# Epigallocatechin Gallate Modulates Cytokine Production by Bone Marrow–Derived Dendritic Cells Stimulated with Lipopolysaccharide or Muramyldipeptide, or Infected with *Legionella pneumophila*

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**Theprimary polyphenol in green tea extract is the catechin epigallocatechin gallate (EGCG). Various studies have shown significant suppressive effects of catechin on mammalian cells, either tumor or normal cells, including lymphoid cells. Previous studies from this laboratory reported that EGCG has marked suppressive activity on murine macrophages infected with the intracellular bacterium *Legionella pneumophila* (Lp), an effect mediated by enhanced production of both tumor necrosis factor-α (TNF-α) and γ-interferon (IFN-γ). In the present study, primary murine bone marrow (BM)–derived dendritic cells (DCs), a phagocytic monocytic cell essential for innate immunity to intracellular microorganisms, such as Lp, were stimulated *in vitro* with the microbial stimulant lipopolysaccharide (LPS) from gram-negative bacteria, the cell wall component from gram-positive bacteria muramyldipeptide (MDP) or infected with Lp. Production of the T helper cell (Th1)-activating cytokine, interleukin-12 (IL-12) and the proinflammatory cytokine, tumor necrosis factor-α (TNF-α),produced mainly by phagocytic cells and important for antimicrobial immunity, was determined in cell culture supernatants by enzyme-linked immunosorbent assay (ELISA). Treatment of the cells with EGCG inhibited, in a dose-dependent manner, production of IL-12. In contrast, enhanced production of TNF-α occurred in a dose-dependent manner in the DC cultures stimulated with either soluble bacterial product or infected with Lp. Thus, the results of this study show that the EGCG catechin has a marked effect in modulating production of these immunoregulatory cytokines in stimulated DCs, which are important for antimicrobial immunity, especially innate immunity. Further studies are necessary to characterize the physiologic function of the effect of EGCG on TNF-α and IL-12 during Lp infection, and the mechanisms involved.** Exp Biol Med 230:645–651, 2005

**Key words:** catechin; cytokines; dendritic cells; *Legionella pneumophilia*

## Introduction

Green tea polyphenols include (–)-epigallocatechin gallate (EGCG), (–)-epigallocatechin, (–)-epicatechin gallate, and (–)-epicatechin. Epigallocatechin gallate is the major polyphenol present in green tea and is commonly known as tea catechin, accounting for greater than 40% of polyphenols in green tea (1).

Previous studies from this laboratory showed that the EGCG catechin has marked modulating effects on cytokine production by immune cells. For example, using the continuous murine alveolar macrophage cell line (MH-S cells), we showed that EGCG induced selective upregulation of the T helper cell (Th1) cytokines, interleukin-12 (IL-12) and tumor necrosis factor-α (TNF-α), important for antimicrobial cell–mediated immunity; and simultaneous downregulation of IL-10, an interleukin associated with biasing towards Th2 helper cells important in humoral antibody–based immunity (2). Other studies from our laboratory demonstrated upregulation of macrophage produced γ-interferon (IFN-γ), including mRNA for IFN-γ, in macrophage cultures treated with EGCG (3).

In the present study, experiments were performed to determine the effects of the EGCG catechin on cytokine production by dendritic cells (DCs), extremely important for innate immunity, especially against intracellular microorganisms. For this purpose, murine bone marrow (BM)–

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derived DCs were stimulated *in vitro* with gram-negative bacteria–derived lipopolysaccharide (LPS), a potent activator of phagocytic cells, such as macrophages and DCs. Muramyldipeptide (MDP) derived from cell walls of gram-positive bacteria was similarly tested for modulatory effects on cytokine responses by DCs. *Legionella pneumophila* (Lp), a ubiquitous opportunistic bacterium that preferentially replicates in phagocytes, including DCs considered essential for innate immunity to microbes, also was examined for effects of the catechin on cytokine production by the cell cultures. The results obtained showed that EGCG inhibited, in a dose-dependent manner, production of IL-12, a cytokine important in activation of antimicrobial cellular immunity. In contrast, the EGCG catechin enhanced, in a similar dose-dependent manner, DC production of TNF-α, a known proinflammatory cytokine. Thus, the results show the EGCG catechin has a marked effect in modulating cytokine profiles in stimulated DCs, which has significant implications for antimicrobial immunity, especially innate immunity.

