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Effect of 6-Gingerol on Pro-Inflammatory Cytokine Production and Costimulatory Molecule Expression in Murine Peritoneal Macrophages[1]

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Background. **Pro-inflammatory cytokines produced primarily by macrophages are key elements in many surgical conditions including sepsis, ischemia-reperfusion injury, and transplant rejection. Herbal products are being used as alternative treatments in such inflammatory conditions. Ginger is known for its ethno-botanical applications as an anti-inflammatory agent. 6-gingerol is one of the active ingredients of ginger that imparts ginger with its anti-inflammatory properties. We hypothesized that the anti-inflammatory effect of 6-gingerol is because of inhibition of macrophage activation, more specifically by an inhibition of pro-inflammatory cytokines and antigen presentation by lipopolysaccharide (LPS) activated macrophages.**

Methods. **To study the effect of 6-gingerol on pro-inflammatory cytokines, we measured the liberation of TNF-α, IL-1β, and IL-12 by murine peritoneal macrophages exposed to several doses of 6-gingerol in the presence of LPS stimulation. We also studied the effect of 6-gingerol on the cell surface expression of B7.1, B7.2, and MHC II. Finally, we examined the APC function of the 6-gingerol treated macrophages by a primary mixed lymphocyte reaction.**

Results. **6-gingerol inhibited the production of pro-inflammatory cytokines from LPS stimulated macrophages but had no effect on the LPS-induced expression of B7.1, B7.2, and MHC II. The APC function of LPS stimulated macrophages was also unaffected by 6-gingerol treatment.**

Conclusion. **Our data indicate that 6-gingerol selectively inhibits production of pro-inflammatory cytokines from macrophages but does not affect either the APC function or cell surface expression of MHC II and costimulatory molecules. We, thus, provide a mechanistic insight into the anti-inflammatory properties of 6-gingerol that may be useful to treat inflammation without interfering with the antigen presenting function of macrophages.**

Key Words: **gingerol; pro-inflammatory cytokines; costimulatory molecules; macrophage.**

INTRODUCTION

Macrophage activation is a key step in mounting an inflammatory response. Activated macrophages play an important role in both the antigen-dependent and antigen-independent inflammatory pathways [1]. The toll like receptors (TLRs) expressed on these cells are responsible for activation of the inflammatory pathways. Interaction of TLRs with their ligands leads to activation of macrophages and in turn initiates an inflammatory reaction [2–5].

Natural products containing bioactive phytochemicals are potentially important sources of anti-inflammatory drugs [6]. Ginger is one such natural product that is known for its therapeutic use in inflammatory disorders [7]. In addition to several studies on the anti-inflammatory properties of ginger, we have found that ginger prolongs survival of mice heart allografts *in vivo* and inhibits several macrophage functions *in vitro*. Whole ginger extract effectively inhibits production of pro-inflammatory lymphokines in macrophages activated through their TLRs by lipopolysaccharide (LPS), and reduces the up-regulation of MHC class II and co-stimulatory molecules in these macrophages. Consequent to these effects, macrophage mediated antigen presentation to T cells seems to be inhibited by ginger (unpublished data). Thus, whole ginger extract appears to have a global inhibitory effect on macrophage function *in vitro* that probably accounts for its anti-inflammatory effect *in vivo*.

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Ginger has several active ingredients that are known to be anti-inflammatory *in vivo* and *in vitro*. 6-gingerol, one of the active vanilloids present in ginger, reduces inflammatory response to carrgeenin *in vivo* [8], and inhibits generation of reactive oxygen and nitrogen species *in vitro* [9]. Because we had observed a global inhibition of macrophage function with whole ginger extract, we wished to determine the effect of 6-gingerol on macrophage function. Our hypothesis was that 6-gingerol has a less global and a more selective effect on macrophage function. To test this hypothesis, we investigated the effect of 6-gingerol on LPS-induced production of the pro-inflammatory cytokines TNF-α, IL-12, and IL-1β, and the pro-inflammatory chemokines RANTES (CCL5) and MCP-1 because production of all these was inhibited by whole ginger extract. We further investigated the effect of 6-gingerol on LPS-induced up-regulation of MHC class II and of the co-stimulatory molecules B7.1 (CD80) and B7.2 (CD86) to determine if 6-gingerol has a selective or a global effect on macrophage activation. Finally, we investigated if 6-gingerol affected the antigen presentation function of LPS activated macrophages.

