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EGCG Induces G-CSF Expression and Neutrophilia in Experimental Sepsis

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Abstract

A major green tea component, epigallocatechin-3-gallate (EGCG), has been proven protective against lethal sepsis in experimental setting, but its protective mechanisms remain incompletely understood. Here we provide evidence to support EGCG's capacities in stimulating G-CSF production and neutrophilia *in vivo*. In an animal model of sepsis, EGCG significantly elevated peritoneal levels of G-CSF and several chemokines (e.g., MCP-1/CCL2, and MIP-1 γ /CCL9), and consequently increased peritoneal neutrophil numbers (neutrophilia) at a late stage. *In vitro*, EGCG divergently affected HMGB1-mediated production of several chemokines: reducing CXCL15 and RANTES/CCL5, but elevating G-CSF and MIP-1 α /CCL3 production by peritoneal macrophages. Similarly, it significantly induced the expression and secretion of G-CSF and MIP-1 α /CCL3 in human peripheral blood mononuclear cells (PBMCs). Based on our preliminary data, it may be important to search for anti-inflammatory and G-CSF-stimulating agents for the clinical management of inflammatory diseases.

Introduction

Circulating monocytes and neutrophils continuously patrol the body to search for invading pathogens, and infiltrate into infected tissues upon detecting exogenous microbial products or endogenous chemokines [1]. Neutrophils are usually the first to arrive at the infection site, and engulf and kill more invading bacteria than other phagocytes [2]. Consequently, neutrophils quickly exhaust intracellular enzymes, and gradually destine to apoptotic cell death. Upon reaching extravascular tissues, monocytes can differentiate into tissue-specific resident macrophages, and ingest and eliminate pathogens or apoptotic neutrophils through reactive oxygen species and hydrolytic enzymes [3]. In addition, monocytes and macrophages are also equipped with pattern recognition receptors (such as Toll-like

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Author contributions

H.W., W.L., R.W., S.Z. and J.D. designed the study; W.L., S. Z., A.W. and R.W. performed the experiments; J.L. provided purified recombinant HMGB1; H.W. analyzed data and wrote the manuscript.

receptor, TLR2, TLR4, and TLR9) [4–6], which specifically recognize molecules shared by a group of related microbes called pathogen-associated molecular patterns (PAMPs). Upon recognition of various PAMPs (such as bacterial peptidoglycan, endotoxin, and CpG-DNA) [6;7], innate immune cells release a wide array of cytokines and chemokines [8–10]. Although an appropriate local inflammation is required to defend against infection, an uncontrolled systemic inflammation may contribute to the pathogenesis of lethal inflammation diseases (such as sepsis).

Sepsis is an overwhelming systemic inflammatory response to severe infections, and remains the primary cause of mortality in medical intensive care units. It afflicts approximately 750,000 Americans each year, and costs the U.S. healthcare system nearly \$17 billion annually [11]. The pathogenesis of sepsis remains poorly understood, but is partly mediated by pathogen- and damage-associated molecular patterns (e.g., bacterial endotoxins or HMGB1) [12], which activate innate immune cells via binding to various pattern-recognition receptors (e.g., TLR4, CD24, or Siglec-10) [13–15]. In animal models of endotoxemia or sepsis, circulating HMGB1 levels are elevated between 24–48 h [16;17], and HMGB1-neutralizing antibodies confer protection [16–18] even when given 24 h after the onset of sepsis, establishing HMGB1 as a late mediator of sepsis [19].

The establishment of HMGB1 as a late mediator of experimental sepsis has prompted the search for inhibitors that can attenuate HMGB1 secretion or action in experimental settings. Green tea contains a class of biologically active polyphenolic catechins including the most abundant epigallocatechin-3-gallate (EGCG). At relatively low concentrations, EGCG (1–20 μ M) dose-dependently abrogates LPS-induced HMGB1 secretion [20] by strategically destroying HMGB1 in the cytoplasm via a cellular degradation process – autophagy (“self-eating”) [21]. More importantly, it has been demonstrated that EGCG conferred significant protection in rodent models of lethal endotoxemia [22] and sepsis [20;21;23].