## Materials and Methods

**Catechins and Stimulants.** EGCG was obtained from Sigma Chemical Co. (St. Louis, MO) and stored as 5 mg/ml stock solutions. Lipopolysaccharide from *Escherichia coli* and MDP were also obtained from Sigma. The vehicle for all solutions was sterile, pyrogen-free water.

**Animals.** Mice used were BALB/c mice (National Cancer Institute, Frederick, MD). Mice were 8–10 weeks of age at the start of an experiment and kept in groups of four in plastic mouse cages with barrier filters, and fed Purina mouse chow and water *ad libitum*. They were housed and cared for in the University of South Florida animal facility, which is fully accredited by the American Association of Laboratory Animal Care.

**Preparation of DCs.** Bone marrow cells were flushed from the femurs and tibias of the mice and the red cells were lysed with ammonium chloride potassium bicarbonate (ACK) lysing buffer. The cells were cultured in RPMI 1640 medium (Sigma Chemical), supplemented with 5% heat-inactivated fetal calf serum, 2 m*M* L-glutamine, 0.1% 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml recombinant murine granulocyte-macrophage colony stimulating factor (BD Pharmingen, San Diego, CA). The cells were cultured for 6–10 days before they were collected.

**Flow Cytometry.** Dendritic cells were harvested as outlined in "Preparation of DCs" and analyzed for the expression of various cell surface molecules by tri-color immunofluorescence with antibodies to CD11b labeled with fluorescein isothiocyanate, (FITC), CD11c labeled with phosphatidylethanolamine (PE), and F4/80 labeled with peridinin chlorophyll protein (PerCP) (all from BD Pharmingen). Cells in phosphate buffered saline (PBS) containing 2% heat-inactivated bovine growth serum were blocked with an anti-Fc receptor antibody (CD16/CD32) for 15 mins. Immunolabeling was performed for 30 mins on ice with the various conjugated antibodies. Cells were fixed with 1% paraformaldehyde, and the fluorescently labeled cells were analyzed by a FACStar flow cytometer (Becton Dickinson, Mountain View, CA).

**Cell Viability.** The XTT assay was used to assess the effects of EGCG on cell viability (*In Vitro* Toxicology Assay Kit XTT Based, TOX-2, Sigma Chemical). This assay is based on the ability of mitochondrial dehydrogenases of viable cells to cleave the tetrazolium ring of XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt), yielding orange formazan crystals, which are soluble in aqueous solutions. Dendritic cells were harvested as outlined in "Preparation of DCs" and dispensed in triplicates at a density of $1 \times 10^6$ cells/ml into a 96-well flat-bottom tissue culture plate. Plates were incubated with EGCG at various concentrations (0, 10, 50, and 100 μg/ml) in 5% $CO_2$ at 37°C for 24 hrs. Because EGCG produced an orange color at higher doses, the culture medium was replaced on Day 2 with fresh culture medium (200 μl) before adding 20 μl of XTT (20% of the medium volume), and cultures were incubated at 37°C for another 4 hrs. The plates were read on an Emax microphage reader (Molecular Devices, Menlo Park, CA), using a wavelength of 450 nm and a reference wavelength of 650 nm. Control wells contained cells alone. Cell survival was calculated as a percentage of MTT inhibition by the following formula: percent survival = (mean experimental absorbance ÷ mean control absorbance) × 100%.

**Bacteria.** Live Lp, a virulent serogroup isolate from Tampa General Hospital (Tampa, FL), was grown on buffered charcoal-yeast extract agar (BCYE; Difco, Detroit, MI) for 48 hrs. The bacterial suspensions were prepared in pyrogen-free saline, and the concentration of bacteria was determined by spectrophotometry.

**Infection.** Dendritic cells were infected for 30 mins with Lp at a ratio of 10 bacteria per cell, washed to remove nonphagocytized bacteria, and incubated in RPMI 1640 medium containing 5% fetal calf serum with no antibiotics. The cultures were then incubated for up to 48 hrs at 37°C in 5% $CO_2$.

