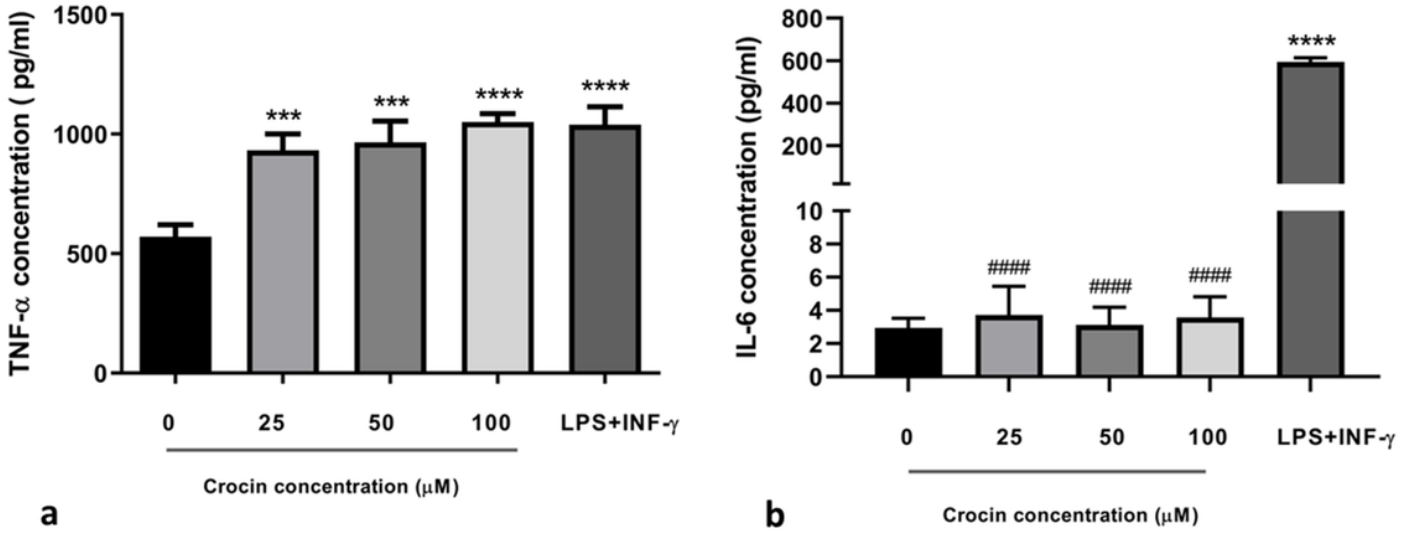

Figure 2

The effects of crocin on IL-6 and TNF-α secretion. Following 24 h treatment with crocin (25- 100 μM), cytokines concentrations in the liquid above the cells were measured using ELISA methods. (a) TNF-α; (b) IL-6. All data were reported as mean ± SEM of 3 independent experiments (n= 3). (****, ####) $p < 0.0001$, (***) $p = 0.0004$ for crocin 25 μM and $p = 0.0002$ for crocin 50 μM. Significant difference with: untreated group (*), and LPS+ IFN-γ (#) (Post hoc test of Tukey–Kramer after One-way ANOVA)