MATERIALS AND METHODS

Materials

6-gingerol was obtained from Calbiochem (San Diego, CA) and dissolved in a 0.1% DMSO solution and further diluted with RPMI 1640 medium. LPS was obtained from Sigma chemicals (St. Louis, MO).

Animals

Male and female C57Bl and Balb/C mice aged 6 to 8 weeks old were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in a pathogen free animal facility at the SUNY Upstate Medical University. Mice had free access to food and water. All experiments were approved by the Committee on the Care and Humane Use of Animals.

Peritoneal Macrophage Isolation

There was 10 mL of ice cold sterile phosphate-buffered saline (PBS) injected into the peritoneal cavity of C57Bl mouse. The harvested peritoneal lavage was washed with PBS and cells were resuspended in RPMI 1640 supplemented with 10% fetal bovine serum (FBS).

Cell Culture

Peritoneal macrophages (10^6 cells/mL) isolated from C57Bl mice were treated with 6-gingerol (1 ng/mL, 10 ng/mL, 100 ng/mL, 1 μg/mL, 10 μg/mL, 100 μg/mL) in the presence of LPS (100 ng/mL) stimulation and incubated for 24 h in a humidified 5% CO_2 incubator. The culture supernatants were stored at -20°C and later assayed for cytokines and chemokines by ELISA. The cells were harvested and stained for flow cytometry analysis of costimulatory molecules and MHC II expression.

Cytokine and Chemokine Assay by ELISA

Culture supernatants from 6-gingerol/LPS treated macrophages were assayed for pro-inflammatory cytokines namely TNF-α, IL-12, and IL-1β and chemokines namely RANTES, MCP-1 by ELISA using assay kits from R&D Systems (Minneapolis, MN) according to manufacturer's instructions.

Flow Cytometry

Murine peritoneal macrophages (10^6 cells/mL) were treated with 6-gingerol (1 μg/mL) for 24 h in the presence of LPS (100 ng/mL) stimulation were harvested and stained with B7.1-PE, B7.2-FITC, and MHC II-APC mAb conjugates (eBiosciences, San Diego, CA) and analyzed in a LSRII flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using the WinMDI software (TSRI Flow Cytometry Core Facility).

Mixed Lymphocyte Reaction

C57Bl peritoneal macrophages (10^6 cells/mL) were treated with LPS in the presence or absence of 6-gingerol for 24 h in a humidified 5% CO_2 incubator. Syngeneic T cells (10^6 cells/mL) purified from the splenocytes were added to the treated macrophages in the presence of alloantigen stimulation. Gamma irradiated splenocytes (10^5 cells/mL) from Balb/C were used as the stimulator cells. After 7 days of incubation T cell proliferation was measured by a non radioactive cell proliferation assay using MTS (CellTiter 96 AQ_{ueous}; Promega, Madison, WI).

NFκB Assay by ELISA

C57Bl peritoneal macrophages (10^6 cells/mL) were treated with 6-gingerol (1 μg/mL) for 24 h in the presence of LPS (100 ng/mL) stimulation. The cells were then harvested and nuclear extract were prepared using nuclear extraction kit from Panomics Inc (Fremont, CA). The extracted nuclear fraction was then assayed for NFκB by ELISA (TransFactor Kit; Clontech, San Diego, CA).

Statistical Analysis

All experiments were done in triplicates and repeated three times. Data are represented as the arithmetic mean $\pm$ SD. Comparison between the groups was analyzed using Student's *t*-test. The accepted level of significance was $P < 0.05$.

