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Comparative Potency of Green Tea and Red Wine Polyphenols in Attenuating Staphylococcal Superantigen-Induced Immune Responses

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Abstract

Proinflammatory cytokines mediate the toxic effects of staphylococcal exotoxins (SE). This study compared the potency of two polyphenols, green tea epigallocatechin gallate (EGCG) and red wine resveratrol (RES), in blocking the pro-inflammatory effects of staphylococcal enterotoxin B (SEB) and toxic shock syndrome 1 (TSST-1). Both EGCG and RES dose-dependently inhibited superantigen-stimulated T-cell proliferation with IC_{50} of 20 μM and 30 μM, respectively. Both polyphenols blocked the production of interleukin 1β, interleukin 6, tumor necrosis factor, gamma interferon, interleukin 2, monocyte chemotactic protein 1, macrophage inflammatory protein (MIP)-1α, and MIP-1β by SE-stimulated human peripheral blood mononuclear cells (PBMC). SEB- and TSST-1-induced NF-κB activation in PBMC was also significantly reduced by EGCG and RES. These results suggest that polyphenols have potent immunosuppressive effects counteracting the host inflammatory cascades induced by superantigens and might be used therapeutically to mitigate the pathogenic effects of microbial superantigens.

Keywords: Anti-inflammatory, polyphenols, superantigens.

1. Introduction

Staphylococcal enterotoxin B (SEB) and the structurally related toxic shock syndrome 1 (TSST-1) are bacterial exotoxins and common etiological agents that cause a variety of autoimmune diseases and shock [1-3]. These exotoxins bind to both the major histocompatibility complex (MHC) class II molecules on antigen-presenting cells and specific Vβ regions of the T-cell antigen receptors [4-6]. The staphylococcal exotoxins (SE) are also known as superantigens because of their ability to

polyclonally activate a considerable proportion of T cells [6]. Their interactions with cells of the immune system also induce a massive production of proinflammatory cytokines and chemokines [1, 2, 7]. The cytokines, tumor necrosis factor (TNF)α, IL-1, and interferon gamma (IFNγ) are pivotal mediators in superantigen-induced toxic shock [8-10]. Both TNFα and IL-1 have potent immuno-stimulating activities and act synergistically with IFNγ to enhance inflammatory and immune reactions and promote tissue injury [11]. Consequently, these cytokines are pathogenic at high concentrations in vivo and are responsible for fever and toxic shock induced by SE [9. 10].

Epigallocatechin-3-gallate (EGCG), the major polyphenol in green tea, has potent anti-inflammatory and anti-oxidant effects in vitro and in vivo [reviewed in 12]. Treatment with EGCG has been reported to provide therapeutic efficacy in animal models of arthritis and glomerulonephritis [12, 13]. In vitro EGCG has multiple biological activities depending on cell type. EGCG decreased the interleukin (IL)-1-induced expression of matrix metalloproteinase (MMP)-13 at 20 μM and MMP-1 at 100 μM in human chondrocytes at the transcriptional level [14]. At these concentrations, EGCG also blocked the IL-1-induced cartilage matrix degradation in cartilage explants and increase in NF-κB translocation and activation in chondrocytes. In a mouse macrophage cell line, millimolar concentrations of EGCG were required to attenuate lipopolysaccharide (LPS)-induced TNFα production and NF-κB activation [15]. EGCG blocked SEB-disrupted epithelial cell barrier function by inhibiting cytokine release [16]. At comparable concentrations, EGCG reduced the IL-1 or TNFα-induced expression of vascular adhesion molecule-1 on endothelial cells and blocked the adhesion of monocytic cells to cytokine-activated endothelial cells [17]. In activated neutrophils, EGCG repressed ROS activity, elastase and chemokine-induced neutrophil chemotaxis [18]. At micromolar concentrations, EGCG induced vasorelaxation in rat aortic rings and increased endothelial nitric oxide synthetase [19]. EGCG is also neuroprotective as it activated the release of non-amyloidogenic protein in human neuroblastoma and rat pheochromocytoma cells at low concentrations (1-10 μM) [20]. In vitro, EGCG inhibited epithelial cancer cell growth with an IC_{50} of 20 μM through the downregulation of p42/p44 and p38 MAPK [21]. Interestingly, the angiogenic factor, vascular endothelial growth factor, was also suppressed in cancer cells [21]. The chemopreventive property of EGCG was linked to its inhibition of cyclooxygenase 2 expression in colon cancer cells [22]. Redox-sensitive transcription factors, NF-κB and AP-1 are molecular targets for chemoprevention with EGCG [23]. In vivo, EGCG (10 mg/kg intraperitoneally) improved hypotension and survival in a rodent model of polymicrobial sepsis [24]. The same dose of this green tea polyphenol given intravenously also reduced myocardial reperfusion injury in rats [25]. These events were associated with inhibition of IκB kinase, AP-1, and c-Jun phosphorylation in the infracted heart. Oral administration of 0.5 g/kg of EGCG given 2 hours before LPS prevented LPS-induced lethality in mice [15] and a reduction in serum TNFα was attributed to the beneficial effects of EGCG. In a diabetic nephropathy model, rats given 0.1 g/kg of EGCG had suppressed hyperglycemia, proteinuria and lipid peroxidation, thereby reducing renal damage [26]. Thus both in vitro and in vivo, EGCG inhibits cytokine release, blocks the effects induced by proinflammatory cytokines and cytokine signaling.