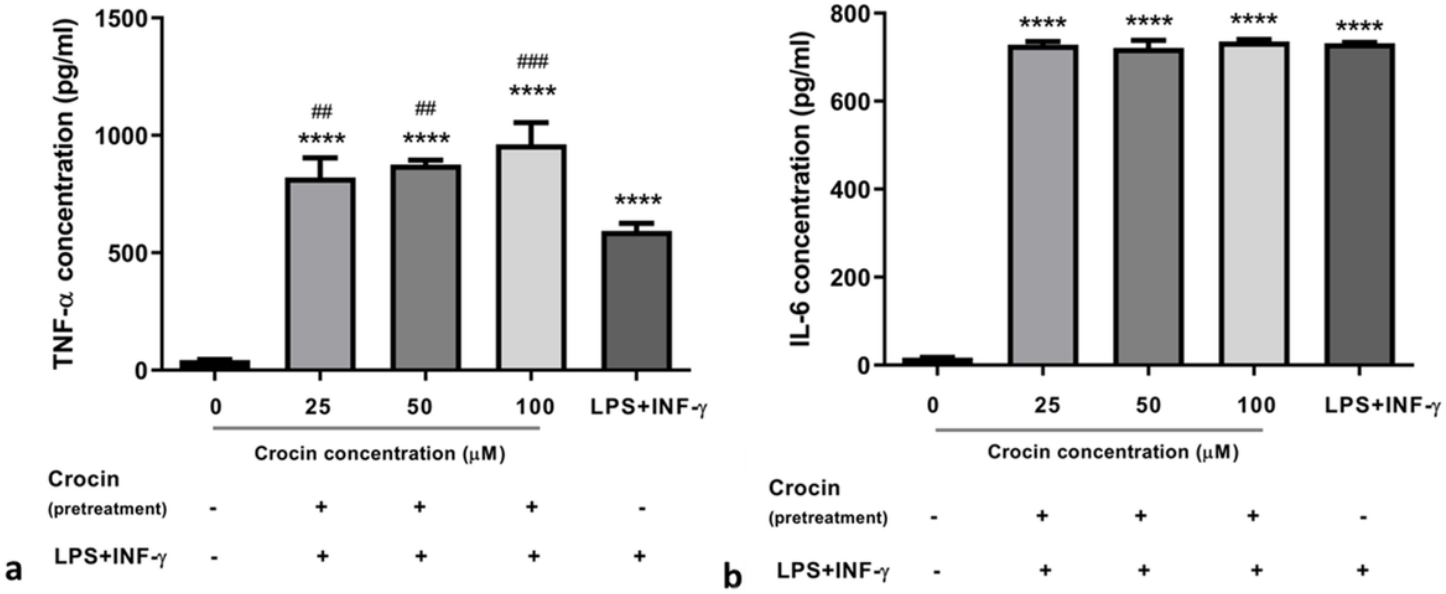

Figure 3

The effects of crocin pretreatment on IL-6 and TNF-α secretion. Following 24 h treatment with crocin (25-100 μM), macrophages were treated with INF-γ (20ng / ml) and LPS (100 ng / ml) for additional 12 h.

Cytokines concentrations in the liquid above the cells were measured using ELISA methods. (a) TNF-α; (b) IL-6. All data were reported as mean ± SEM of 3 independent experiments (n= 3). (****) $p < 0.0001$, (###) $p = 0.0001$, (##) $p = 0.0051$ for crocin 25 µM and $p = 0.0010$ for crocin 50 µM in a. Significant difference with: untreated group (*), and LPS+ IFN-γ (#) (Post hoc test of Tukey–Kramer after One-way ANOVA)