In this study, we report another possible mechanism by which EGCG may confer protection against lethal sepsis. EGCG was capable of enhancing macrophage/monocyte-mediated production of G-CSF and chemokines (e.g., MIP-1 α /CCL3 or MIP- γ /CCL9), thereby enhancing possible neutrophil recruitment or proliferation required for microbial elimination.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM, Cat. No. 11995-065), penicillin/streptomycin (Cat. No. 15140-122) and fetal bovine serum (FBS, Cat. No. 26140079) were from Invitrogen. Human serum (Cat. No. H4522), epigallocatechin-3-gallate (EGCG, Cat. No. E4143, purity 95%) and thioglycollate (Cat. No. 70157) were obtained from Sigma. Thioglycollate solution (4%) was prepared in sterile water, and aged for > 6 months at room temperature in the dark. Goat anti-G-CSF antibodies (Cat. No. sc-49679, K-15), and rabbit anti-MIP-1 α /CCL3 antibodies (Cat. No. sc-33203, FL-92) were from Santa Cruz. HRP conjugated donkey anti-rabbit or anti-goat IgG was from Santa Cruz (Cat. No. sc-2020) and GE Healthcare (Cat. No. NA934), respectively. Recombinant calmodulin-binding protein

tagged HMGB1 (CBP-HMGB1) was expressed in *E. coli* BL21 (DE3) pLysS cells [16], and purified to remove contaminating bacterial endotoxin by Triton X-114 extraction as previously described [24].

Animal models of sepsis

This study was approved by the Institutional Animal Care and Use Committee (IACUC protocol #: 2008-033), and performed in accordance with the guidelines for the care and use of laboratory animals at the Feinstein Institute for Medical Research, Manhasset, New York. BALB/c mice (male, 7–8 weeks, 20–25 g) were subjected to lethal sepsis by cecal ligation and puncture (CLP) as previously described [21;25;26]. Briefly, the cecum of BALB/c mice was ligated at 5.0 mm from the cecal tip, and then punctured once with a 22-gauge needle. At 24 and 48 h post CLP, EGCG or control saline solution was intraperitoneally injected into septic mice, and peritoneal cytokine levels and leukocyte counts were determined at 52 h post CLP. In parallel experiment, thioglycollate broth (4%, 2.0 ml, being aged at room temperature in the dark for at least 6 months) was intraperitoneally administered to mice at 3-day prior to CLP surgery, and animal survival rates were monitored for two weeks thereafter.

Peritoneal leukocytes counts

At 52 h post CLP, the peritoneal lavage fluid was harvested, and peritoneal leukocytes were collected by centrifugation (8500 g, 1 min, 4°C), and resuspended in $1 \times$ PBS. The total numbers of peritoneal leukocytes were counted with a hemocytometer, and expressed as leukocytes (10^6) per cavity. After H&E staining, the ratio of different leukocytes was estimated from counting 750–1000 cells from multiple (6–12) high-powered fields. This ratio was then used to estimate the absolute number of neutrophils (“N”) and mononuclear cells (“M”, macrophages and monocytes) per mouse.

Cell culture

Primary peritoneal macrophages were isolated from healthy BALB/c mice (male, 7–8 weeks, 20–25 g) at 2–3 days after intraperitoneal injection of thioglycollate broth (4%, 2.0 ml) as previously described [20;27]. Human blood was purchased from the Long Island Blood Bank (Melville, NY), and human peripheral blood mononuclear cells (HuPBMCs) were isolated by density gradient centrifugation through Ficoll (Ficoll-Paque PLUS, Pharmacia, Piscataway, NJ) as previously described [28–30]. Following overnight culture, floating lymphocytes were carefully removed, and the remaining monocytes (attached to the bottom of culture plates) were used for further experiment. Primary macrophages and monocytes were cultured (on six-well plate, 3.5×10^6 cells/well) in DMEM supplemented with 1% penicillin/streptomycin and 10% FBS or 10% human serum (HS). Adherent macrophages or monocytes were gently washed with, and cultured in, DMEM supplemented with 10% FBS or 10% HS before stimulating with HMGB1 (1.0 μ g/ml) in the absence or presence of EGCG (20 μ M) for 16 h. Subsequently, the cell-conditioned culture media were analyzed for cytokine levels by using Cytokine Antibodies Arrays as previously described [21;25].