Another structurally distinct polyphenol, resveratrol (3, 4', 5-trihydroxy-trans-stilbene) from red wine also shows chemoprotective effects against heart diseases, inflammation and cancers [27]. Resveratrol (RES) attenuated TNFα-induced activation of coronary arterial endothelial cells [28] and TNFα induced MCP-1 in adipocytes [29]. RES downregulated gamma interferon-induced inflammatory genes by decreasing STAT-1 activation in a mouse macrophage cell line [30]. In addition, RES blocked PI3 kinase and mTOR signaling in human glioma cells [31, 32]. RES is also neuroprotective as it prevented neurotoxicity induced by beta-amyloid in rat hippocampal neurons [33]. RES inhibited the proliferation of human renal cancer cells and exerted its antitumor effect by suppressing the expression of the VEGF

gene [34]. RES has potent anti-oxidative effects and inhibited the production of reactive oxygen species and reactive nitrogen species during respiratory burst [35]. In lipopolysaccharide-stimulated murine peritoneal macrophage, inflammatory gene expression of inducible nitric oxide synthetase and cyclooxygenase was also down-regulated [35]. RES regulated metabolism by stimulating glucose uptake in muscle cells through a mechanism involving sirtuins and AMPK [36].

Based on their similar immunomodulating properties, the relative potency of these two diverse polyphenols in attenuating staphylococcal superantigen-induced T cell activation and cytokine production from human peripheral blood mononuclear cells (PBMC) was evaluated.

2. Material and Methods

2.1. Materials

Purified TSST-1 and SEB were obtained from Toxin Technology (Sarasota, FL). The endotoxin content of these preparations was < 1 ng of endotoxin/mg protein as determined by the Limulus amoebocyte lysate gelation test (BioWhittaker, Walkersville, MD). Human (h) recombinant (r) TNFα, antibodies against hTNFα, peroxidase-conjugated anti-rabbit IgG, and peroxidase-conjugated anti-goat IgG were obtained from Boehringer-Mannheim (Indianapolis, IN). Human rIFNγ and rIL-6 were obtained from Collaborative Research (Boston, MA). Antibodies against IFNγ, IL-2, and MCP-1 were obtained from BDPharMingen (San Diego, CA). Recombinant IL-2, MCP-1, MIP-1α, MIP-1β; antibodies against IL-1β, IL-6, MIP-1α, and MIP-1β were purchased from R&D Systems (Minneapolis, MN). EGCG was obtained from Calbiochem (San Diego, CA) and dissolved in phosphate buffer saline (PBS). RES was purchased from Sigma (St. Louis, MO) and dissolved in dimethylsulfoxide. All other common reagents were from Sigma.

