# EGCG Impairs Chemokine Production in Human Colon Epithelial Cell Lines

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**Running title: EGCG inhibits chemokine synthesis in HT29 and T84 cells**

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**Abbreviations used in the text:**

CFb, complement factor b; COX, cyclooxygenase; EGCG, epigallocatechin-3-gallate; FRA-1, fos-related antigene-1; GRO, growth regulated oncogene; IEX-1L, congenic gene of inhibitor apoptosis protein; IL , interleukin; β 5 integrin, beta 5 integrin; IP-1, interferon-inducible protein-10; 5-LOX, 5-lipoxygenase; MIP, macrophage inflammatory protein; MMP-14, matrix metalloproteinase-14; NF-κB, nuclear factor-kappa B; $PGE_2$, prostaglandin $E_2$; PGES, prostaglandin E synthase; PML, polymorphonuclear leukocytes; RANK, receptor activator of NF-κB; RANTES, regulated on activation normal T cell expressed and secreted; TNF-α, tumor necrosis factor-alpha

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## ABSTRACT

A major component in green tea, epigallocatechin-3-gallate (EGCG), is reported to interfere with different steps of a number of inflammatory pathways. After oral administration, EGCG is retained in the gastro-intestinal tract, where it is thought to exert preventive functions against inflammatory bowel disease (IBD) and colon cancer. In this study, the human colon adenocarcinoma cell lines HT29 and T84 were used to investigate the effect of EGCG on intestinal inflammation. HT29 and T84 cells were stimulated with tumor necrosis factor (TNF)-α to induce the "inflammatory" condition and to trigger the inflammatory cascade *in vitro*, and treated with EGCG to study its effect on inflammatory processes. The secretion of the chemokines interleukin (IL)-8 and macrophage inflammatory protein (MIP)-3α and prostaglandin $E_2$ ($PGE_2$) was determined by ELISA. The gene expression level was measured by quantitative real-time (RT)-PCR. Treatment of TNF-α-stimulated HT29 cells with EGCG dose-dependently inhibited the synthesis of IL-8, MIP-3α and $PGE_2$. Treatment with EGCG also inhibited the production of IL-8 and MIP-3α in TNF-α-stimulated T84 cells. Gene expression analysis in both HT29 and T84 cells revealed that EGCG down-regulates genes involved in inflammatory pathways. This study shows that EGCG acts broadly on the production of chemokines and $PGE_2$ in the chemokine and eicosanoid-pathways of colon epithelial cells. Therefore, EGCG might prove useful for the prevention and/or attenuation of colonic disorders.

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## INTRODUCTION

The constant exposure of the intestinal epithelium to diverse types of nutrients and microorganisms present in the natural flora leads to a permanent “stress state” for the enterocytes. Mucosal surfaces of the intestinal tract form one of the main routes for microbial pathogens to enter a host, and are important sites of microbially-induced diseases. The intestinal mucosa becomes compromised in some food allergies or after invasion of the epithelium by pathogenic bacteria. This might lead to acute inflammation, characterised by an excessive production of inflammatory mediators. These are, in part, responsible for the recruitment of specific cell types, for example, macrophages/monocytes, lymphocytes or neutrophils (Kagnoff, 1996).

During intestinal inflammation, polymorphonuclear leukocytes (PML), including neutrophils, migrate into the mucosa. In the intestine, an exacerbation of destructive processes occurs due to different pro-inflammatory and chemoattractant molecules. The latter are chemokines, a large family of small proteins, which are involved in innate immune and inflammatory responses by chemoattracting to, and activating leukocytes at the site of inflammation, and up-regulating adhesion molecules important for leukocyte trafficking (Baggiolini and Moser, 1997; Dwinell et al., 1999). Biological actions of chemokines are mediated through G-protein-coupled receptors which are present on the surface of target cells (Balkwill, 1998; Murphy et al., 2000). These receptors exhibit overlapping specificity for chemokines within each subfamily, and the cellular expression of respective receptors determines which cell types respond to a given chemokine. Based on the arrangement of their N-terminal cysteine residues, chemokines are subdivided into four subfamilies, including CXC and CC. Cells responsive to chemokines recognize concentration gradients and migrate towards the source of chemokine secretion. IL-8, MIP-2 and growth-regulated oncogene (GRO)-$\alpha$ and -$\gamma$ are CXC chemokines which attract and activate neutrophils, whereas the CC chemokines, such as MIP-3$\alpha$, activate leukocyte populations, including monocytes, T lymphocytes, dendritic cells

and, to a lesser extent, neutrophils (Ajuebor and Swain, 2002). Chemokines contribute to the perpetuation of inflammatory processes, increasing chronic intestinal inflammation and mucosal destruction. Secreted chemokines and their specific chemokine cell-surface receptors play a crucial role in the final composition of leukocytes present in the inflamed intestine (MacDermott, 1999; Banks et al., 2003).

Prostaglandins are also involved in the regulation of a variety of physiological and pathological processes in the immune response and in inflammation. $PGE_2$ and other prostanoids are generated through two bifunctional enzymes, cyclooxygenases-1 and -2 (COX-1 and COX-2) (Kim et al., 1998). In general, COX-1 is constitutively expressed in a wide range of tissues including the gastro-intestinal tract and plays a role in the tissue homeostasis, *e.g.* maintenance of gastro-intestinal integrity (Singer et al., 1998). The inducible form, COX-2, which regulates prostaglandin synthesis, is overexpressed in several epithelial cancers and at sites of inflammation (Parker et al., 1997; Kim et al., 1998; Singer et al., 1998; Poligone and Baldwin, 2001; Martel-Pelletier et al., 2003). The expression of this enzyme is induced by various stimuli, *e.g.* TNF-α, IL-1β, in a variety of cell lines.

Natural compounds, such as components of green tea, influence inflammation and cancer (Chen et al., 2003). EGCG, the major catechin of green tea, has many biological functions, including anti-inflammatory and chemopreventive effects (Lin and Lin, 1997; Ahn et al., 2004; Park and Surh, 2004). In China, Korea and Japan, where a large proportion of the population consumes green tea daily, the incidence of colon cancer is relatively low compared with that found in Western societies. EGCG interferes in several steps of inflammatory processes, *e.g.* in the synthesis of eicosanoids, and in the chemokine-mediated recruitment of polymorphonuclear leukocytes (PML) to the injured site (Chen et al., 2002). Furthermore, EGCG was shown to suppress the maturation of murine dendritic cells through the inhibition of ERK, p38 kinase, JNK and the NF-κB signaling pathway (Ahn et al., 2004). *In vivo* studies show that green tea polyphenols decrease inflammation in animal models (Varilek et al., 2001)

Several reports have shown the benefits of EGCG in a variety of inflammatory conditions. In the present study, we evaluate the anti-inflammatory effects of EGCG *in vitro*. Human colon adenocarcinoma cell lines HT29 and T84 were stimulated with TNF-α and the potential of EGCG to improve gastro-intestinal inflammation, by modulating expression of cytokines and chemokines, was studied. Here we show that EGCG effectively modulates a number of mediators involved in different inflammatory diseases on protein and gene expression level. Our results suggest that EGCG may be of preventive and, furthermore, therapeutic value to treat intestinal inflammation.