Cytokine Antibody Array

Murine Cytokine Antibody Arrays (Cat. No. M0308003, RayBiotech Inc., Norcross, GA, USA), which respectively detect 62 cytokines on one membrane, were used to determine cytokine levels in murine peritoneal fluid or macrophage-conditioned culture medium as previously described [20;29]. Briefly, the membranes were sequentially incubated with equal volumes of murine peritoneal lavage fluid (100 µl) or cell culture medium (200 µl), primary biotin-conjugated antibodies, and horseradish peroxidase-conjugated streptavidin. After exposing to X-ray film, the relative signal intensity was determined using the Scion Image software.

Western blotting

The levels of G-CSF and MIP-1α/CCL3 in the culture medium were determined by western blotting analysis following a standard protocol. Briefly, equal volume of culture medium [conditioned by equal number ($\sim 3.5 \times 10^6$) of human monocytes] was subjected to ultrafiltration using centricon-3 (M.W. cutoff of 3 kDa), and proteins in the retentate fraction were normalized to equal volume. After resolving the concentrated proteins on sodium dodecyl sulfate (SDS)-polyacrylamide gels, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% non-fat milk, the membrane was incubated with respective antibodies (anti-G-CSF or anti-MIP-1α/CCL3, 1:1000) overnight. Subsequently, the membrane was incubated with the appropriate secondary antibody, and the immunoreactive bands were visualized by chemiluminescence technique.

Real-time RT-PCR

Total RNA was isolated from human monocytes using the Qiagen RNeasy kit as per the manufacturer's instructions, and reversely transcribed into the first-strand cDNA using Random primers. Following reverse transcription, qPCR primers for human G-CSF (forward: 5'-AGGGATCTCCCCGAGTTG-3', and reverse: 5'-CCATTCCCAGTTCTTCCATCTG-3'), 18S rRNA, and GAPDH (forward: 5'-CCTGCACCACCAACTGCTTAG-3', and reverse: 5'-TGAGTCCTTCCACGATACCAA-3') were used to quantify the mRNA expression levels using a Roche LightCycler 480 detection system (Roche Diagnostics). Amplification was performed using Taqman® Universal PCR Master Mix using the following conditions: 50°C, 2'; 95°C 10'; followed by 40 cycles of 95°C for 15'' and 60°C for 1'. This cycle was followed by a melting curve analysis, baseline and cycle threshold values (Ct values) were automatically determined using Roche LightCycler 480 software. The relative G-CSF mRNA expression was calculated by comparison to relative standard curves of untreated monocytes and normalized to GAPDH gene expression.

Statistical Analysis

Data are expressed as mean $\pm$ SD of at least 2–4 independent experiments ($n = 2-4$). One-way ANOVA was used for comparison among all different groups. When the ANOVA was significant, post-hoc testing of differences between groups was performed using Tukey's test. The Kaplan-Meier method was used to compare the differences in mortality rates between groups. A P value < 0.05 was considered statistically significant.

Results

EGCG elevated peritoneal levels of G-CSF and several chemokines in septic mice

To further elucidate the mechanisms underlying EGCG-mediated protection against lethal sepsis, we determined the impact of EGCG on peritoneal accumulation of various cytokines/chemokines at a late stage of experimental sepsis (52 h post CLP), when many animals started to die in the saline group while EGCG conferred significant protection [20;23]. At the doses that conferred significant protection against lethal sepsis [20;23], EGCG (4.0 mg/kg) did not affect peritoneal levels of most cytokines at a late stage of sepsis (Fig. 1A). However, it significantly elevated peritoneal levels of G-CSF and several chemokines (e.g., MCP-1/CCL2 and MIP-1 γ /CCL9) (Fig. 1A, 1B). Although the relative levels of few other chemokines (e.g., MIP-2) were occasionally elevated in a subset of EGCG-treated mice (Fig. 1A), their reproducibility was not comparable to G-CSF, MCP-1/CCL2, MIP-1 γ /CCL9 (data not shown).