**Treatment.** Dendritic cells, either infected or noninfected, were added at a concentration of $2 \times 10^5$ cells/ml to 24-well plastic plates (CoStar, Cambridge, MA) and various concentrations of EGCG (0, 10, 50, and 100 μg) were added to each well. For stimulation of noninfected cells, 10 ng/ml of LPS or 10 μg/ml of MDP were added to each well with the various concentrations of EGCG.

**Enzyme-Linked Immunosorbent Assay (ELISA).** The amount of IL-12 p40/p70 and TNF-α in the culture supernatants of DC cultures, 24 hrs after treatment, was determined by sandwich ELISA, using matched antibody pairs and protein standards for ELISA (BD Pharmingen) for IL-12 and Duoset ELISA development system (R&D Systems, Minneapolis, MN) for TNF-α. For this purpose, medium-bind, 96-well Costar enzyme immunoassay (EIA) plates were coated with a specific mono-

clonal cytokine antibody for IL-12 p40/p70 or TNF-α overnight at 4°C. Plates were blocked for 1 hr at 37°C with PBS plus 3% bovine serum albumin (BSA) for IL-12 p40/p70 (or 1% lipid-free BSA for TNF-α), and 0.05% Tween 20. Culture supernatants or serial dilutions of murine cytokine standard were added for 1 hr, followed by biotinylated anti-murine IL-12 p40/p70 or TNF-α, and then streptavidin-alkaline phosphatase (1:1000; BD Pharmingen) for 30 mins. After the substrate was added, plates were allowed to develop. The plates were washed between additions with three to five changes of nanopure water. The plates were read at 450 nm on an Emax microphage reader (Molecular Devices). Units were calculated from the cytokine standard curve, which was performed for each plate.

**Statistics.** The results were expressed as means ± SD of indicated number of experiments. Statistical significance was determined using Student's *t* test for unpaired observations. A value of $P < 0.05$ was considered significant.

## Results

Bone marrow–derived DCs were greater than 97% CD11c-FITC and CD11b-PE immunoreactive as determined by fluorescence-activated cell sorter (FACS). As apparent in Figure 1, murine-derived DCs stimulated with 10 ng/ml LPS, produced detectable levels of TNF-α in the culture supernatants 24 hrs after stimulation. The DC cultures treated with increasing amounts of EGCG showed marked enhancement of TNF-α when treated with a concentration of 50 μg/ml catechin. In contrast, a higher concentration of EGCG (100 μg/ml) markedly inhibited TNF-α production in the LPS-stimulated cultures (Fig. 1).

The effects of EGCG were examined further to determine effects on responses to other microbial immunomodulators. For this purpose, similar DC cultures were treated with 10 μg/ml MDP. The results of these experiments showed DCs stimulated with MDP and treated with the 50 μg/ml concentration of EGCG had an approximately 3-fold increase in TNF-α production. Furthermore, a 100 μg/ml EGCG concentration also resulted in a significant increase, but less than that induced by the lower concentration (Fig. 2).

Next, we examined the effect of EGCG on cytokine production by DCs after infection with Lp, a ubiquitous microorganism that causes severe opportunistic disease. The effects of EGCG on the pattern of production of TNF-α in cultures of DC infected with Lp were similar to those observed after stimulation with the soluble bacterial components LPS or MDP (Figs. 1 and 2, respectively), in which the 50 μg/ml EGCG concentration enhanced production of TNF-α to approximately 2.5 ng/ml, a level several-fold higher than observed in Lp-infected DCs treated with 100 μg/ml EGCG (Fig. 3).