2.2. Cell culture

Human PBMC were isolated by Ficoll-Hypaque density gradient centrifugation of heparinized blood from normal human donors. PBMC (10^6 cells/mL) were cultured at 37^0C in RPMI 1640 medium supplemented with 10% inactivated fetal bovine serum in 24-well plates as previously described [37]. Cells were stimulated with SEB (200 ng/mL) or TSST-1 (200 ng/mL) for 16 hr. Varying concentrations (2, 20, 50, 100 μM) of EGCG or RES were added simultaneously with SEB or TSST-1. Comparisons of inhibitory activity were made with PBMC from the same donors to reduce effects due to donors' variability and experiments were repeated at least three times. Culture supernatants were collected and analyzed for IL-1β, TNFα, IL-6, IFNγ, IL-2, MCP-1, MIP-1α, and MIP-1β. Cell viability was determined by the trypan blue dye exclusion method. At the end of the experiment, cells were recovered and the number of trypan blue-positive cells was counted. Cells were 93-98% viable in the presence or absence of polyphenols with SEB using concentrations of polyphenols described.

T-cell proliferation was assayed with PBMC (10^6 cells/well), which were plated in triplicate with SEB or TSST-1 (200 ng/mL), with or without EGCG or RES, for 48 hr at 37^0C in 96-well microtiter plates. Cells were pulsed with 1 μCi/well of [^{3}H]thymidine (New England Nuclear, Boston, MA) during the last 5 h of culture as described previously [37]. Cells were harvested onto glass fiber filters, and incorporation of [^{3}H]thymidine was measured by liquid scintillation.

2.3. Measurement of cytokines and chemokines

Cytokines and chemokines were measured by an enzyme-linked immunosorbent assay (ELISA) with cytokine- or chemokine-specific antibodies according to the manufacturer's instructions, as previously described [37, 38]. Human recombinant cytokines and chemokines (20-1000 pg/mL) were used as standards for calibration on each plate. The detection limit of each assay was 20 pg/mL. The cytokine and chemokine data were expressed as the mean concentration (pg/mL) ± SD of duplicate samples.

2.4. NF-κB activation assay

NF-κB activation was measured with a Trans-AM NF-κB kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions.

Oligonucleotides containing the NF-κB consensus sequence (5'-GGGACTTTCC-3') were bound to a 96-well plate. Only the active form of NF-κB in cell extracts can specifically bind to the plate-bound-oligonucleotides. Nuclear extracts (10 µg) containing NF-κB protein from PBMC were added to the wells, followed by the primary antibody against p65 subunit of NF-κB and the horseradish peroxidase-conjugated secondary antibody. Optical density was determined on an absorbance plate reader at 450 nm.