An abstract containing part of these data has been presented (Porath et al., 2004).

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## METHODS

### Reagents

Recombinant human TNF-α was purchased from Peprotech (London, UK). Recombinant human IL-8, purified mouse anti-human IL-8 and biotinylated mouse anti-human IL-8 were obtained from BD Pharmingen (San Diego, CA, USA). Recombinant human MIP-3α/CCL20, anti-human MIP-3α/CCL20 and biotinylated anti-human MIP-3α/CCL20 were purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany). Cell culture reagents were obtained from GIBCO (Basel, Switzerland). EGCG (Teavigo&[trade]) was obtained from DSM Nutritional Products (Basel, Switzerland). Primers and probes were purchased from Sigma-Genosys (Homefield Road, Haverhill, UK).

### Cell culture

HT29 and T84 cells were obtained from ATCC (LGC Promochem, Molsheim, France). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin, 50 μg/ml streptomycin, L-glutamine and nonessential amino acids (NEAA, Invitrogen, Basel, Switzerland). Cells were maintained in a water-saturated atmosphere of 95% $O_2$ and 5% $CO_2$ at 37°C. Cells were used between passages 20 to 50. For experiments, cells were seeded into 12-well-plates at $1\times10^6$ cells per well and used after 3 or 4 days of pre-culture. They were starved in DMEM containing 0.25% FBS for 18 hours before the experiments. Cells were stimulated with TNF-α (100 ng/ml) in phenol-free DMEM containing 0.25% FBS. EGCG was dissolved in DMSO and added to the culture medium concomitantly with the TNF-α stimulus. Where appropriate, DMSO was added to the cell culture at a final concentration of 0.5% (v/v).

### Cytotoxicity

Released lactate dehydrogenase (LDH) was measured in culture supernatants immediately after harvesting, using LDH enzyme controls as standard (Sigma, St. Louis, USA).

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In a microtiter plate, 20 μL of undiluted culture supernatants or standard were mixed with β-NAD solution (172 mM) and Tris acetate buffer (13.6 g/L Tris base, 12.8 g/L KCl, 5.08 g/L L-lactate, 1 g/L $NaN_2$, pH 9.3). The kinetics of the reaction was measured at 340 nm at 30°C for 5 min. To determine the total LDH contents, cells were lysed in the presence of a buffer containing 0.1 M NaCl, 1 mM EDTA, 10 mM Tris/HCl and 1% Triton X-100, and protease inhibitors. This protocol was adapted from Korzeniewski and coworker (Korzeniewski and Callewaert, 1983).

**Enzyme-linked immunosorbent assay (ELISA) and Enzyme Immunoassay (EIA)**

*$PGE_2$ assay.* The amount of $PGE_2$ in culture supernatants was assayed with EIA kits (obtained from Cayman Chemicals, Ann Arbor, MI, USA) according to the manufacturer's instructions.

*IL-8 assay.* Nunc Maxisorp 96-well microtitre plate (Fisher Scientific, Wohlen, Switzerland) was coated with 3 μg/ml goat anti-human IL-8 antibody (Pharmingen, Becton Dickinson, Heidelberg, Germany) in 50 μL binding buffer (0.1 M $Na_2HPO_4$, pH 9.0) overnight at 4°C. After washing with PBS containing 0.05% Tween 20 (PBS-T), wells were blocked with PBS containing 10% FBS and incubated for two hours at room temperature. After further washing with PBS-T, 100 μL aliquots of culture supernatant or recombinant IL-8 were added and incubated overnight at 4°C. To detect bound IL-8, 0.25 μg/ml biotinylated anti-human IL-8 antibody (Pharmingen, Becton Dickinson, Heidelberg, Germany), mixed in blocking buffer, was added for one hour at room temperature. Streptavidin and alkaline phosphatase (DAKO, Glostrup, Denmark), diluted 1:50 in PBS, were pre-incubated for 30 min at room temperature to form conjugates. These conjugates were then added to the plate and incubated at 37°C for 1 hour. After washing, 50 μL of p-nitrophenyl phosphate substrate (1 mg/ml) (Sigma, Steinheim, Germany) was added to each well and incubated at 37°C for 20 min. The optical density at 405 nm was read using a microtitre plate photometer (Molecular Devices, USA). All determinations were performed in triplicates.

*MIP-3α assay*. Nunc immunosorb microtitre plate (Fisher Scientific, Wohlen, Switzerland) was coated overnight at 4°C with 2 μg/ml goat anti-human MIP-3α/CCL20 antibody (R&D Systems, Wiesbaden-Nordenstadt, Germany) diluted in 50 μL of carbonate coating buffer (pH 9.6). After

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washing with PBS-T, wells were blocked with PBS, containing 1% BSA, 5% sucrose and 0.05% $NaN_3$, for one hour at room temperature. After further washing with PBS-T, 50 μL aliquots of culture supernatants or recombinant MIP-3α were added for two hours at room temperature. To detect bound MIP-3α, 0.5 μg/ml biotinylated anti-human MIP-3α antibody (R&D Systems, Wiesbaden-Nordenstadt, Germany), mixed in blocking buffer, was added and the plate was incubated for one hour at room temperature. Streptavidin-biotin alkaline phosphatase complexes (DAKO, Glostrup, Denmark) were formed and immune complexes visualised and measured as described above. All determinations were performed in triplicates.

**RNA isolation and reverse transcription**

Total RNA was isolated using the RNeasy® Mini Kit from Qiagen (Hilden, Germany). Extraction was performed according to the manufacturer's directions. Matrix-bound RNA was eluted with 30 μL of RNase-free water. The quantity and purity of RNA was determined by measuring the optical density at 260 nm and 280 nm. Subsequently, 1.5 to 3.5 μg of total RNA were converted to first strand cDNA using SuperScript II™ reverse transcriptase (Invitrogen, Basel, Switzerland) and 50 ng/μL random hexamers (Microsynth, Balgach, Switzerland). The conditions for the cDNA synthesis were: 5 min at 70°C for random primer annealing followed by cooling on ice; 10 min at room temperature, 50 min at 42°C and 15 min at 70°C for the denaturation step; and 20 min at 37°C for the RNase H digestion. The cDNA was subsequently diluted to 100 μL in water and stored at −80°C.

