

Triptolide Attenuates Endotoxin- and Staphylococcal Exotoxin-Induced T-Cell Proliferation and Production of Cytokines and Chemokines

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Proinflammatory cytokines mediate the toxic effects of superantigenic staphylococcal exotoxins (SE) and bacterial lipopolysaccharide (LPS). Triptolide, an oxygenated diterpene derived from a traditional Chinese medicinal herb, *Tripterygium wilfordii*, inhibited SE-stimulated T-cell proliferation (by 98%) and expression of interleukin 1 β , interleukin 6, tumor necrosis factor, gamma interferon, monocyte chemotactic protein 1, macrophage inflammatory protein (MIP)-1 α , and MIP-1 β by human peripheral blood mononuclear cells (PBMC). It also blocked the production of these cytokines and chemokines by LPS-stimulated PBMC in a dose-dependent manner. These results suggest that triptolide has potent immunosuppressive effects even counteracting the

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14. ABSTRACT Proinflammatory cytokines mediate the toxic effects of superantigenic staphylococcal exotoxins (SE) and bacterial lipopolysaccharide (LPS). Triptolide, an oxygenated diterpene derived from a traditional Chinese medicinal herb, Tripterygium wilfordii, inhibited SE-stimulated T-cell proliferation (by 98%) and expression of interleukin 1beta, interleukin 6, tumor necrosis factor, gamma interferon, monocyte chemotactic protein 1, macrophage inflammatory protein (MIP)-1alpha, and MIP-1beta by human peripheral blood mononuclear cells (PBMC). It also blocked the production of these cytokines and chemokines by LPS-stimulated PBMC in a dose-dependent manner. These results suggest that triptolide has potent immunosuppressive effects even counteracting the effects of superantigens and LPS. It also may be therapeutically useful for mitigating the pathogenic effects of these microbial products by downregulating the signaling pathways activated by both bacterial exotoxins and endotoxins.					
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effects of superantigens and LPS. It also may be therapeutically useful for mitigating the pathogenic effects of these microbial products by downregulating the signaling pathways activated by both bacterial exotoxins and endotoxins.

Keywords Cytokine, SEB, TSST-1, LPS, Immunosuppression, Triptolide.

INTRODUCTION

Staphylococcal exotoxins (SE) and bacterial lipopolysaccharide (LPS) are the most common etiological agents causing shock.^[1–3] Although these bacterial products interact with host cells through different receptors, they both trigger the release of inflammatory cytokines and chemokines, inducing inflammation and resulting in tissue injury. LPS from Gram-negative bacteria binds directly to CD14 that facilitates its interaction with Toll-like receptor 4 (TLR4), and MD2 of monocytes/macrophages and other cells.^[4] Subsequent transmembrane signaling then activates multiple pathways including the NF- κ B and p38 MAP kinase pathways resulting in cellular activation and expression of inflammatory cytokines and chemokines. LPS induces excessive levels of the proinflammatory cytokines, interleukin 1 (IL-1), and tumor necrosis factor α (TNF α); the key mediators of septic shock and more chronic inflammatory reactions.^[5]

Staphylococcal toxic shock syndrome toxin 1 (TSST-1) and the distantly related staphylococcal enterotoxin A and B (SEA and SEB) also are potent activators of the immune system and cause a variety of human diseases, ranging from food poisoning to toxic shock.^[1,2,6,7] These exotoxins bind to both the major histocompatibility complex (MHC) class II molecules on antigen-presenting cells and specific V β regions of the T-cell antigen receptors.^[8–10] These toxins are called superantigens because of their ability to polyclonally activate a considerable proportion of T cells.^[8] Their interactions with cells of the immune system also induce a massive production of proinflammatory cytokines and chemokines.^[10–12] The cytokines, TNF α , IL-1, and interferon gamma (IFN γ) are pivotal mediators in superantigen-induced toxic shock.^[2,7,10,13]

Both TNF α and IL-1 have potent immunostimulating activities and act synergistically with IFN γ to enhance inflammatory and immune reactions and promote tissue injury.^[14] Consequently, these cytokines are pathogenic at high concentrations in vivo and are responsible for fever and toxic shock induced by SE.^[2,7,10]

Triptolide is a diterpenoid triepoxide isolated from the Chinese medicinal herb *Tripterygium wilfordii* Hook F (TWHF). TWHF has been used for centuries in traditional Chinese medicine to treat rheumatoid arthritis, nephritis, and pulmonary diseases.^[15] Extracts of TWHF suppress type II collagen-induced arthritis and effectively prevent allograft rejection.^[16,17] In

vitro, TWHF extracts inhibited T-cell activation by phytohemagglutinin (PHA) or anti-CD3 antibody.^[18] Triptolide has been identified as the major active constituent responsible for the anti-inflammatory and immunosuppressive effects of TWHF.^[19–21]

Triptolide has been reported to inhibit many biological processes in a wide variety of cell types. Triptolide inhibits LPS-stimulated COX-2 mRNA and synthesis of PGE₂ in LPS-stimulated monocytes.^[22]