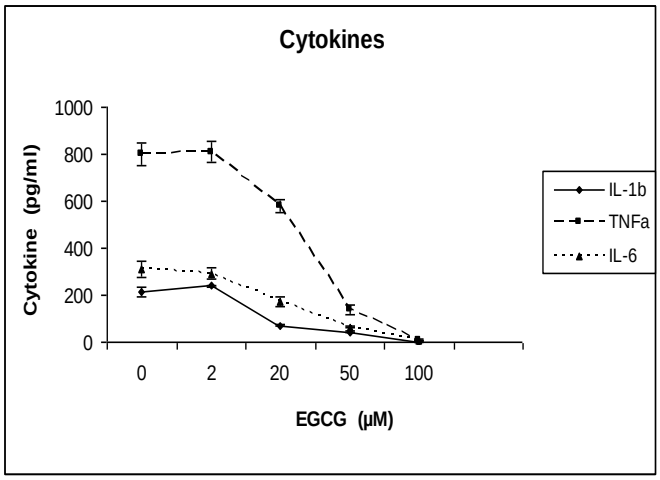
2.5. Data analysis

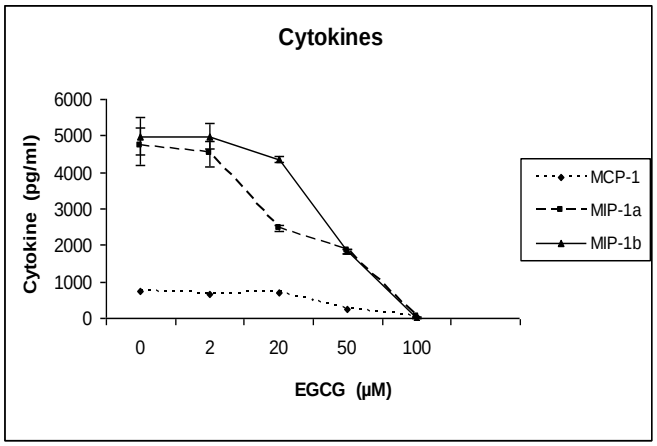
Data were expressed as the mean ± SD and were analyzed for significant differences by the Student's *t*-test with Stata (Stata Corp., College Station, TX, USA). Differences between polyphenol-treated and untreated control groups were considered significant if P was < 0.05.

3. Results

3.1. EGCG attenuated cytokine and chemokine production from SEB-stimulated PBMC

Based on reports that EGCG has anti-inflammatory effects, we tested its potency in attentuating cytokine and chemokine production by the superantigen, SEB. Figure 1A shows that EGCG blocked the production of IL-1β, TNFα and IL-6 in SEB-stimulated PBMC in a dose-dependent manner. EGCG at 50 µM reduced the IL-1β, TNFα and IL-6 levels to 20%, 17% and 21% in culture supernatants, respectively. The production of chemokines (MCP-1, MIP-1α, MIP-1β) were also suppressed considerably (Figure 1B). Reduction of cytokines and chemokines were statistically significant (P <0.05) between SEB and SEB plus EGCG samples at concentrations of 20 to 100 µM. T-cell cytokines, IFNγ and IL-2 were attenuated substantially even at a lower concentration of EGCG. At 20 µM EGCG, IFNγ and IL-2 were reduced to 1% and 4%, respectively (Figure 1C). Dose response inhibition curves of EGCG were similar at both high SEB (1000 ng/mL) and low SEB (10 ng/mL) concentrations (data not shown).





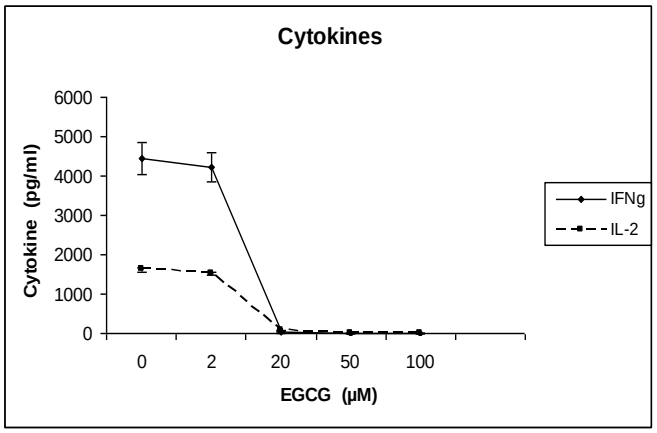


Figure 1. Dose-response inhibition of *(A)* IL-1β, TNFα, and IL-6, *(B)* MCP-1, MIP-1α, MIP-1β *(C)* IFNγ and IL-2 production by PBMC stimulated with 200 ng/mL of SEB in the presence of various concentrations of EGCG. Values represent the mean ± SD of duplicate samples and results represent three experiments. Results are statistically significant (P <0.05) between SEB and SEB plus EGCG samples at concentrations of 20 to 100 µM.