Catechin also had marked effects on production of IL-

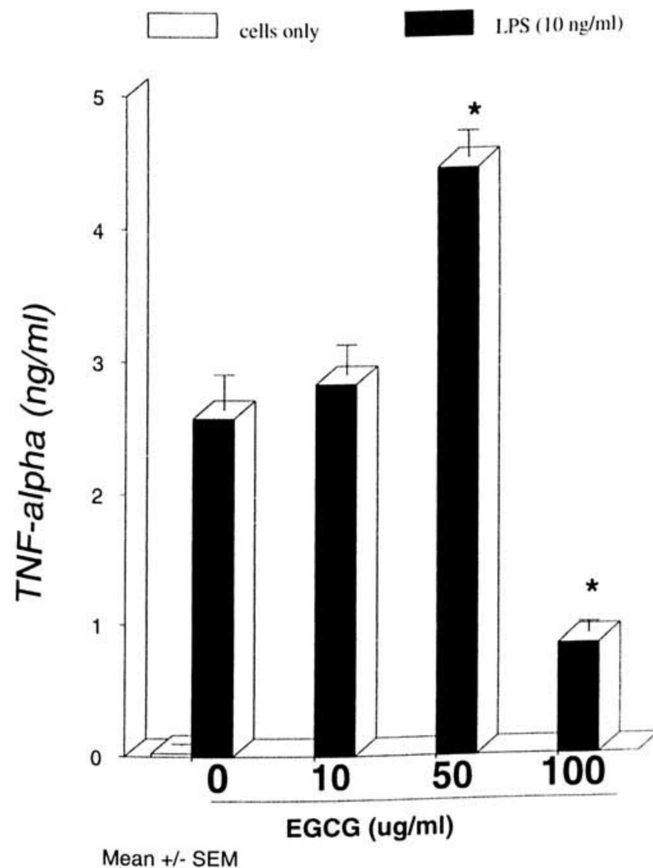


**Figure 1.** Effects of increasing concentrations of EGCG on TNF-α production in cultures of BM-derived DCs stimulated with LPS. Results expressed as mean value ± SEM (ng/ml) from five independent experiments. The asterisks indicate statistically significant differences of $P < 0.05$ from values of the non–EGCG-treated, LPS-stimulated cells.

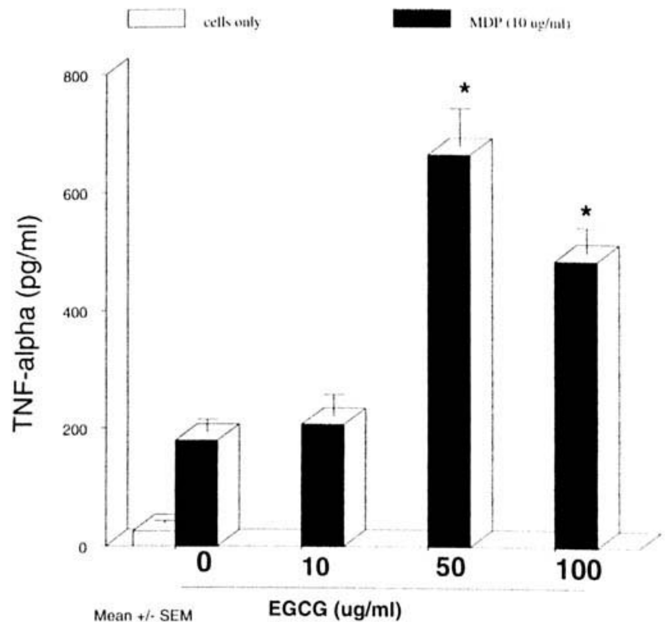


**Figure 2.** Effects of increasing concentrations of EGCG on TNF-α production in cultures of BM-derived DCs stimulated with MDP. Results expressed as mean value ± SEM (pg/ml) from five independent experiments. The asterisks indicate statistically significant differences of $P < 0.05$ from values from non–EGCG-treated, MDP-stimulated cells.