RESULTS

6-Gingerol Inhibits Pro-Inflammatory Cytokine Production by LPS Stimulated Macrophages

An increase in TNF-α, IL-12, and IL-1β production was observed in macrophages treated with LPS. We observed a decrease in the secretion of all these cytokines by LPS stimulated macrophages in presence of 6-gingerol. TNF-α production was increased in the presence of LPS stimulation to 64.57 pg/mL in comparison to control cells (not detected), whereas in the presence of 6-gingerol it was decreased to 55.25 pg/mL ($P \leq 0.1$). IL-12 production was also increased in the presence of LPS stimulation (23.8 pg/mL) in comparison to control (8.3 pg/mL) and in the presence of 6-gingerol no IL-12 was detected ($P \leq 0.0001$). IL-1β production was also decreased in the presence of 6-gingerol and LPS (10.22 pg/mL, $P \leq 0.002$) in comparison to only LPS

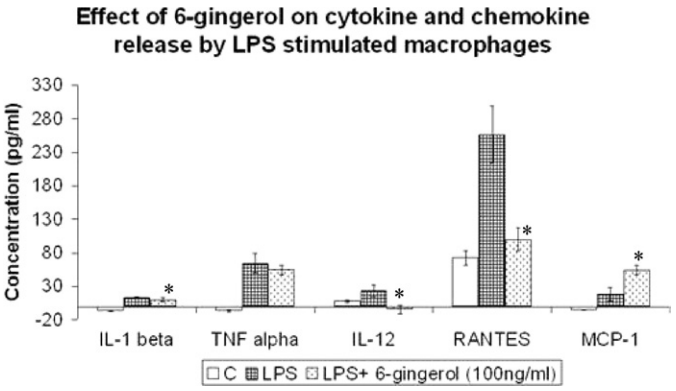

FIG. 1. Effect of 6-gingerol on cytokine and chemokine secretion by murine peritoneal macrophages in the presence of LPS stimulation. Peritoneal macrophages from C57Bl/6 mouse were stimulated with 100 ng/mL LPS in the presence or absence of 6-gingerol for 24 h. The cell supernatants were collected and cytokine and chemokine production was determined by ELISA. C, Control; LPS, LPS stimulated; LPS +6-gingerol, LPS stimulation in the presence of 6-gingerol. Data represent mean ± SD ($n = 9$). *$P \leq 0.05$ in comparison to LPS stimulated macrophages.

stimulation (13.97 pg/mL). No IL-1β was detected in control cells. To further investigate the effect of 6-gingerol on chemokine production by LPS stimulated macrophages we measured RANTES and MCP-1 production by ELISA in the cell culture supernatant. We observed an inhibitory effect of 6-gingerol on RANTES production by LPS stimulated macrophages (control, 72.96 pg/mL; LPS, 256.9 pg/mL; LPS +6-gingerol-100.38 pg/mL; $P \leq 0.0001$), but in the case of MCP-1 production we observed an opposite, stimulatory, effect (control-not detected, LPS-18.92 pg/mL, LPS +6-gingerol-54.79 pg/mL, $P \leq 0.0001$) as shown in Fig. 1.

Effect of 6-Gingerol on the Expression of Costimulatory Molecules and MHC II on Peritoneal Macrophages

LPS induced activation of macrophages results in up-regulation of costimulatory molecules along with an increase in inflammatory cytokine production. Therefore we further investigated the effect of 6-gingerol on the expression of costimulatory molecules namely, B7.1 and B7.2 on LPS stimulated macrophages. At the same time we also examined MHC II expression as it also indicates that activation state of macrophages. 6-gingerol did not have any effect on LPS induced expression of B7.1 and B7.2 on peritoneal macrophages as shown in Figs. 2a and 2b. Figure 2c shows the effect of 6-gingerol on LPS induced MHC II expression on the macrophages which was also not affected by 6-gingerol.