**Quantitative Real Time (RT)-PCR analysis**

The analysis of mRNA expression profiles was performed with multiplex quantitative RT-PCR. In a 50 μL PCR reaction, 3 μL cDNA (corresponding to 30-50 ng of total RNA input) were amplified in an ABI Prism 7700 Sequence Detector (PE Biosystems, Foster City, CA, USA), using 2× Taqman Universal PCR Master Mix (Applied Biosystems, Rotkreuz, Switzerland), 50 nM primers and 100 nM probe for the 18S rRNA reference gene, and 300 nM primers and 100

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nM probe for genes of interest. The probe for 18S rRNA was fluorescently labelled with VIC™ on the 5′ end and TAMRA on the 3′ end (Applied Biosystems, Rotkreuz, Switzerland), whereas probes for the genes of interest were labelled with 6-carboxy-fluorescein (FAM) on the 5′ end and TAMRA on the 3′ end. The PCR amplification conditions consisted of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 and 60 sec at 95°C and 60°C, respectively.

mRNA abundance was defined using the $\Delta C_T$ method according to the manufacturer's protocol (ABI Prism 7700 Sequence Detection System, Rotkreuz, Switzerland). Briefly, the $\Delta C_T$ for the gene of interest was determined as the difference between the $C_T$ values for the gene of interest and 18S rRNA, where the $C_T$ value is the cycle threshold. The standard deviation (SD) was obtained from $\Delta C_T$ of cDNA samples assayed in duplicate, where the upper and lower errors were defined as $2^{-(\Delta C_T - SD)}$ and $2^{-(\Delta C_T + SD)}$, respectively. $\Delta\Delta C_T$ was determined as the difference in $\Delta C_T$ of unstimulated cells compared to that of each treatment group. The mRNA level for the gene of interest was determined as $2^{-\Delta\Delta C_T}$ and, therefore, reflects changes relative to unstimulated cells. Each cDNA sample was assessed in duplicates.

**Statistical analysis**

The upper and lower limits of mRNA expression levels were calculated as described above. All other values are presented as mean±SD and all statistics were evaluated double-sided using a two-sample equal variance Student's *t*-test, assuming independent variance. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ were considered to be significant.

## RESULTS

### Impact of EGCG on the viability of intestinal epithelial cells

The cytotoxicity of cell treatments and EGCG were evaluated by measuring the LDH activity in cell culture supernatants. Table 1 shows percentages of LDH released in HT29 and T84 cells. For HT29, the amount of released LDH was similar in the different treatments, indicating that TNF-α and EGCG did not affect the cell viability to a higher extent than TNF-α. This, however might induce apoptosis (Aggarwal, 2000). T84 cells treated with 25 μM of EGCG displayed higher LDH release than untreated cells, suggesting that EGCG might induce apoptosis.

### TNF-α induces the secretion of chemokines

TNF-α is an important pro-inflammatory mediator involved in gastro-intestinal inflammation and able to activate the synthesis of chemokines in epithelial cells (MacDermott, 1999). Therefore, we verified its effects on the production of IL-8 and MIP-3α in HT29 and T84 cell lines. Cells were cultured in the presence or absence of TNF-α for 24 hours and the secretion of these chemokines was examined. Unstimulated cells did not produce significant levels of IL-8 and MIP-3α. In contrast, TNF-α-activated cells demonstrated time- and dose-dependent synthesis of IL-8 and MIP-3α in HT29 (Fig. 1A and C). Similar data for the dose-dependent secretion of both chemokines were also obtained in T84 cells (data not shown). The time-dependent production of IL-8 and MIP-3α in TNF-α-stimulated T84 cells is shown in Figure 1B and D. Compared to T84 cells, the production of IL-8 in activated HT29 cells was 1.5 fold increased after 24 hours. However, no significant differences were observed on the MIP-3α production between both cell lines (Fig. 1).

### EGCG inhibits the production of IL-8 and MIP-3α in intestinal epithelial cells

The effects of EGCG on the IL-8 and MIP-3α secretion were investigated after 24 and 72 hours in TNF-α-stimulated HT29 and T84 cells. EGCG significantly reduced the production of IL-8 at 24 hours (Fig. 2A) and virtually abolished it at 72 hours (Fig. 2B). Increasing concentrations of EGCG gradually reduced IL-8 secretion in activated cells, indicating that the secretion of IL-8 from the cell to the culture medium was more affected in the presence of EGCG in HT29 than in T84 cells (Fig. 2C). This indicates a difference in the sensitivity of these cells to EGCG.

To investigate the impact of EGCG on MIP-3α secretion, TNF-α-activated cells were incubated with varying concentrations of EGCG for 24 hours. EGCG dose-dependently decreased the synthesis of MIP-3α in both cell lines (Fig. 3A). The production of MIP-3α was also investigated after 72 hours of culture. As expected, TNF-α strongly increased the synthesis of MIP-3α in both HT29 and T84 cells. This increase was significantly reduced in the presence of EGCG at a concentration of 50 μM (Fig. 3B).

## EGCG inhibits the production of $PGE_2$ in epithelial cells

Since it is known that intestinal epithelial cells produce $PGE_2$ (Eckmann et al., 1997), we decided to investigated the influence of EGCG on its synthesis in activated HT29 cells. Unstimulated cells produced low amounts of $PGE_2$. Upon TNF-α stimulation a significant increase on the $PGE_2$ secretion was observed. Figure 4A shows that TNF-α dose-dependently augmented the secretion of $PGE_2$ after 24 hours. The highest amount of $PGE_2$ was obtained in cells stimulated in the presence of 100 ng/ml TNF-α. In cells treated with 50 μM EGCG, $PGE_2$ synthesis was diminished by 80±6% (data not shown). EGCG dose-dependently reduced the production of $PGE_2$ (Fig. 4B).

## EGCG modulates genes involved in inflammatory responses

In order to clarify whether EGCG modulates the expression of TNF-α-induced genes, we used quantitative RT-PCR. Since activated HT29 and T84 cells up-regulated inflammatory genes within 2-24 hours (Fig. 5), the influence of EGCG on these genes was analyzed after 6 hours of culture. Basal gene expression levels in unstimulated HT29 cells were different, with

weakly (*e.g.* TNF-α, MIP-2, GRO-α, IL-1α, IL-1β, IP-10), moderately (IL-8, MIP-3α, GRO-γ, PGES, MMP-14, RANK, IEX-1L, CFb) and abundantly (*e.g.* COX-2, 5-LOX, β 5 integrin, RANTES, FRA-1) expressed genes (Fig. 6). In T84 cells, however, basal gene expression levels differed from those of HT29 cells with respect to moderately (*e.g.* IL-8, MIP-3α, GRO-γ, PGES, MMP-14, RANK, IEX-1L, RANTES, CFb) and abundantly (*e.g.* COX-2, 5-LOX, β 5 integrin, FRA-1) expressed genes (data not shown).

TNF-α induced a substantial increase in mRNA levels of cytokines and chemokines (TNF-α, IL-8, MIP-2, MIP-3α, GRO-α, GRO-γ, IP-10), in addition to COX-2. However, mRNA levels of other genes, including 5-LOX, RANK, and β 5 integrin, were unaltered by TNF-α stimulation. EGCG (50 μM) reduced the mRNA levels of IL-8, TNF-α, MIP-3α, MIP-2, GRO-α, GRO-γ and COX-2 by 40 to 85% in both cell lines (Fig. 7). This effect was specific, but not restricted, to these genes. Conversely, EGCG did not augment the expression of TNF-α-induced genes. TNF-α strongly augmented the expression of IL-8 mRNA levels in HT29 and T84 cells, while it moderately and weakly increased the expression of TNF-α, MIP-3α, and GRO-γ mRNA (Fig. 7A, B and E), COX-2 and GRO-α mRNA (Fig. 7C and D), respectively. Notably, these genes were significantly down-regulated by EGCG, an observation which is consistent with our results of produced chemokines (IL-8 and MIP-3α) (Fig. 2 and 3).