To understand the possible consequence of EGCG-mediated elevation of G-CSF and chemokines, we measured the total leukocyte number in the peritoneal lavage fluid. At the doses that EGCG conferred protection against lethal sepsis, EGCG did not significantly affect the total number of mononuclear cells ("M", including monocytes and macrophages, Fig. 2A, 2B), but doubled the total number of neutrophils ("N") in the peritoneal cavity (Fig. 2A, 2B). It suggests that EGCG is capable of modulating neutrophil recruitment and/or proliferation by enhancing G-CSF and chemokine release during experimental sepsis.

EGCG divergently modulated the release of G-CSF and several chemokines by peritoneal macrophages

To gain insight into the mechanisms underlying EGCG-mediated G-CSF and chemokine regulation, we investigated how EGCG affected HMGB1-induced cytokine and chemokine release by peritoneal macrophages [16;31–33]. Consistent with previous reports [24], HMGB1 effectively induced the release of several chemokines (e.g., CXCL15, MCP-1/CCL2, MIP-1 α /CCL3, and RANTES/CCL5) in peritoneal macrophage cultures (Fig. 3A). Surprisingly, at the concentrations comparable to its protective doses *in vivo*, EGCG divergently affected the release of G-CSF and several chemokines. While reducing HMGB1-induced release of CXCL15 and RANTES/CCL5, EGCG inversely elevated the release of G-CSF and MIP-1 α /CCL3 by peritoneal macrophages (Fig. 3A, 3B).

To confirm these cytokine-modulating properties, we further examined the effect of EGCG on HMGB1-induced G-CSF and chemokine expression using primary human monocytes. Consistent with previous findings [34], HMGB1 slightly induced the expression of G-CSF and MIP-1 α /CCL3 in human monocyte cultures (Fig. 4A). However, EGCG increased HMGB1-induced production and release of G-CSF and MIP-1 α /CCL3 (Fig. 4A). Even by itself, EGCG also induced the production of G-CSF and MIP-1 α /CCL3 by monocytes (Fig. 4A). To elucidate the mechanism G-CSF regulation, we determined G-CSF mRNA expression levels in HMGB1- and/or EGCG-treated HuPBMCs by real-time RT-PCR technique. As predicted, the level of G-CSF mRNA was barely detectable in quiescent huPBMCs (Fig. 4B). Following stimulation with HMGB1 and/or EGCG, the G-CSF mRNA

levels were dramatically increased in human monocytes, suggesting that EGCG could indeed induce G-CSF expression by innate immune cells.

A leukocyte-eliciting agent, thioglycollate, rendered animals resistant to septic insult

To appreciate the importance of leukocyte recruitment in sepsis, we determined the effect of a leukocyte-eliciting agent, thioglycollate, on sepsis-induced animal lethality. Pre-treatment with thioglycollate rendered animal resistant to lethal sepsis, significantly increasing animal survival rates from 50% to 90% (Fig. 5). In light of the findings that EGCG dramatically reduced bacterial loads in the liver [35], it is possible that elevation of peritoneal neutrophils may facilitate elimination of invading pathogens in the peritoneal cavity.

Discussion

Innate immune cells (such as macrophages, monocytes and neutrophils) are responsible both for eliminating invading pathogens, and for initiating inflammatory responses [19]. Because excessive release of proinflammatory cytokines may contribute to the pathogenesis of lethal systemic inflammation, anti-inflammatory agents capable of facilitating leukocyte recruitment and/or proliferation may be needed for the clinical management of infectious diseases. Here we report a novel mechanism by which an anti-inflammatory agent, EGCG, conferred protection against lethal peritonitis. It stimulates or enhances HMGB1-mediated production and release of G-CSF and several chemokines by macrophages or monocytes, thereby facilitating possible neutrophil proliferation and/or recruitment to infected sites.