In human synovial fibroblasts, triptolide suppresses the production and expression of prometalloproteinases 1 and 3 and inhibits the expression of COX-2 and IL-1-induced PGE₂ production.^[23] Triptolide also inhibits vascular endothelial cell growth factor expression in phorbol 12-myristate 13-acetate (PMA)-activated endothelial cells^[24] and attenuates the expression of IL-6, IL-8, and cell adhesion molecule ICAM-1 by PMA-stimulated human bronchial epithelial cells.^[25] The effects of triptolide on other cell types include the inhibition of the expression of C3, CD40, and B7H in TNF α -activated human proximal tubular epithelial cells^[26] and suppression of LPS-induced TNF α , IL-1 β , and nitric oxide production by microglial cells.^[27] Additionally, triptolide inhibits T-cell IL-2 expression at the purine-box/NF-AT and NF κ B target sequence after specific DNA binding.^[28]

A proposed mechanism of action for triptolide is inhibition of NF κ B transcriptional activation. Additional studies indicated that triptolide also blocks constitutive expression of cell-cycle regulators, cyclins D1, B1, and A1 in bronchial epithelial cells.^[25] Triptolide also has antineoplastic activity and sensitizes cells to TNF α -induced apoptosis in tumor cells via the activation of caspase 3.^[29,30] Recently, cDNA array analysis indicated that triptolide inhibits the expression of genes associated with cellular inflammation, cell-cycle progression, and cell survival.^[31] A soluble derivative of triptolide (PG490-88) was effective in suppressing obliterative airway disease in a mouse allograft model and blocks bleomycin-induced lung fibrosis.^[32]

This study was undertaken to determine the effect of triptolide on staphylococcal superantigen-induced T-cell activation and cytokine production by human peripheral blood mononuclear cells (PBMC) to determine whether it may be used to suppress toxic shock syndrome. These effects were compared with those of triptolide on LPS-stimulated PBMC, as previous studies used LPS or cytokines as the stimulating agents.

MATERIALS AND METHODS

Reagents

Purified TSST-1 and SEB were obtained from Toxin Technology (Sarasota, FL, USA). 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, USA). Human (h) recombinant (r) TNF α , antibodies against hTNF α , peroxidase-conjugated antirabbit IgG, and peroxidase-conjugated antigoat IgG were obtained from Boehringer-Mannheim (Indianapolis, IN, USA). Human rIFN γ and rIL-6 were obtained from Collaborative Research (Boston, MA, USA). Antibodies against IFN γ and MCP-1 were obtained from BDPharMingen (San Diego, CA, USA). Recombinant MCP-1, MIP-1 α , MIP-1 β , and antibodies against IL-1 β , IL-6, MIP-1 α , and MIP-1 β were purchased from R&D Systems (Minneapolis, MN, USA). Lipopolysaccharide (*Escherichia coli* 055:B5) was purchased from Difco (Detroit, MI, USA). Triptolide was obtained from Calbiochem (San Diego, CA, USA) and dissolved in DMSO. All other common reagents were from Sigma (St. Louis, MO, USA).

Cell Culture

Human PBMC were isolated by Ficoll-Hypaque density gradient centrifugation of heparinized blood from normal human donors. PBMC were cultured at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum at a concentration of 10⁶ cells/mL in 24-well plates. Cells were stimulated with TSST-1 (200 ng/mL), SEB (200 ng/mL), or LPS (5 ng/mL) for 16 hr. Various concentrations of triptolide were added simultaneously with TSST-1, SEB, or LPS. Supernatants were harvested and analyzed for IL-1 β , TNF α , IL-6, IFN γ , MCP-1, MIP-1 α , and MIP-1 β . Cytotoxicity was measured by the uptake of trypan blue.

T-cell proliferation was assayed with PBMC (10⁵ cells/well) that were plated in triplicate with TSST-1 or SEB (200 ng/mL), with or without triptolide, for 48 hr at 37°C in 96-well microtiter plates. Cells were pulsed with 1 μ Ci/well of [³H]thymidine (New England Nuclear, Boston, MA, USA) during the last 5 hr of culture as described previously.^[33] Cells were harvested onto glass fiber filters, and incorporation of [³H]thymidine was measured by liquid scintillation.

Cytokine Assays

Cytokines and chemokines were measured by an enzyme-linked immunosorbent assay (ELISA) with cytokine- or chemokine-specific antibodies as previously described.^[33,34] 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.

Ribonuclease Assays

Total RNA was isolated 4 hr after SE or LPS treatment from cells by using a guanidinium isothiocyanate/chloroform-based technique (TRIZOL,

GIBCO, Grand Island, NY, USA) per the manufacturer's instructions. The RNase protection assay was performed as follows: total cellular RNA (5–10 µg) was hybridized with a ³³P UTP-labeled RNA probe (mck-1, mck-2b, mck-3b, mck-5 utilizing the BDPharmingen RiboQuant In Vitro Transcription kit, 1 × 10⁶ cpm/RNA sample) using the BDPharmingen hybridization buffer, according to the manufacturer's directions (BDPharmingen). After hybridization, the samples were treated with RNase A and T1 according to the procedure provided by BDPharmingen; the RNase was inactivated; and the protected RNA was precipitated with a master cocktail containing 200 µL of Ambion (Austin, TX, USA) Rnase inactivation reagent, 50 µL of ethanol, 5 µg of yeast tRNA, and 1 µL of Ambion GycoBlue co-precipitate per RNA sample. The samples were mixed well, incubated at – 70°C for 30 min, and centrifuged at 14,000 rpm for 15 min at room temperature. The pellets were resuspended in 3 µL of BDPharmingen sample buffer and subjected to polyacrylamide gel electrophoresis as recommended by the manufacturer (BDPharmingen).