Finally, the dose-dependent impact of EGCG on gene expression was evaluated. In HT29 and T84 cells, EGCG down-regulated IL-8, TNF-α, MIP-3α and COX-2 mRNA levels in the concentration range of 6.25 to 50 μM (Fig. 8). In T84 cells, EGCG at 12.5 and 25μM did not have any significant effect on IL-8 mRNA levels, although a trend for down-regulation was observed (Fig. 8B). In summary, expression levels of tested chemokines were significantly diminished following EGCG treatment in these cell lines.

## DISCUSSION

In this report, we present evidence that EGCG beneficially affects the inflammatory response in the colon adenocarcinoma cell lines HT29 and T84. These cell lines were used as models for intestinal mucosal diseases and have different origins and phenotypes. *In vitro*, differentiated HT29 cells express characteristic features of mature intestinal cells, such as microvilli, small intestinal digestive enzymes, lipid droplets, primary and secondary lysosomes (Velcich et al., 1995). T84 is a transplantable human carcinoma cell line derived from a lung metastasis of colon carcinoma (Murakami and Masui, 1980) which possess secretory capacity such as small intestinal crypt cells (Mun et al., 1998).

Our experimental approach dealt with the identification of the anti-inflammatory function of EGCG in TNF-α-activated colon adenocarcinoma cell lines. The effects of EGCG were determined at two levels: the expression of genes involved in inflammation and the production of chemokines (IL-8 and MIP-3α) and $PGE_2$. EGCG showed potent effects on genes of the inflammatory pathway, including those of the cytokine/chemokine network (*e.g.* IL-8, TNF-α, MIP-3α, MIP-2, GRO-α and GRO-γ). CXC chemokines, such as IL-8, MIP-2, GRO-α and -γ, are responsible for chemoattraction of neutrophils, while CC chemokines (*e.g.* MIP-3α) play a role in the recruitment of lymphocytes and dendritic cells to the inflamed tissue. Cytokines and chemokines are induced by TNF-α stimulation and are abundantly expressed and secreted in IBD (Fiocchi, 1997; Ajuebor and Swain, 2002; Banks et al., 2003; Kaser et al., 2004; Papadakis, 2004). We demonstrated that EGCG markedly diminished the expression and secretion of IL-8 and MIP-3α in colon epithelial cells.

IBD and colon cancer are associated with an increased activity of intestinal immune cells, which augments the production of pro-inflammatory cytokines, including TNF-α. Therefore, TNF-α was used to induce an inflammatory state *in vitro*. TNF-α is involved in the mediation of the sustained inflammatory response and high amounts of this cytokine can be found in IBD patients (D'Haens, 2003). The expression and production of IL-8 was studied in macrophages isolated

from normal and inflamed colonic tissue resected for IBD (Grimm et al., 1996). Macrophages from IBD expressed more IL-8 mRNA than those from the normal mucosa, and lipopolysaccharide treatment further increased it. In addition, the recruitment of macrophages may be responsible for the IL-8 secretion which leads to neutrophil attraction in IBD (Grimm et al., 1996). This suggests that in the intestine activated macrophages migrate into the epithelium and secrete IL-8 which recruits PML to the injured site. This is supported by our macrophage *in vitro* model (data not shown) where RAW 264.7 cells were stimulated with LPS in the presence or absence of EGCG. In this study, we have identified EGCG as an inhibitor of IL-8. Moreover, after TNF-α stimulation, epithelial cells also produce IL-8, and consequently, recruit PML. The inhibition of the expression and production of IL-8, either by EGCG or other natural compounds (*i.e.* phenolic compounds), interferes with the recruitment of PML and thus may impede progression and aggravation of inflammation (Sugimoto et al., 2002; Park and Surh, 2004). In conclusion, EGCG has the potential to reduce the IL-8 production in both epithelial cells and macrophages.

With regard to MIP-3α, it is synthesized by the human colon epithelial cells and therefore by HT29 cells which also express CCR6, the cognate receptor for MIP-3α (Izadpanah et al., 2001). MIP-3α mRNA and protein levels were found to be increased in colonic tissues from patients with IBD (Kwon et al., 2002). In the present study, EGCG inhibited the synthesis of MIP-3α in colon cancer cell lines and also down-regulated gene expression. Via MIP-3α production, intestinal epithelial cells recruit dendritic cells and memory T cells to the site of inflammation. Whereas the normal intestinal mucosa is home of dendritic and T cells, a specific T cell population immigrates during IBD and contributes to the generation of inflammation (Fiocchi, 1998). Therefore, an excess of these cells in the inflamed intestine should be avoided. By reducing MIP-3α production, EGCG could prevent homing of these cells and consequently IBD symptoms.

Furthermore, we observed that EGCG inhibits $PGE_2$ production. In the gastro-intestinal environment, prostaglandins are important regulators of gastro-intestinal fluid secretion. In food

allergies or after invasion of microorganisms, water secretion and TNF-α-induced electrolyte production lead to an increase of $Ca^{2+}$-dependent $PGE_2$ in the intestinal epithelium. Exogenous $PGE_2$ up-regulated IL-8 gene expression and protein production in human colonic epithelial cells (Yu and Chadee, 1998). $PGE_2$ production was increased in colonic fibroblasts and in IBD patients. Intestinal fibroblasts, beside epithelial cells, could be targets for $PGE_2$ and sites of colonic prostanoid biosynthesis *in vivo* (Kim et al., 1998). The expression of COX-2 has also been observed after Salmonella infection in intestinal epithelial cells (Singer et al., 1998), and COX-2 protein was reported to be expressed in three of eight colon cancer cell lines, including HT29 (Parker et al., 1997). Our results demonstrated that EGCG diminished pro-inflammatory $PGE_2$ production and expression in HT29 cells. Studies have shown that EGCG targets COX-2 by inhibiting the $PGE_2$ production and possibly reduces the risk of colon cancer and inflammation in humans (August et al., 1999; Hong et al., 2001; Park and Surh, 2004). Due to differences between HT29 and T84 cells, TNF-α-stimulated T84 cells did not produce detectable amounts of $PGE_2$ and COX-2 protein (data not shown). With regard to this observation, in HT29 cells, EGCG had shown an influence on the three parameters tested: COX-2 gene, COX-2 protein expression (data not shown) and $PGE_2$ generation, whereas in T84 cells, only the COX-2 mRNA levels were affected by EGCG.