3.2. RES blocked cytokine and chemokine production from SEB-stimulated PBMC

RES, another polyphenol known for its anti-oxidant activity, was also investigated. RES dose dependently inhibited IL-1β, TNFα and IL-6 production by SEB-stimulated PBMC, reducing IL-1β, TNFα and IL-6 by 29%, 65%, and 51%, respectively, at 50 μM of RES (Figure 2A). At the same concentration of RES, the production of chemokines (MCP-1, MIP-1α, MIP-1β) and T-cell cytokine (IFNγ and IL-2) was blocked to the same extent (Figure 2B and 2C). Higher concentrations of RES blocked the production of these cytokines and chemokines more completely. Inhibition of cytokines and chemokines by RES were statistically significant (P <0.05) between SEB and SEB plus RES samples at concentrations of 20 to 100 μM. RES did not affect the viability of the cells over the concentration range used in these studies (2-100 μM), as confirmed by trypan blue dye exclusion test. Thus comparatively, using the same cell culture system and PBMC from the same blood donors, EGCG was more potent in blocking TNFα, IFNγ and IL-2 than RES.

3.3. EGCG and RES inhibited TSST-1 induced cytokines and chemokines

Figure 3 compares the inhibition by 50 μM EGCG and RES on cytokine and chemokine production by PBMC cultures stimulated with another staphylococcal exotoxin, TSST-1. The level of inhibition of TSST-1-stimulated cells by EGCG and RES was similar to that of SEB suggesting that both polyphenols are effective inhibitors of superantigen-activated inflammatory pathways. The suppressive effects of EGCG were higher on T-cell cytokines IFNγ and IL-2. EGCG was also a stronger inhibitor than RES using TSST-1-stimulated cells.

3.4. EGCG and RES inhibited superantigen-induced T-cell proliferation

Because superantigen polyclonally activates T cells, the effect of the polyphenols, EGCG and RES on SE-induced T-cell proliferation was next investigated. Figure 4 shows that both EGCG and RES potently inhibited SEB- and TSST-1-stimulated T-cell proliferation in a dose-dependent manner; achieving 6% and 16% inhibition at 50 μM of EGCG and RES, respectively. The inhibition of T-cell proliferation by either EGCG or RES were statistically significant (P <0.05) between superantigen and superantigen plus polyphenol (20-100 μM) samples.