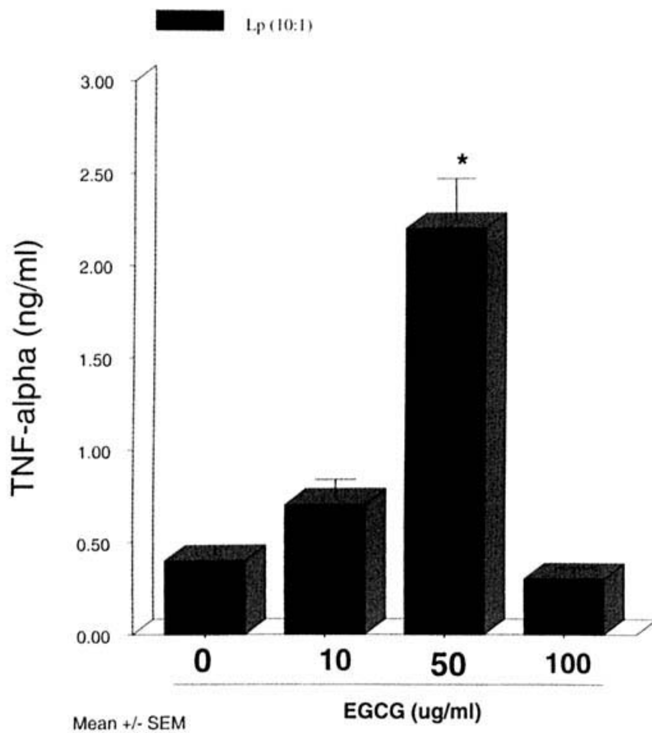


**Figure 3.** Effects of EGCG on TNF-α production by DCs infected for 24 hrs with Lp. The TNF-α levels in culture supernatants were determined by ELISA and results expressed as mean value ± SEM (ng/ml) from three independent experiments. The asterisk indicates statistically significant differences of $P < 0.05$ from values obtained with non–EGCG-treated, Lp-infected DCs.

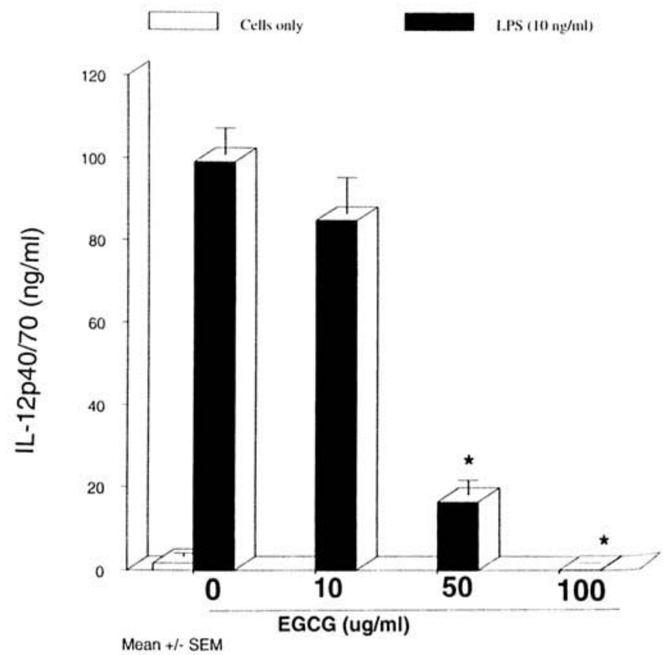


**Figure 4.** Effects of EGCG on IL-12 p40/p70 production by BM-derived DCs stimulated by LPS. Results expressed as mean value ± SEM (ng/ml) from five independent experiments, 24 hrs after stimulation of cells. The asterisk indicates statistically significant differences of $P < 0.05$ from values obtained with non–EGCG-treated, LPS-stimulated cells.

12 p40/p70 in the stimulated or infected DC cultures. As is apparent in Figure 4, LPS-treated cells without catechin evinced marked production of the IL-12 p40/p70cytokine. However, addition of EGCG to the cultures markedly inhibited production of IL-12 p40/p70. The 10 μg/ml concentration of EGCG had a slight inhibitory effect. Moreover, the 50 μg/ml concentration markedly depressed IL-12 p40/p70 production, whereas the 100 μg/ml concentration made it undetectable (Fig. 4). Similar suppressive effects were observed by catechin treatment of MDP-stimulated DCs. The 10 μg/ml catechin concentration reduced the production of this cytokine by half, whereas the 50 and 100 μg/ml concentrations essentially abolished the response (Fig. 5). Similarly, DCs infected with Lp and treated with EGCG showed a marked reduction of (50 μg/ml) or essentially abolished (100 μg/ml) the response (Fig. 6).