Quantitative RT-PCR analysis identified several genes that were induced in TNF-α-stimulated HT29 and T84 cells, including IL-8, TNF-α, MIP-3α, MIP-2, GRO-α and GRO-γ. These genes were down-regulated by EGCG and have NF-κB regulatory binding sites. Consequently, they are regulated by the transcription factor NF-κB. Similar to the effects observed for IL-8, the expression levels of MIP-2, GRO-α and GRO-γ were also diminished by EGCG in both cell lines. These chemokines, like IL-8, act through the chemokine receptor CXCR2 (Murphy et al., 2000) and are also elevated after TNF-α stimulation (Fig. 7D and E). The concerted effect of EGCG on four chemokines binding to a common receptor indicates that this flavonoid impedes most or all biological activities that are mediated by CXCR2. Furthermore, 5-LOX, an enzyme expressed in differentiated HT29 cells (Cortese et al., 1995) and responsible for the synthesis of leukotrienes,

was also expressed in HT29 and T84 cell lines, but no differences was observed between unstimulated and TNF-α-activated cells (Fig. 6). In our experiments, the expression of 5-LOX was not influenced by EGCG (data not shown). A possible explanation for this observation is that TNF-α is not able to induce 5-LOX.

Collectively, we provide evidence that EGCG attenuates the inflammatory response in the colon adenocarcinoma cell lines HT29 and T84 by inhibiting the production of chemokines and $PGE_2$. However, this study considers the effect of EGCG in the *in vitro* gastro-intestinal inflammation and, thus, it should be followed by *in vivo* experiments to consolidate this statement. In addition, *in vivo* studies will also corroborate the use of EGCG in preventing and attenuating gastro-intestinal disorders.

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## LEGENDS FOR FIGURES

Figure 1: **Induction of IL-8 and MIP-3α production by TNF-α-stimulated HT29 and T84 cells**

Unstimulated and TNF-α-stimulated HT29 and T84 cells were cultured for 4 to 24 hours. IL-8 and MIP-3α secretion were measured in culture supernatants by ELISA. Representative data of one of three similar experiments are given. A and B, dose- and time-dependent increase of IL-8 production in HT29 and T84 cells, respectively. C and D, dose- and time-dependent increase of MIP-3α secretion in HT29 and T84 cells, respectively.

Figure 2: **EGCG inhibited the production of IL-8 in epithelial cells**

Levels of IL-8 in supernatants of unstimulated and TNF-α-stimulated HT29 and T84 cells cultured for 24 (A) and 72 (B) hours in the presence of 50 μM of EGCG. EGCG dose-dependently inhibited the synthesis of IL-8 in these epithelial cells (C). TNF-α-activated HT29 and T84 cells produced 21704 pg/ml and 20962 pg/ml of IL-8, respectively. The secretion of IL-8 was determined by ELISA. * $p < 0.05$ and ** $p < 0.01$. Representative data of one of three similar experiments are shown.

Figure 3: **Effect of EGCG on TNF-α-induced MIP-3α production in HT29 and T84 cells**

Cells were stimulated with 100 ng/ml of TNF-α in the presence of varying concentrations of EGCG for 24 hours (A). Unstimulated and stimulated cells were cultured for 72 hours in the presence of 50 μM of EGCG (B). The synthesis of MIP-3α was determined by ELISA. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Representative data of one of three similar experiments are shown.

Figure 4: **EGCG inhibited the secretion of $PGE_2$ in TNF-α-activated HT29 cells**

HT29 cells were cultured with different concentrations of TNF-α for 24 hours (A). Stimulated cells were incubated with varying concentrations of EGCG for 24 hours (B). Activated cells produced 420 pg/ml of $PGE_2$. The production of $PGE_2$ was determined by ELISA. ** $p < 0.01$ and *** $p < 0.001$. Similar results were obtained in two other series of experiments.

Figure 5: **Time-dependent up-regulation of specific genes in HT29 cells**

Cells were cultured without or with 100 ng/ml of TNF-α for 6 hours. RNA was isolated and gene expression levels of IL-8, TNF-α, MIP-3α and COX-2 were measured using the quantitative RT-PCR. Values are expressed relative to unstimulated cells at arbitrary units ± SD. Similar results were obtained in two other series of experiments.

Figure 6: **Expression levels of selected genes in HT29 cells**

RNA of unstimulated and TNF-α-activated HT29 cells which were cultured for 6 hours were isolated and the levels of mRNA of the indicated genes determined by quantitative RT-PCR. Values are expressed relative to 18S rRNA and are indicated as means ± SD of 2 to 4 experiments.

Figure 7: **EGCG modulates the gene expression level of inflammatory genes**

Activated HT29 and T84 cells were cultured in the presence of 50 μM of EGCG for 6 hours. The effects of EGCG on mRNA expression levels of IL-8, TNF-α and MIP-3α in HT29 and T84 cells, respectively, were analyzed (A and B). The influence of EGCG on COX-2, GRO-α and GRO-γ mRNA levels in HT29 and T84 cells, respectively, are given (C, D and E). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Representative data of one of four similar experiments are shown.

Figure 8: **EGCG dose-dependently down-regulated some inflammatory genes**

TNF-α-stimulated HT29 (A) and T84 cells (B and C) were cultured in the presence of varying concentrations of EGCG for 6 hours. The effect of EGCG on the mRNA level of IL-8, TNF-α, MIP-3α and COX-2 is given. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Representative data of one of three similar experiments ± SD are shown.

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## TABLES

Table 1: **Determination of cell viability in HT29 and T84 cells treated with EGCG**

Cells were stimulated with 100 ng/ml of TNF-α and incubated in presence or absence of varying concentrations of EGCG. After 24 hours, LDH was determined in culture supernatants immediately after harvest. Results are given as percentage of total LDH contents ± SD. * $p < 0.05$. Similar data have been obtained in at least two independent sets of experiments.

| **Cell viability** | **HT29** **(% of LDH contents)** | **T84** **(% of LDH contents)** |
|---|---|---|
| Unstimulated cells | 2.5 ± 0.0 | 1.3 ± 0.1 |
| TNF-α (100 ng/ml) | 5.8 ± 1.7 | 2.4 ± 0.4 |
| EGCG (50 μM) | 5.1 ± 1.3 | 0.6 ± 0.2 * |
| EGCG (25 μM) | 2.3 ± 1.0 | 2.5 ± 0.7 |
| TNF-α + EGCG (50 μM) | 4.6 ± 0.4 | 1.9 ± 0.0 |
| TNF-α + EGCG (25 μM) | 6.3 ± 0.4 | 6.2 ± 0.7 * |