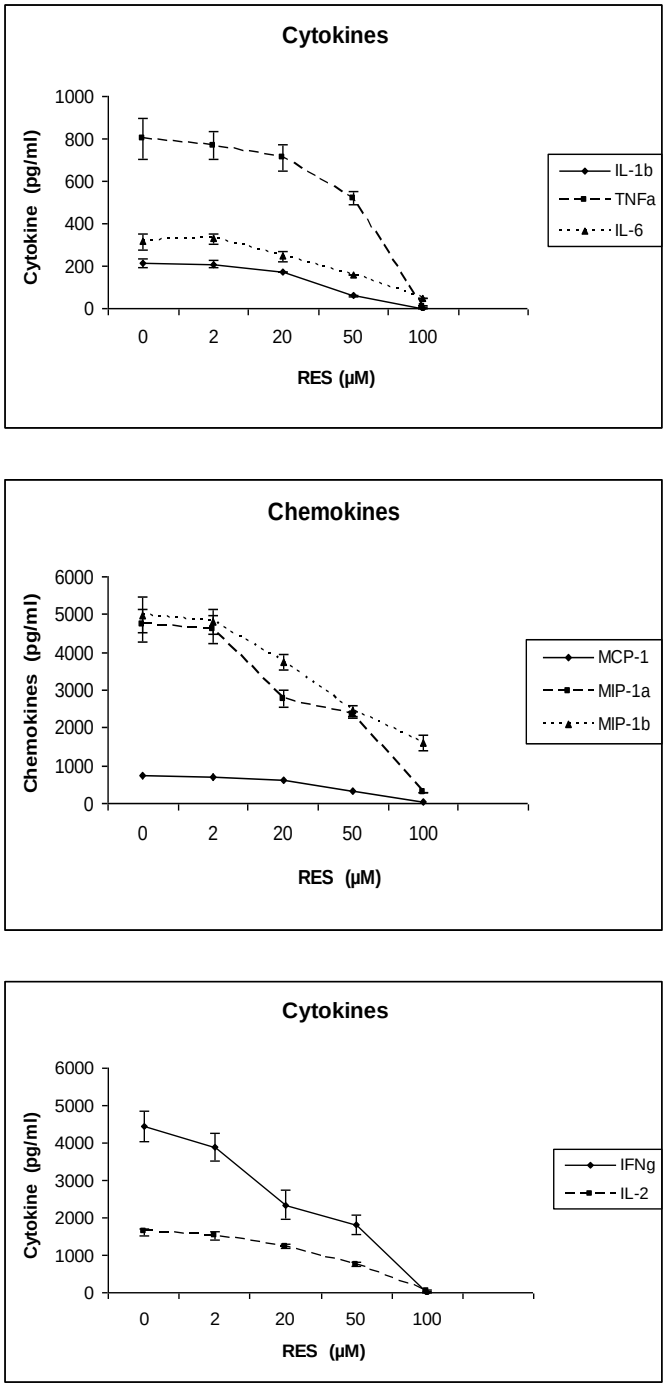
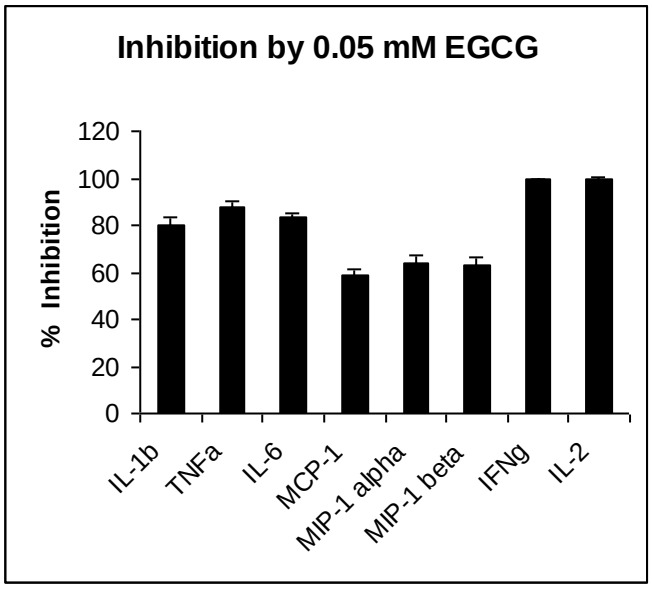


Figure 2. Dose-response inhibition of *(A)* IL-1β, TNFα, and IL-6, *(B)* MCP-1, MIP-1α, MIP-1β *(C)* IFNγ and IL-2 production by PBMC stimulated with 200 ng/mL of SEB in the presence of various concentrations of RES. Values represent the mean ±

SD of duplicate samples and results represent three experiments. Results are statistically significant (P <0.05) between SEB and SEB plus RES samples at concentrations of 20 to 100 μM.