Even by itself, EGCG can induce G-CSF mRNA expression and protein production by macrophage/monocyte cultures. It may underlie the observed elevation of peritoneal G-CSF levels at a late stage of experimental sepsis. Remarkably, the elevation of peritoneal G-CSF levels was associated with a specific increase in neutrophil counts in the peritoneal fluid. It remains to be determined whether the elevated G-CSF might have facilitated the proliferation of neutrophil, which may contribute to an effective bacterial elimination during a late stage of sepsis [35]. Indeed, G-CSF has been shown to stimulate the proliferation of neutrophils [36;37], and widely used to accelerate recovery from neutropenia (an abnormal neutrophil deficiency) in cancer patients undergoing chemotherapy [38], preventing cancer patients from subsequent infections and sepsis. Intriguingly, we found that EGCG induced G-CSF mRNA expression and protein production by human monocytes. Under physiological conditions (37°C, pH 7.0), G-CSF exists in equilibrium with altered conformations balancing between monomers and dimers [39]. In agreement with EGCG's capacity in cross-linking proteins [21;40;41], we detected the presence of G-CSF dimers in EGCG-stimulated human monocytes. Because G-CSF signals through the formation of tetrameric complex comprising two G-CSF and two receptor molecules, there is on-going effort in generating G-CSF dimers for prolonged half-life and elevated biological activities (US Patent 20140248234A1 and China Patent 102380090A). It may be important to explore the therapeutic efficacy of EGCG for treating neutropenia in cancer patients undergoing extensive chemotherapies.

In addition, the EGCG-mediated elevation of chemokines (e.g., MCP-1/CCL2 and MIP-1 γ /CCL9) possibly by macrophages/monocytes may contribute to neutrophilia possibly by

facilitating neutrophil recruitment. Indeed, MCP-1/CCL2, MIP-1 γ /CCL9 and MIP-1 α /CCL3 can all stimulate neutrophil recruitment during bacterial infection [42], and CCL3/CCL9 can even engage the same high-affinity receptor on neutrophils [43]. Thus, we propose that EGCG can directly stimulate or indirectly enhance HMGB1-mediated expression of G-CSF and several chemokines by other innate immune cells (e.g., macrophages or monocytes), thereby facilitating possible neutrophil proliferation and/or recruitment to infected sites. Nevertheless, it is not likely that EGCG itself directly stimulates neutrophil infiltration, because it has been shown that EGCG itself cannot stimulate, but rather inhibit, neutrophil infiltration into inflamed skin tissues in animal models of UV radiation-induced inflammation [44] and ovalbumin-induced allergy [45]. Although EGCG itself does not directly stimulate neutrophil infiltration, it can slow down apoptotic cell death of activated neutrophils [46], thereby sustaining a pool of infiltrating neutrophils at the inflammation site.

In addition to chemokines, the advanced glycation end products (AGEs) formed by nonenzymatic reactions between proteins and reducing sugars in the aged thioglycollate broth can also elicit peritoneal neutrophils and macrophages. Upon intraperitoneal injection of thioglycollate, circulating neutrophils are rapidly recruited into peritoneal cavity within 6–24 h. Subsequently, monocytes/macrophages are recruited, and fulfill a central role in eliminating apoptotic neutrophils and orchestrating inflammatory responses [47;48]. Interestingly, we found that prophylactic treatment with thioglycollate solution rendered animals resistant to subsequent septic insult, which was consistent with the findings that stimulation of leukocyte recruitment (by thioglycollate) confers protection against lethal endotoxemia [49;50].

At present, it is not known whether facilitation of peritoneal leukocyte recruitment underlies the protection conferred by prophylactic thioglycollate or delayed EGCG treatments. After all, EGCG also exhibits a wide spectrum of anti-bacterial [35;51;52], anti-viral [53;54], anti-fungal [55], and anti-inflammatory [20;21;23] properties, which may collectively account for its protective effects in animal models of lethal sepsis. Given the essential roles of the innate immune cells (such as macrophages, monocytes and neutrophils) in eliminating invading pathogens and initiating inflammatory responses [19], it may be important to develop anti-inflammatory agents capable of facilitating microbial elimination for the treatment of lethal inflammatory diseases.