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## FIGURES

Fig. 1

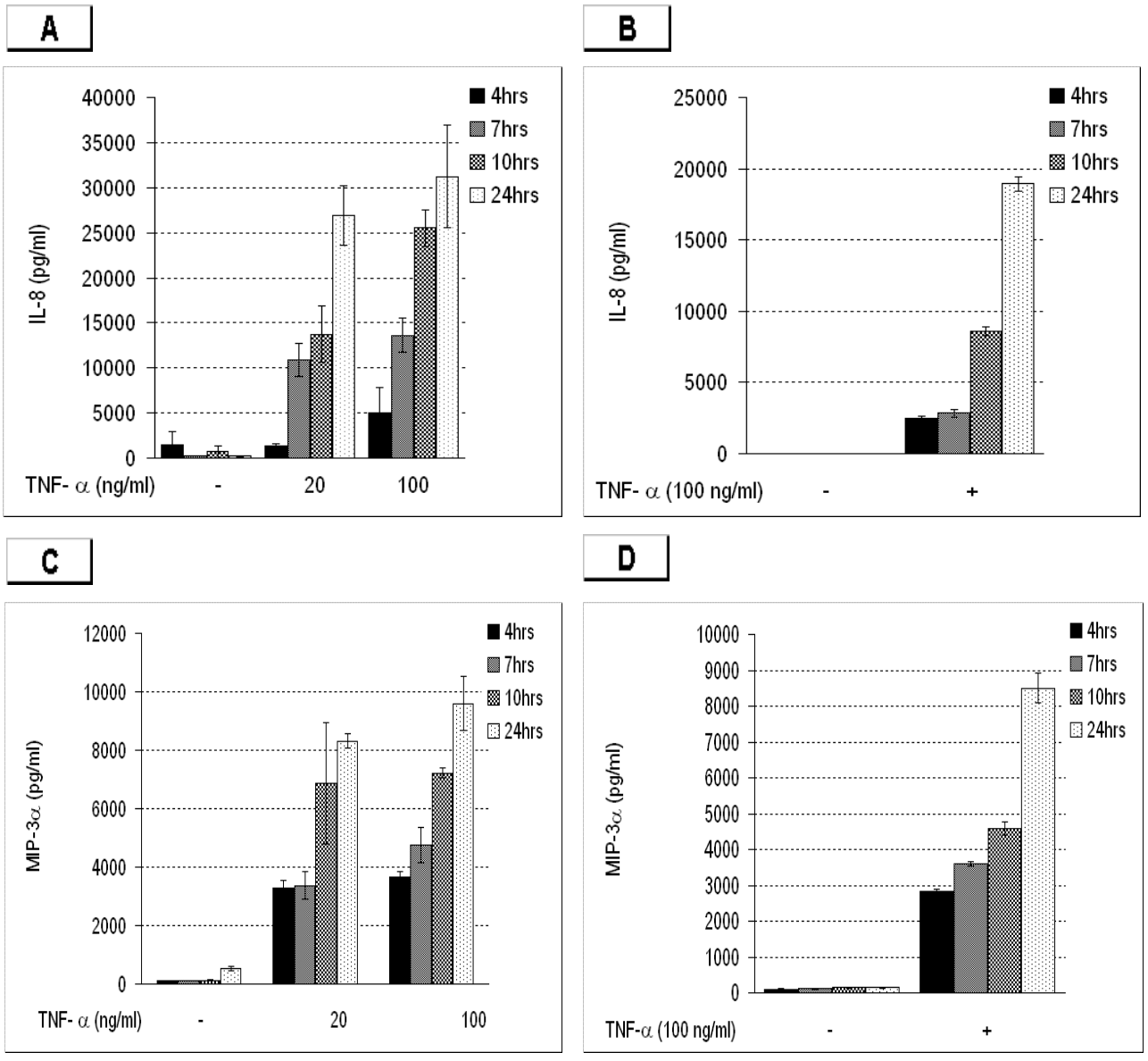

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Fig. 2

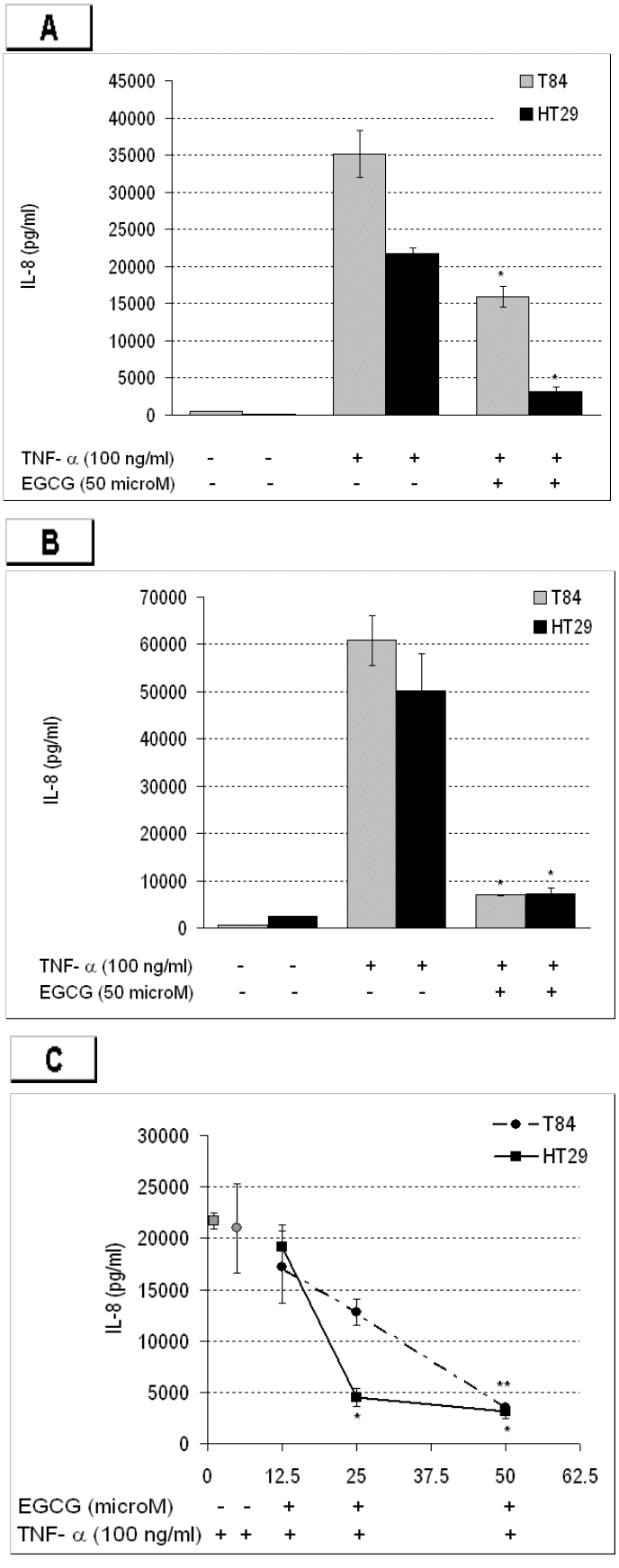

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Fig. 3