6-Gingerol Did Not Affect the Antigen Presenting Abilities of Macrophages

To further examine the effect of 6-gingerol on the APC function of macrophages we determined the effect of 6-gingerol on macrophage mediated alloantigen presentation. LPS stimulated C57Bl/6 macrophages in the presence or absence of 6-gingerol were used as antigen presenting cells and CD4+ cells (C57Bl/6) were used as responder cells. Irradiated BALB/c splenocytes were used as the source of alloantigen. As expected, we observed an increase in C57Bl/6 T cell proliferation when LPS activated macrophages were used as the sole source of APCs in comparison to non stimulated C57Bl/6 macrophages. 6-gingerol did not inhibit the activated macrophage induced T cell proliferation indicating that it did not have any effect on the APC function of LPS activated macrophages. With non stimulated macrophages as APC the proliferation index (PI) was 13.92 that increased to 16.9 with LPS stimulated macrophages. In the presence of 6-gingerol the PI with LPS stimulated macrophage was the same as in its absence [LPS +6-gingerol (100 ng/mL), 15.59; LPS +6-gingerol (1 μg/mL), 16.35].

6-Gingerol Inhibits LPS Induced NFκB Induction

Because we observed that 6-gingerol inhibits the LPS induced up-regulation of inflammatory cytokine production, we studied the effect of 6-gingerol on NFκB induction. We observed an inhibition in NFκB induction by 6-gingerol as shown in Fig. 3.

DISCUSSION

The present study on 6-gingerol, an active component of whole ginger, was prompted by our observation that whole ginger extract has a profound and global inhibitory effect on macrophage activation. Our results demonstrate that, in contrast to the profound and global effect of whole ginger, 6-gingerol inhibits only certain selective components involved in macrophage activation. It inhibits LPS induced cytokine and lymphokine production in macrophages, similar to ginger but unlike whole ginger, it has no effect on the up-regulation of either MHC class II, costimulatory molecules or macrophage antigen presentation. Taken together, these results demonstrate for the first time that 6-gingerol selectively inhibits pro-inflammatory lymphokine production in activated macrophages.

Although several studies have addressed the immunological effect of whole ginger, there have been relatively few studies on the immunological effects of 6-gingerol. Only one study by Young and co-workers has investigated the *in vivo* effects of 6-gingerol on inflammation and found that it suppresses the inflammatory response to carrageenin *in vivo* [8]. Our *in vitro* study complements their *in vivo* findings and suggests that the mechanism behind 6-gingerol's *in vivo* anti-inflammatory effect could be from its inhibition of pro-inflammatory lymphokine production in activated macrophages.