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Abbreviations used in this paper

CLP	cecal ligation and puncture
EGCG	epigallocatechin-3-gallate
G-CSF	granulocyte colony stimulating factor

HMGB1	high mobility group box 1
MIP-1	macrophage inflammatory protein 1
TLR	toll-like receptor

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Figure 1. EGCG elevated peritoneal levels of G-CSF, MCP-1/CCL2, and MIP-1γ/CCL9 in septic animals

BALB/c mice were subjected to lethal sepsis by CLP, and intraperitoneally administered with control saline (0.2 ml/mouse) or EGCG solution (4.0 mg/kg) at +24, +48 hours post CLP. At 52 hours post the onset of sepsis, peritoneal levels of cytokines were determined by cytokine antibody arrays. **A). Representative Cytokine Antibody Arrays.** Peritoneal lavage fluid was pooled from 3 septic mice, and incubated with a membrane. **B) Peritoneal levels of G-CSF and several chemokines.** The average cytokine levels were expressed as

mean $\pm$ SD (n = 12). Shown are results that were consistently different between “+CLP” and “CLP+EGCG” groups in three independent experiments. *, $P < 0.05$ versus “+ CLP” group.

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Figure 2. EGCG increased peritoneal neutrophil counts in septic mice

BALB/c mice were subjected to lethal sepsis by CLP, and intraperitoneally administered with saline (0.2 ml/mouse) or EGCG solution (4.0 mg/kg) at +24, +48 hours post CLP. At 52 hours post the onset of sepsis, total peritoneal leukocyte number in each mouse were counted, and expressed as mean $\pm$ SD (n = 6). **A). Peritoneal leukocyte counts.** The number of major leukocytes (neutrophils, “N”; and mononuclear cells, “M”) was counted after H&E staining from 6–12 randomly selected fields. The insert showed the typical smaller (10–12 μ m) neutrophils (“N”) with multilobed nucleus, as well as larger (14–17 μ m)

monocytes (“M”) with kidney-shaped nucleus. Occasionally, even larger ($> 20 \mu\text{m}$) macrophages (“M”) with rounded nucleus were also found. *, $P < 0.05$ versus “vehicle” group for the number of total leukocytes and neutrophils. **B). Representative photographs of leukocytes.** Bar, $10 \mu\text{m}$.

Figure 3. EGCG divergently modulated HMGB1-stimulated release of G-CSF and various chemokines by peritoneal macrophages

Thioglycollate-elicited primary macrophages were isolated from BALB/c mice, and stimulated with recombinant HMGB1 (1.0 µg/ml) in the absence or presence of EGCG (20 µM) for 16 h, and the extracellular levels of cytokines and chemokines were determined by Cytokine Antibody Arrays. **A). Representative Cytokine Antibody Arrays. B) Relative levels of G-CSF and several chemokines.** The average levels of G-CSF and chemokines

were expressed as mean $\pm$ SD (n = 4). *, $P < 0.05$ versus “-HMGB1”; #, $P < 0.05$ versus “+HMGB1”.

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Figure 4. EGCG induced G-CSF and chemokine expression in human monocytes

Primary human monocytes were isolated from blood of health volunteers, and stimulated with HMGB1 in combination with EGCG at indicated concentrations for 16 h, and the levels of G-CSF and MIP-1α/CCL3 protein (**Panel A**) and G-CSF mRNA (**Panel B**) were determined by western blotting analysis and real-time RT-PCR, respectively. **A).**

Representative western blots. Arrows indicate a faint band corresponding to the molecular weight of G-CSF monomer (M). **B).** **Relative G-CSF mRNA expression levels in HuPBMCs.** The relative G-CSF mRNA level was expressed as mean ± SD of GAPDH

mRNA levels (in arbitrary units, AU). *, $P < 0.05$ versus untreated control (“-HMGB1 - EGCG”).

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Figure 5. Thioglycollate pre-treatment rendered mice resistant to septic insult

Thioglycollate broth was intraperitoneally administered into mice to elicit leukocytes into peritoneal cavity. At 3 days post thioglycollate injection, mice were subjected to CLP-induced sepsis, and animal survival rates were monitored for more than two weeks (two independent experiments with ten mice per group). *, $P < 0.05$ versus “saline” group.