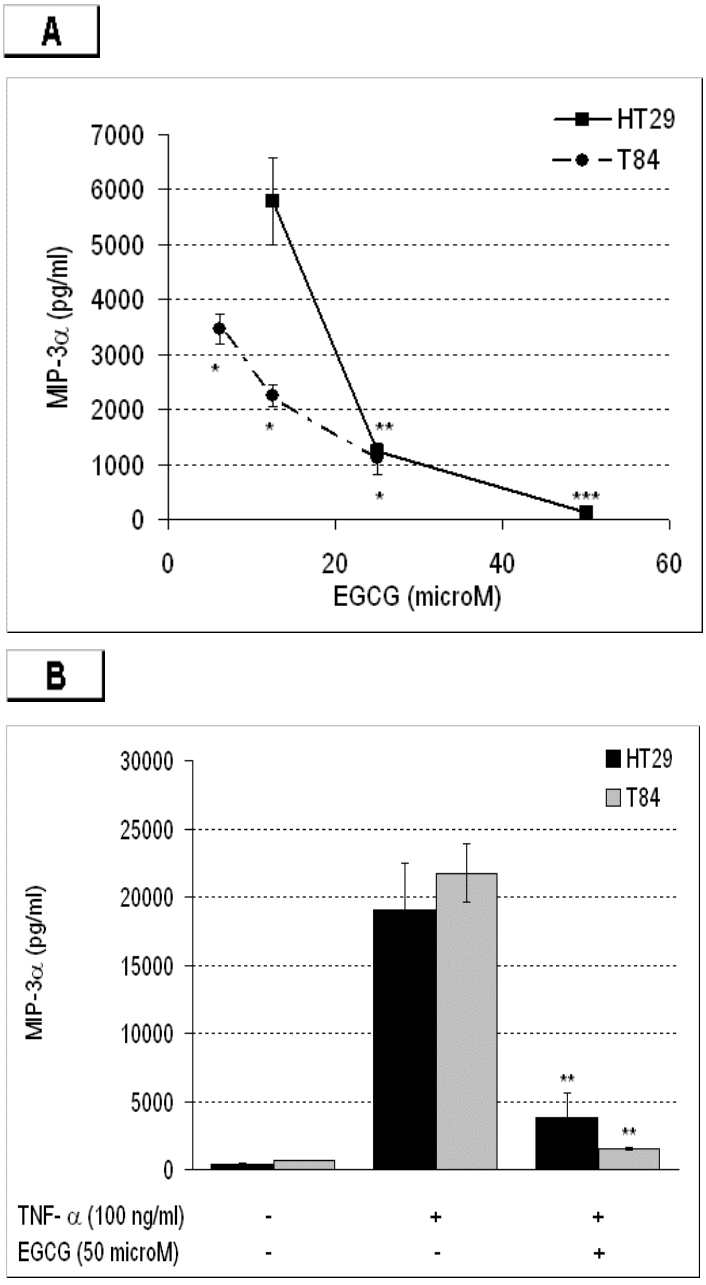

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Fig. 4

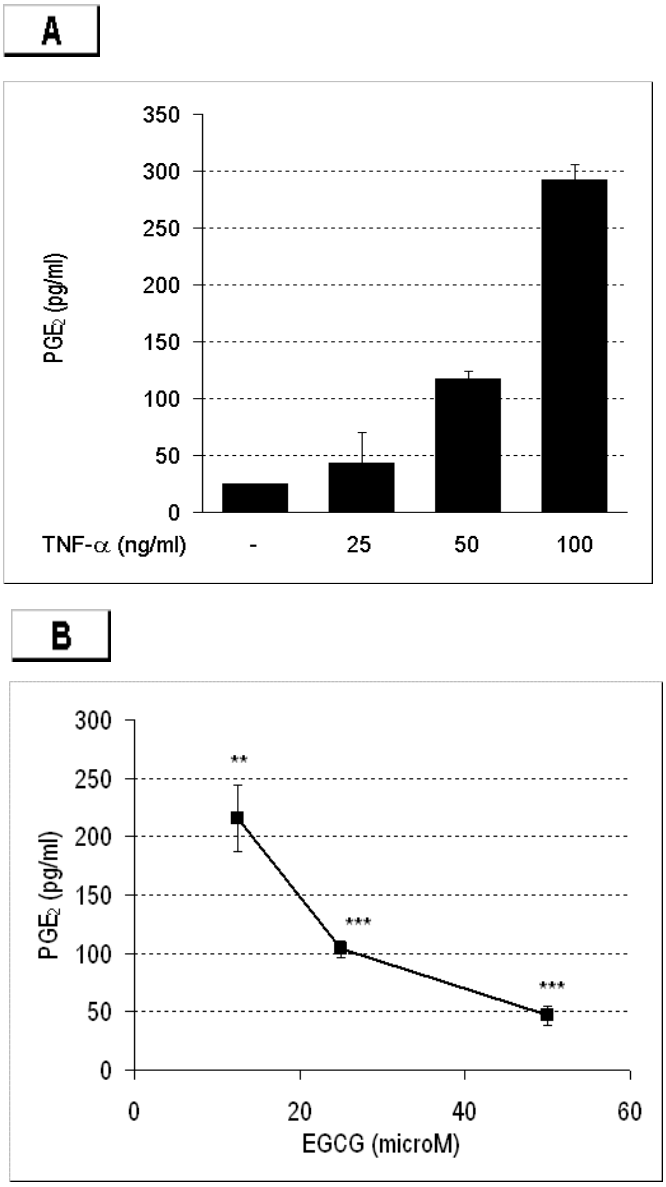

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Fig. 5

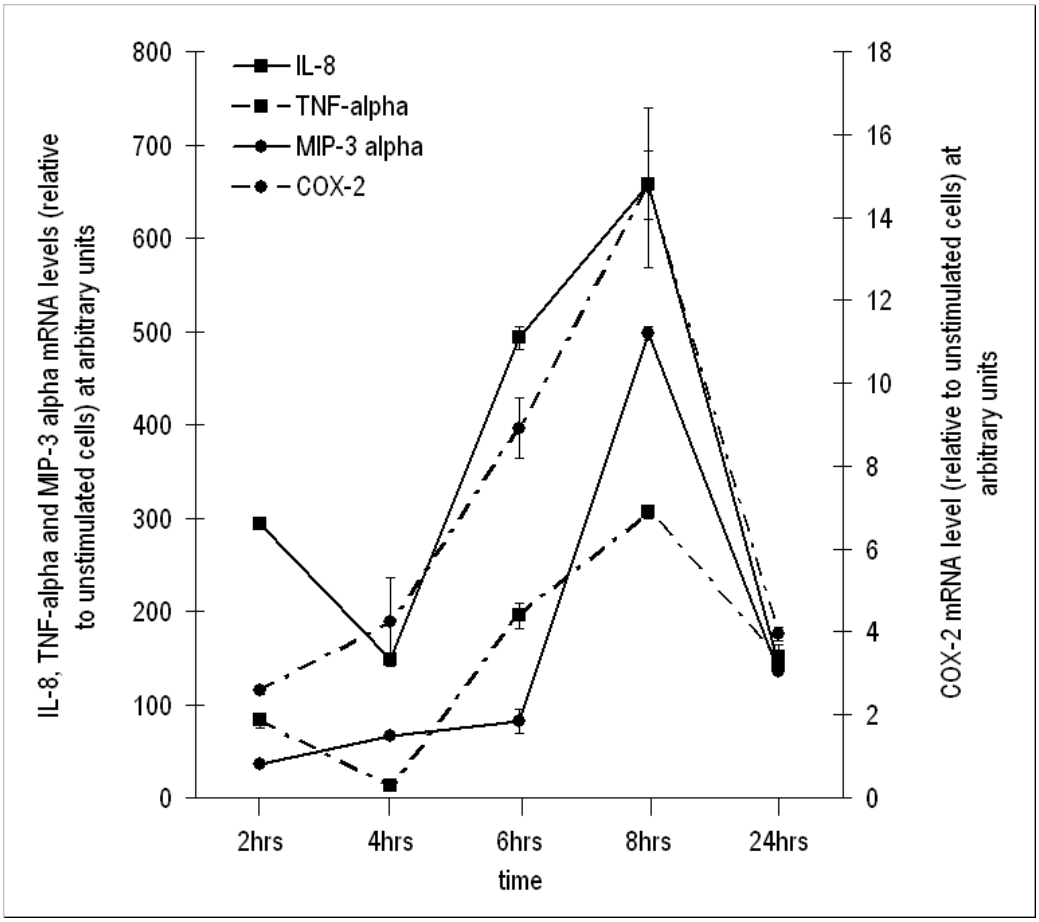