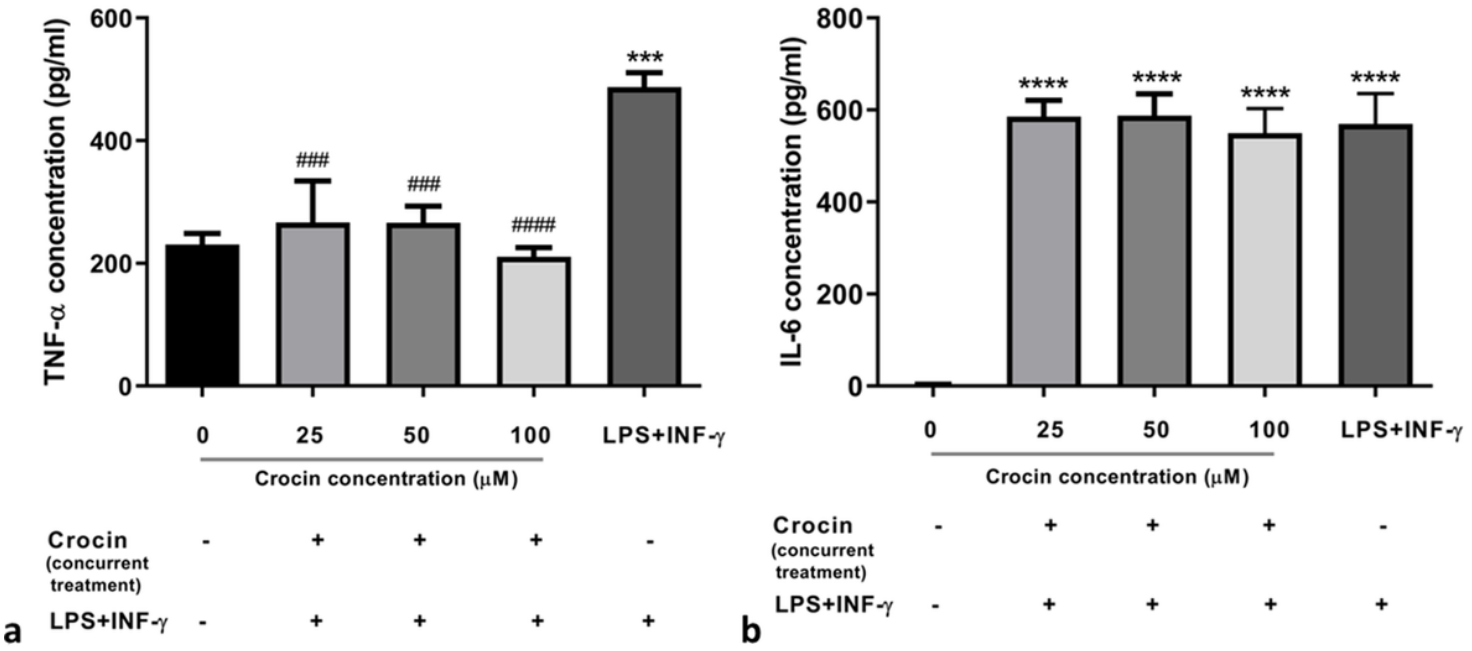

Figure 4

The effects of concurrent treatment of crocin and LPS + INF-γ on IL-6 and TNF-α secretion. Following 24 h treatment with crocin (25- 100 µM), and INF-γ (20ng / ml) and LPS (100 ng / ml), cytokines concentrations in the liquid above the cells were measured using ELISA methods. (a) TNF-α; (b) IL-6. All data were reported as mean ± SEM of 3 independent experiments (n= 3). (####, ****) $p < 0.0001$, (###,***) $p = 0.0001$. Significant difference with: untreated group (*), and LPS+ IFN-γ (#) (Post hoc test of Tukey–Kramer after One-way ANOVA)

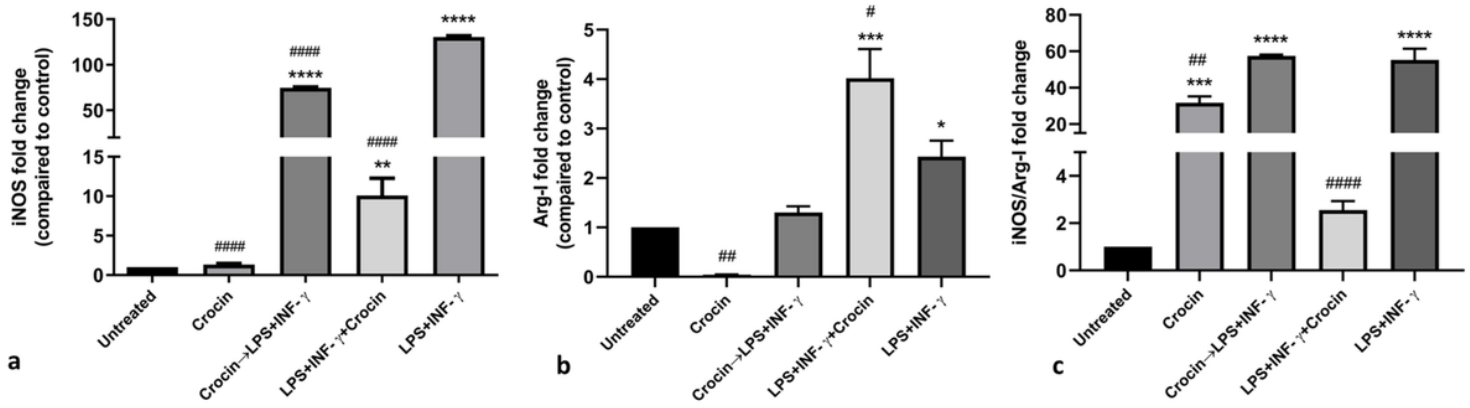

Figure 5

Evaluation of Arg-I and iNOS mRNA expression levels. Following treatment, pretreatment and posttreatment with crocin 50 μM for 24 h and INF-γ (20ng / ml) and LPS (100 ng / ml) for 12 h. Arg-I and iNOS mRNA expression were measured using RT-qPCR method. (a) iNOS relative mRNA expression; (b) Arg-I relative mRNA expression; (c) iNOS/Arg-I expression ratio. All data were presented as mean ± SEM of 3 independent experiments (n= 3). (####, ****) $p < 0.0001$, (***) $p = 0.0003$ in a and $p = 0.0004$ in b, (**) $p = 0.0032$, (##) $p = 0.0019$ in b and $p = 0.0030$ in c , (*) $p = 0.0489$, (#) $p = 0.0280$. Significant difference with: untreated group (*), and LPS+ IFN-γ (#) (Post hoc test of Tukey–Kramer after One-way ANOVA)