To determine whether the change in cytokine production levels might be caused by cytotoxic effects of EGCG, cell viability of DCs treated with various concentrations of EGCG (0, 50, and 100 μg/ml) was assessed using the XTT assay. As shown in Figure 7, treatment of DCs with EGCG at 10 and 50 μg/ml did not decrease cell viability, which indicates that increased TNF-α and decreased IL-12 production levels were not caused by EGCG toxicity at these concentrations. However, a significant ($P < 0.05$) decrease in cell viability (75% of control) was observed when DCs were treated with the higher concentration, of

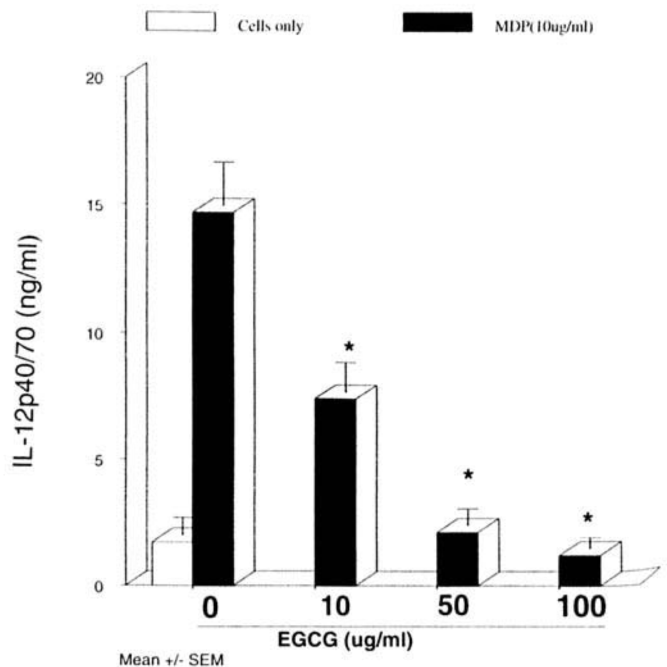


**Figure 5.** Effects of increasing concentrations of EGCG on IL-12 p40/p70 production in cultures of BM-derived DCs stimulated with MDP. Results expressed as mean value ± SEM (ng/ml) from five independent experiments. The asterisks indicate statistically significant differences of $P < 0.05$ from the values of the non–EGCG-treated, MDP-stimulated cells.

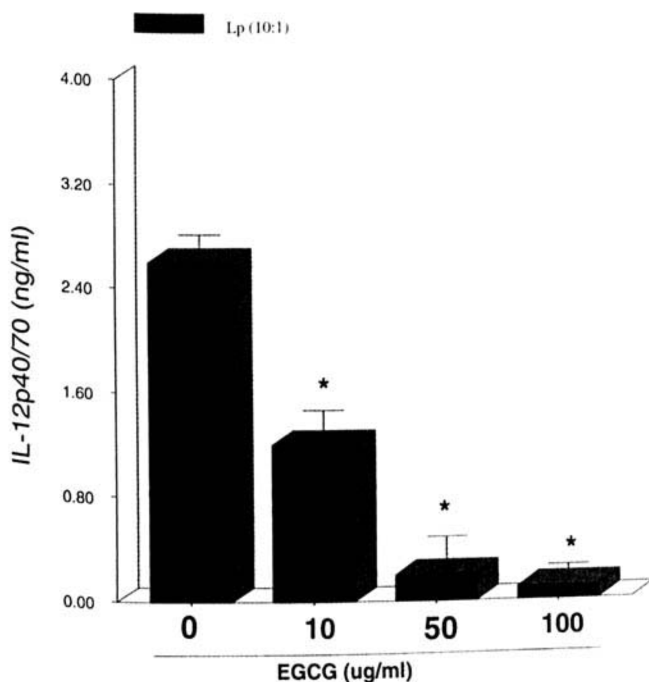


**Figure 6.** Effects of EGCG on IL-12 p40/p70 production by DCs infected for 24 hrs with Lp. Results expressed as mean value ± SEM (ng/ml) from three independent experiments. The asterisks indicate statistically significant differences of $P < 0.05$ from values obtained with non–EGCG-treated, Lp-infected DCs.