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Fig. 6

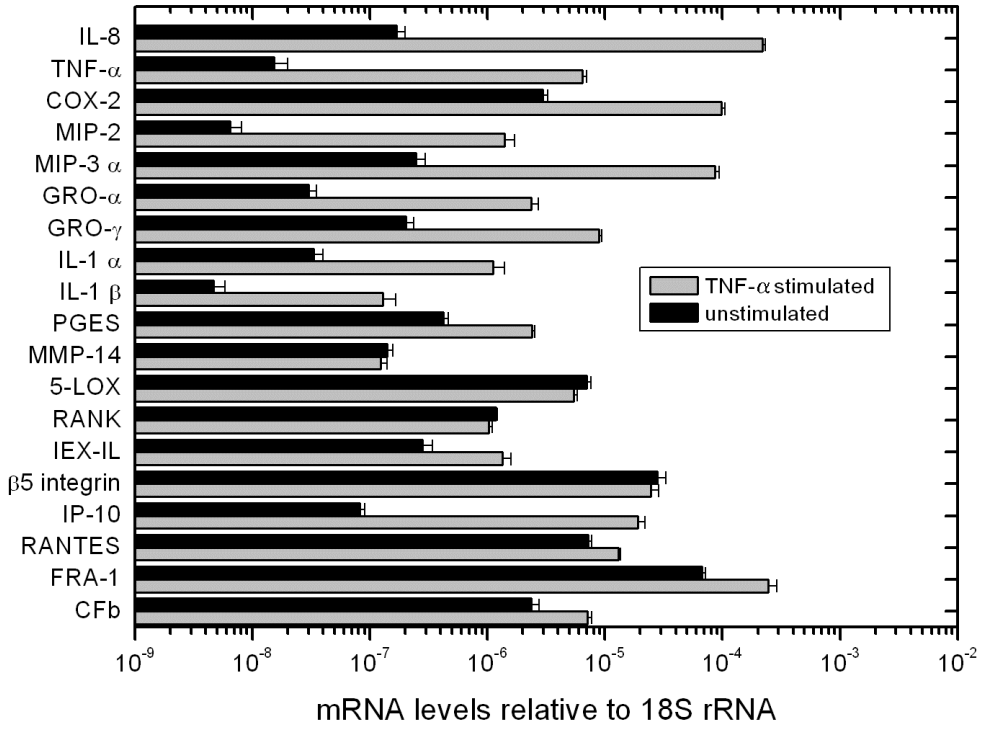

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Fig. 7

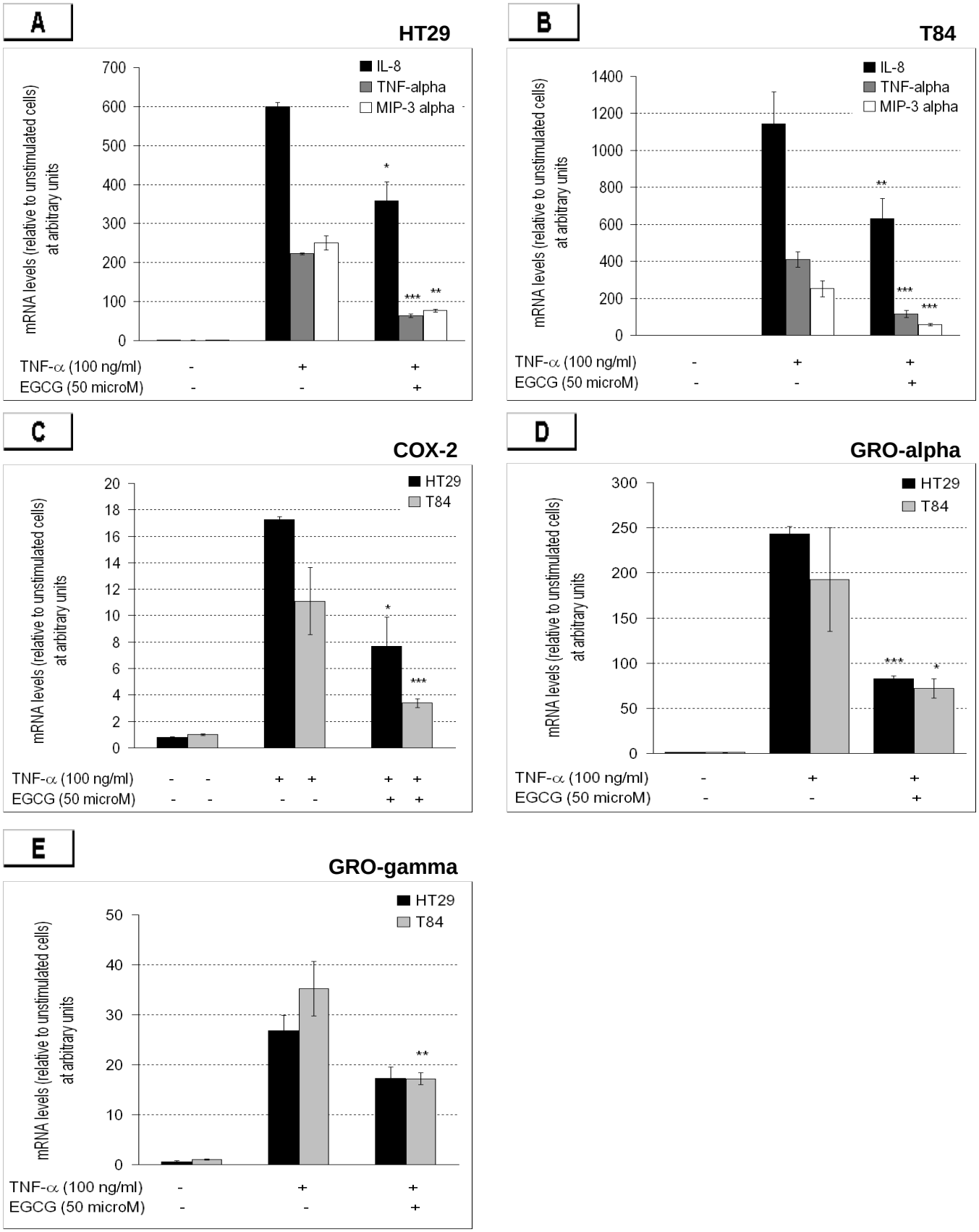

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Fig. 8