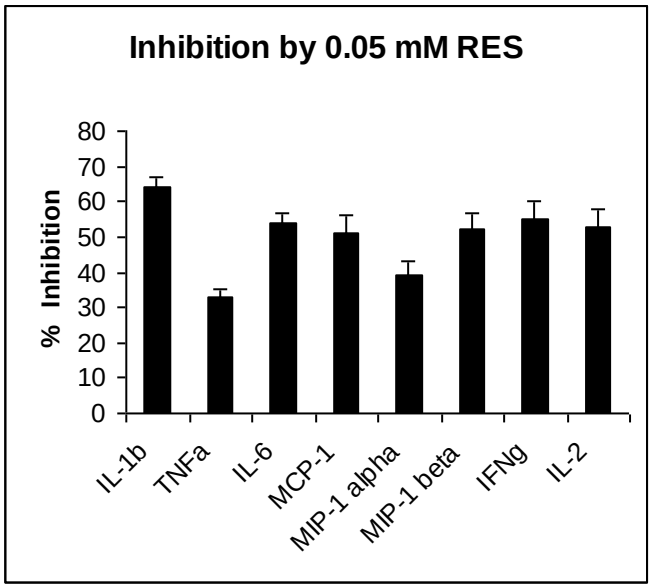
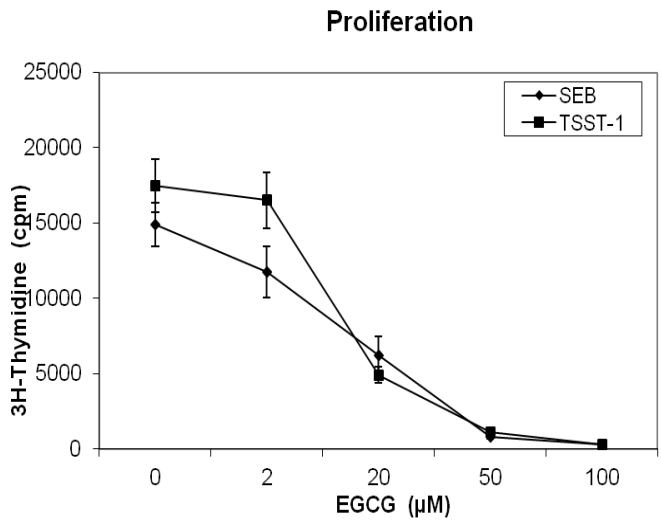


Figure 3. Inhibition of *(A)* IL-1β, TNFα, IL-6, MCP-1, MIP-1α, MIP-1β, IFNγ and IL-2 production by PBMC stimulated with 200 ng/mL of TSST-1 in the presence of 50 μM EGCG, *(B)* IL-1β, TNFα, IL-6, MCP-1, MIP-1α, MIP-1β, IFNγ and IL-2 production by PBMC stimulated with 200 ng/mL of TSST-1 in the presence of 50 μM RES. Values represent the mean ± SD of three samples and results represent three experiments. Results are statistically significant (P <0.05) between TSST-1 and TSST-1 plus EGCG and TSST-1 plus RES.



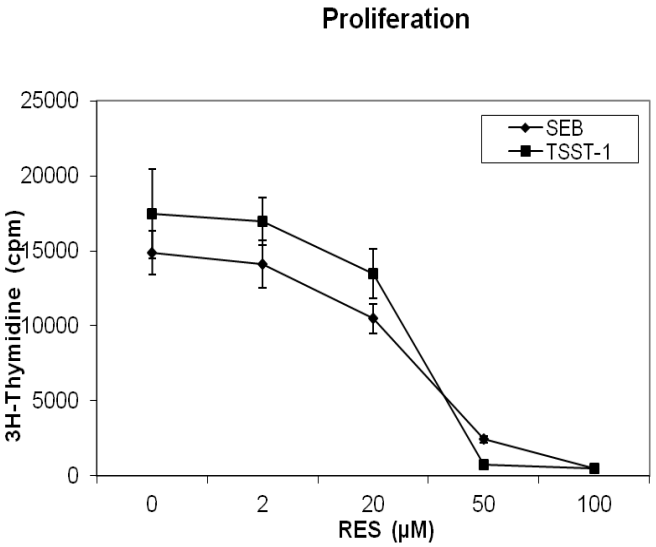


Figure 4. Inhibition of T-cell proliferation in PBMC stimulated with 200 ng/mL of SEB or TSST-1 in the presence of various concentrations of *(A)* EGCG, *(B)* RES. Values represent the mean ± SD of triplicate samples and results represent three experiments. Results are statistically significant (P <0.05) between stimulant (TSST-1 or SEB) and stimulant plus polyphenol (20-100 μM) samples.