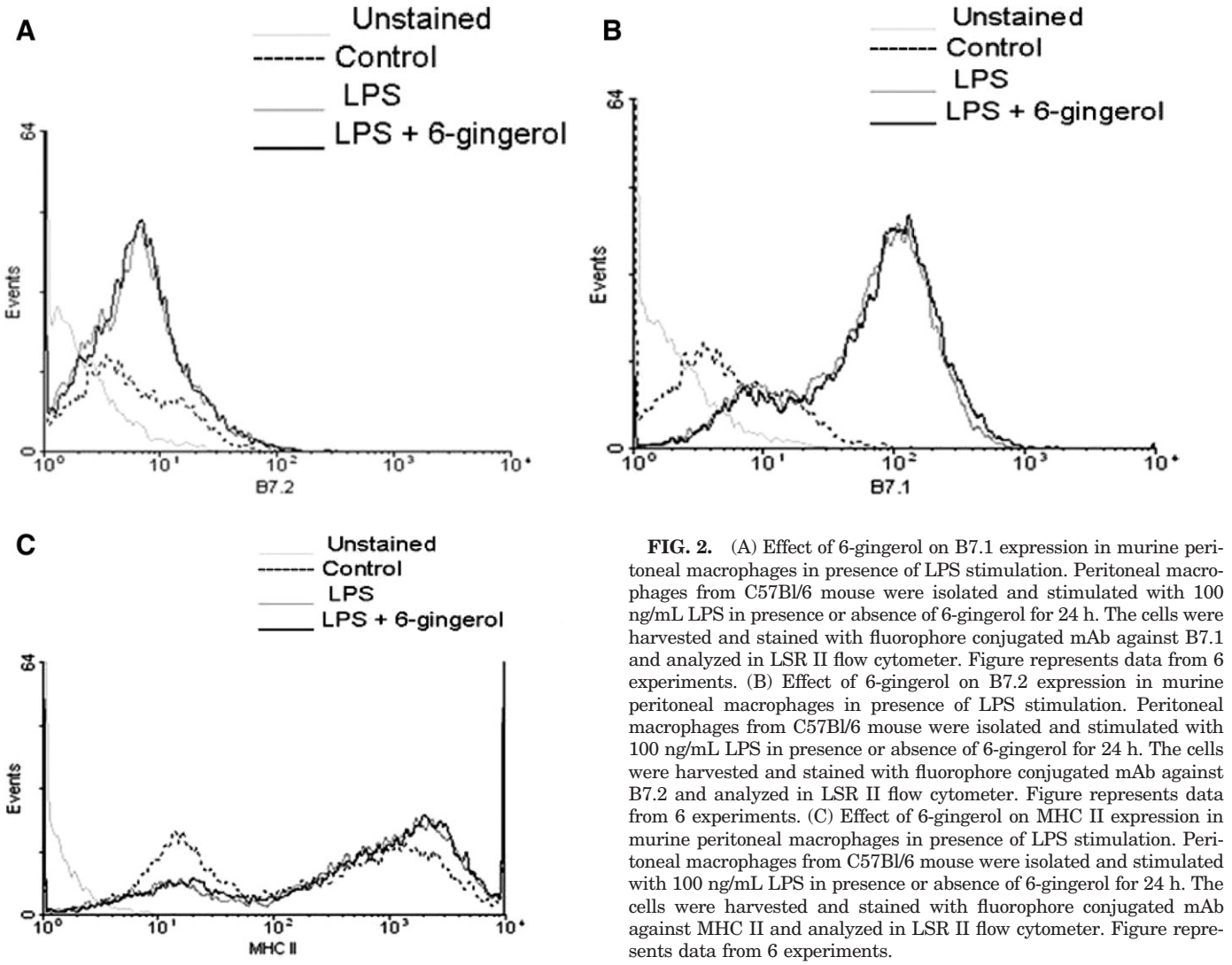

FIG. 2. (A) Effect of 6-gingerol on B7.1 expression in murine peritoneal macrophages in presence of LPS stimulation. Peritoneal macrophages from C57Bl/6 mouse were isolated and stimulated with 100 ng/mL LPS in presence or absence of 6-gingerol for 24 h. The cells were harvested and stained with fluorophore conjugated mAb against B7.1 and analyzed in LSR II flow cytometer. Figure represents data from 6 experiments. (B) Effect of 6-gingerol on B7.2 expression in murine peritoneal macrophages in presence of LPS stimulation. Peritoneal macrophages from C57Bl/6 mouse were isolated and stimulated with 100 ng/mL LPS in presence or absence of 6-gingerol for 24 h. The cells were harvested and stained with fluorophore conjugated mAb against B7.2 and analyzed in LSR II flow cytometer. Figure represents data from 6 experiments. (C) Effect of 6-gingerol on MHC II expression in murine peritoneal macrophages in presence of LPS stimulation. Peritoneal macrophages from C57Bl/6 mouse were isolated and stimulated with 100 ng/mL LPS in presence or absence of 6-gingerol for 24 h. The cells were harvested and stained with fluorophore conjugated mAb against MHC II and analyzed in LSR II flow cytometer. Figure represents data from 6 experiments.