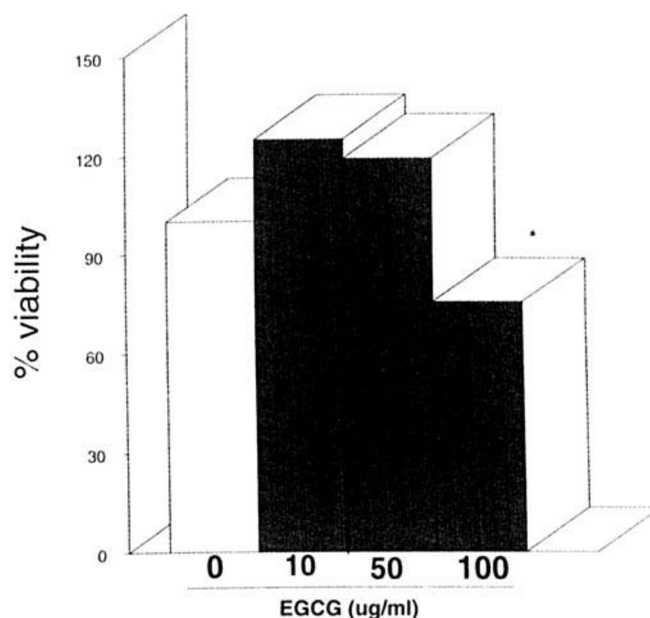


**Figure 7.** Bone marrow–derived DCs were exposed to various concentrations (0, 50, and 100 μg/ml) of EGCG for 24 hrs. Cell viability was analyzed with XTT assay. Percent viability was determined by measuring the optical density at 450 nm and a reference wavelength of 650 nm in a microplate reader. The results are expressed as an average of three independent experiments performed in triplicate. Asterisks indicate statistically significant differences of $P < 0.05$ from values obtained with non–EGCG-treated DCs.

100 μg/ml, which may explain why TNF-α production levels did not continue to increase at 100 μg/ml. This suggests that at least some of the decrease of IL-12 production at the highest concentration used in the present study may also be caused by cytotoxic effects of EGCG.

## Discussion and Conclusions

In related studies, it has been reported that EGCG inhibited IL-12 production by LPS-treated DCs (4). This study confirms these recent results and further shows that EGCG also inhibits IL-12 production by DCs that have been treated with MDP as well as infected with Lp.

The effects of catechins, such as EGCG, on stimulated IL-12 production by other subsets of immune cells are opposite from these studies on DCs. For example, we previously reported that EGCG selectively upregulated IL-12 production in a murine alveolar macrophage cell line (2). Topical application of EGCG before UVB exposure has also been reported to increase IL-12 production in draining lymph nodes of C3H/HeN mice (5). Thus, the effects of catechins appear to vary depending upon the type of immune host cell studied, and perhaps even on tissue localization.

The cytokine modulatory effects of catechins, such as EGCG, on pro-inflammatory cytokines also appears to be host-cell specific. EGCG has been reported to decrease LPS-induced TNF-α production in a dose-dependent manner in the murine macrophage cell line, RAW 264.7, and to similarly inhibit LPS-induced TNF-α production in elicited BALB/c mouse peritoneal macrophages; effects attributed, in part, to blocking NF-κβ activation (6). Previous studies from this laboratory have also shown that EGCG diminished nicotine-induced inhibition of TNF-α production by alveolar macrophages infected with Lp, and reversed cigarette smoke condensate downregulation of TNF-α in response to Lp infection (3). In cultured human peripheral blood mononuclear cells, EGCG was also reported to stimulate production of TNF-α (7). All of these reports again suggest that cytokine modulatory effects of catechins have varied effects, depending on the immune cell subpopulation studied.

Although not examined in the present study, previous studies have shown that EGCG in combination with other catechins found in plant products, such as green tea, can have synergistic effects. For example, EGCG in combination with epicatechin, a catechin also found in green tea, was reported to have an enhanced inhibitory effect on TNF-α release from BALB/3T3 cells treated with okadaic acid, a known tumor promoter, in comparison with EGCG alone (8).

Various mechanisms for the biologic effects of catechins have been reported. With respect to known anti-tumor activity of catechins, EGCG has been reported to selectively induce apoptosis in carcinoma cells but not in normal cells (9), an effect attributed to $G_0/G_1$ phase arrest of the cell cycle (10). Various other studies have reported that the anti-tumor effects of EGCG are caused by its effects on

cell-cycle regulatory proteins, such as its induction of the cyclin kinase inhibitor, WAF1/p21 (11). Other reports have linked the anti-tumor effects of catechins such as EGCG to the inhibition of proteolytic enzymes such as urokinase, an enzyme needed by cells to invade and form metastases, based on computer modeling and an amidolytic assay (12). Other studies have linked anti-carcinogenic effects of catechins such as EGCG to their modulatory effects on cytokines. For example, EGCG was reported to dose-dependently inhibit TNF-α production from a human stomach cancer cell line (KATO-III) treated with okadaic acid (13). Tumor necrosis factor-α is thought to be an endogenous tumor promoter (14).