3.5. EGCG and RES blocked NF-κB activation in superantigen-stimulated PBMC

The transcription factor NF-κB is a key regulator of inflammation and acts downstream of many cell surface receptors including MHC class II molecules and toll-like receptors. The effects of EGCG and RES on NF-κB activation were next investigated. Cell extracts from SEB-treated

PBMC in the presence or absence of EGCG or RES were prepared and NF-κB activation was measured by DNA-binding on oligonucleotide-coated plates. As shown in Figure 5, EGCG blocked SEB-induced increase in activated NF-κB to 27% at 50 μM (P <0.05) whereas RES reduced NF-κB activation to 39% (P <0.05). Thus the inhibitory dose of both EGCG and RES was directly correlated to the potency of these polyphenols in reducing cytokine and chemokine production.

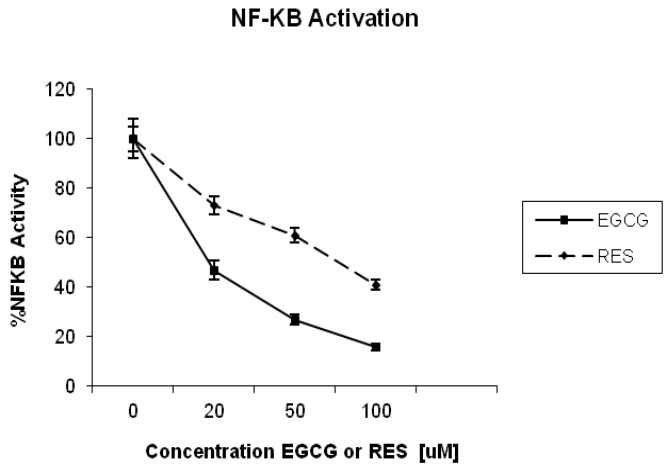


Figure 5. Inhibition of NF-κB activation in PBMC stimulated with 200 ng/mL of SEB by varying concentrations of *(A)* EGCG and *(B)* RES. Values are the mean ± SD of duplicate samples from three experiments. Results are statistically significant (P <0.05) between SEB and SEB plus polyphenols from 20-100 μM.

4. Discussion

Toxic shock caused by staphylococcal superantigens is a serious disease with a high mortality rate, and so far, successful treatment remains elusive despite great efforts invested in drug development. Anti-inflammatory and immunosuppressive therapeutics represent a potentially useful treatment by targeting many downstream signaling pathways affecting multiple cytokines and chemokines. The results presented here indicate that green tea EGCG and red wine RES suppressed the induction of proinflammatory cytokines and chemokines by TSST-1- and SEB-stimulated human PBMC. The production of these mediators by monocytes/macrophages and T cells in response to superantigens initiates leukocyte activation and migration, contributing directly to inflammation and tissue injury associated with shock. The effect of EGCG and RES was more pronounced on T- cell proliferation. EGCG also had more inhibitory effect on the T- cell cytokines, IL-2 and IFNγ whereas higher doses were required to suppress other cytokines and chemokines to the same extent. RES also dose dependently reduced the production of chemokines MCP-1, MIP-1α, and MIP-1β and all cytokines from superantigen-stimulated PBMC. This indicates that polyphenols inhibited both cytokines and chemokines produced in vitro by both T cells and monocytes in response to superantigens.

Attenuated T cell activation with decreased elaboration of key proinflammatory cytokines by EGCG and RES suggests that polyphenols from green tea and red wine may prove useful in treating superantigen-induced shock. Our studies showed that both EGCG and RES suppress a broad range of cytokines and chemokines, induced by superantigens, suggesting that these polyphenols target several intracellular signaling pathways. One prominent pathway is the transcriptional activation of NF-κB which regulates the expression of inflammatory cytokines, cyclooxygenase 2, and cell adhesion molecules [39]. This interference of NF-κB activation by both polyphenols likely accounts for their immunosuppressive effects. Our result is in agreement with previous observations that polyphenols downregulate NF-κB activity which accounts for their pleiotropic effects [12, 27]. In conclusion, due to the broad spectrum of cytokines antagonized, and based on its beneficial therapeutic effects in autoimmune diseases, polyphenols may prove useful as therapeutics for the treatment of superantigen-induced toxic shock.

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