Further, it is known that pro-inflammatory lymphokines stimulate generation of reactive oxygen and nitrogen species in macrophages [10, 11]. Thus, when the production of pro-inflammatory lymphokines in macrophages is inhibited, the generation of the noxious reactive species is likely to be reduced. Indeed, 6-gingerol has been shown to suppress the generation of reactive oxygen and nitrogen species in activated J774.1 mouse macrophages [9] that could be secondary to its inhibition of pro-inflammatory lymphokines as demonstrated in our study. Thus, our data suggest another mechanism by which 6-gingerol inhibits inflammation *in vivo* via a secondary effect on the generation of reactive oxygen species.

Another difference between the effect of whole ginger and 6-gingerol on activated macrophages is that the former inhibited the production of MCP-1 while 6-gingerol appeared to increase its production.

Our findings that 6-gingerol inhibits production of pro-inflammatory lymphokines but has no effect on the upregualtion of costimulatory molecules provides some clues to the intracellular signal transduction pathway inhibited by 6-gingerol. Upon macrophage activation by LPS, signals are transduced via two pathways: one dependent on MyD88, the other dependent on TRIF. The former activates the transcription factor NFκB that stimulates the transcription of pro-inflammatory lymphokine genes. The latter, the TRIF dependant pathway, is involved in the up-regulation of costimulatory molecules. Since 6-gingerol selectively inhibits lymphokine production, it is likely that this effect is from inhibition of the MyD88 dependent pathway; perhaps at the level of NFκB. Support for this concept comes from the observation that 6-gingerol inhibits NFκB in phorbol ester-stimulated mouse skin [12, 13]. Although these latter effects on NFκB are seen in a different tissue, additional experiments to test this hypothesis could uncover NFκB as a molecular target of 6-gingerol in the treatment of inflammatory conditions.

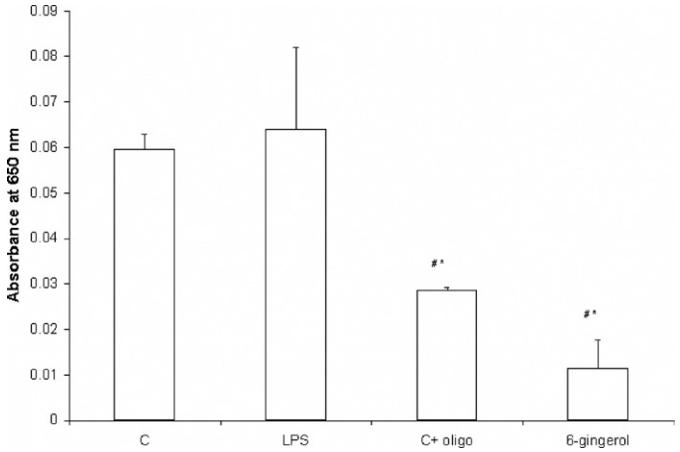

FIG. 3. Effect of 6-gingrol on LPS induced NFκB activity in murine peritoneal macrophages. Peritoneal macrophages were incubated with LPS in the presence or absence of 6-gingerol for 24 h. NFκB activity was assayed in the nuclear fraction by ELISA method. Control, macrophages were not pre-treated; LPS, macrophages pre-treated with LPS only; C + Oligo, control nuclear extract + competitive oligo; 6-gingerol, macrophages pre-treated with LPS and 6-gingerol. Data represent mean ± SD ($n = 3$). #$P \leq 0.05$ in comparison with LPS. *$P \leq 0.05$ in comparison with Control.

Our results indicate that 6-gingerol decreases the LPS induced activation of NFκB, however, to identify the precise targets of 6-gingerol in the MyD88 dependent pathway needs a more detailed molecular approach.

In summary, we have demonstrated that 6-gingerol selectively inhibits lymphokine production in LPS stimulated macrophages. Because LPS is one of the strongest activators of macrophages, our findings have relevance to macrophage activation, a central event in inflammation. This selective inhibition of lymphokine production by 6-gingerol has the potential to be used in designing molecular targeted therapies in inflammatory diseases.

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