Despite all of these reports demonstrating that catechins have modulatory effects on cytokine production by various host cells, it is not yet clear whether cytokine production alterations are responsible for the biologic effects of catechins, especially the antimicrobial effects. Furthermore, none of the experimental reports to date have collectively examined how immune cells are influenced by catechins such as EGCG or correlated such biologic effects.

In addition to the effects of EGCG on TNF-α and IL-12 examined in the present study, catechins have been reported to inhibit production of other important immunoregulatory factors, including vascular endothelial growth factor and IL-8, in normal human keratinocytes stimulated with TNF-α (15).

Assessment of the physiologic relevance of the findings presented here must take into account the maximum achievable EGCG concentrations attainable *in vivo*. For example, a 1600 mg oral dose of EGCG under fasting conditions has been reported to achieve a maximum human plasma level of 3.4 μg/ml (16). This level is three times less than the lowest concentration used in this *in vitro* study (10 μg), which is equivalent to eight times the highest reported daily intake from tea (16), making it likely that only pharmaceutical prepared formulations of green tea would reach plasma levels of the catechin used in this study. However, it is likely that concentrations of catechins at tissue sites are higher then in the blood. For example, 400-1000 times greater concentrations of EGCG in the oral cavity as compared with plasma have been obtained when green tea solution (1.2 g of green tea solids per 200 ml of water) is held in the mouth without swallowing (17). It is also important to note that EGCG has a relatively short half-life *in vivo*, ranging from 1.87–4.58 hrs for a 50–1600 mg dose (16), whereas DCs used in the present study were cultured with EGCG for a period of 24 hrs. The short half-life of catechins *in vivo* might be overcome, however, by repeated administration, which is feasible given the reported low toxicity of this catechin and given that even high doses, such as 1600 mg, are well tolerated by human subjects (16).

Intracellular growth of Lp has been reported to be severely restricted in DCs (18), which precluded an assessment of catechin modulatory effects on IL-12 and TNF-α on Lp growth in DCs in this study. However, infection with Lp clearly has significant cytokine modulatory effects on DCs, which likely mediates host response to intracellular pathogenic organisms such as Lp. Future studies are necessary to determine tea catechin modulatory effects on other cytokines known to be important for host responses to Lp, the mechanisms involved, and how such cytokine modulation affects the host response to infection with intracellular pathogenic organisms, such as Lp.

The seemingly opposed effects of EGCG on IL-12 and TNF-α production are not without precedent. Ho *et al.* (19) reported a similar effect, downregulation of IL-12 and upregulation of TNF-α, on human DCs treated with aspirin. Ahn *et al.* (4) found reduced expression of NF-κB p65 in murine DCs treated with EGCG, suggesting that inhibition of IL12 could be the result of lack of formation or translocation of the p65/p50 heterodimer, resulting in downregulation of IL-12 expression. In addition, Hackstein *et al.* found aspirin-induced downregulation of IL12 in murine DCs and attributed this to decreased NF-κB DNA-binding activity by targeting p50 (20).

One possible explanation for the effects observed on TNF-α may include an NF-κB–independent pathway in BM-derived DCs. Such an independent pathway would be capable of inducing TNF-α transcription despite the inhibitory effects of EGCG on NF-κB (p65) production. Several studies have described NF-κB–independent signal transduction pathways that result in LPS-induced TNF-α activation, including activation of the mitogen-activated protein kinases (21), ERK1 and ERK2 (22, 23); c-Jun N-terminal kinases (24, 25); and p38 (25, 26).

In conclusion, we present evidence that EGCG can inhibit IL-12 production while enhancing TNF-α production in BM-derived DCs simulated with LPS or MDP, or infected with Lp. Elucidation of the molecular mechanisms for the divergent effects of EGCG on IL-12 and TNF-α production, as well as how such a dichotomy may be important to Lp infection will require further study.

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