Statistical Analysis

Data were expressed as the mean ± SD and were analyzed by the Student's *t*-test with Stata (Stata Corp., College Station, TX). Differences between triptolide-treated groups and untreated controls were considered significant if *p* was < .05.

RESULTS

Triptolide Blocked Cytokine and Chemokine Production

Based on reports that triptolide has anti-inflammatory effects, we tested its potency in blocking cytokine and chemokine production by two different stimulants, the superantigen TSST-1 and LPS. Figure 1A shows that triptolide blocked the production of IL-1β and IL-6 in TSST-1-stimulated PBMC in a dose-dependent manner. A low dose of triptolide (10 nM) reduced the IL-1β and IL-6 levels to 12% and 17% in culture supernatants, respectively. The production of other inflammatory cytokines (TNFα and IFNγ) and chemokines (MCP-1, MIP-1α, MIP-1β) also were blocked by triptolide (Fig. 1B and 1C). Higher concentrations of TSST-1 (500 ng/mL) failed to reverse the suppressive effects of triptolide. Dose response inhibition curves of triptolide were similar at both high TSST-1 (1000 ng/mL) and low TSST-1 (10 ng/mL) concentrations (data not shown).

The suppressive effects of triptolide were further examined by using LPS as a stimulant that activates a different receptor. Triptolide also inhibited

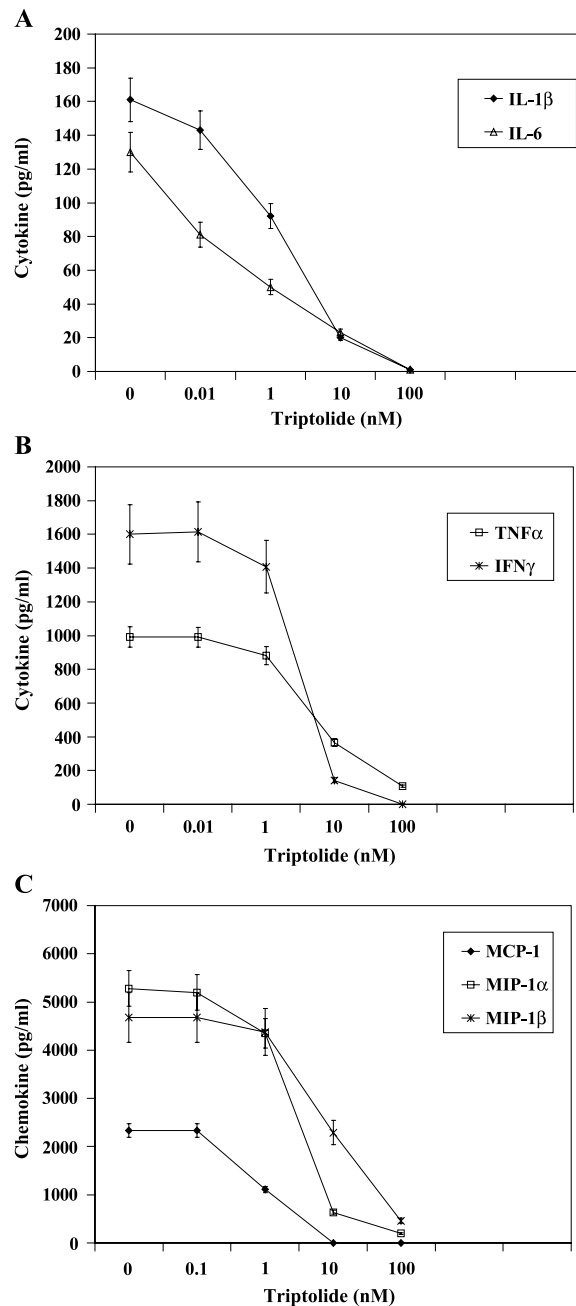


Figure 1: Dose-response inhibition of (A) IL-1 β and IL-6, (B) TNF α and IFN γ , (C) MCP-1, MIP-1 α , and MIP-1 β production by PBMC stimulated with 200 ng/mL of TSST-1 in the presence of various concentrations of triptolide. Values represent the mean $\pm$ SD of duplicate samples and results represent three experiments. Results are statistically significant ($p < .05$) between TSST-1 and TSST-1 plus triptolide samples at concentrations of 1 to 100 nM of triptolide for IL-1 β , IL-6, and MCP-1. For TNF α , IFN γ , MIP-1 α , and MIP-1 β results are statistically significant ($p < .05$) between TSST-1 and TSST-1 plus triptolide samples at 10 to 100 nM.

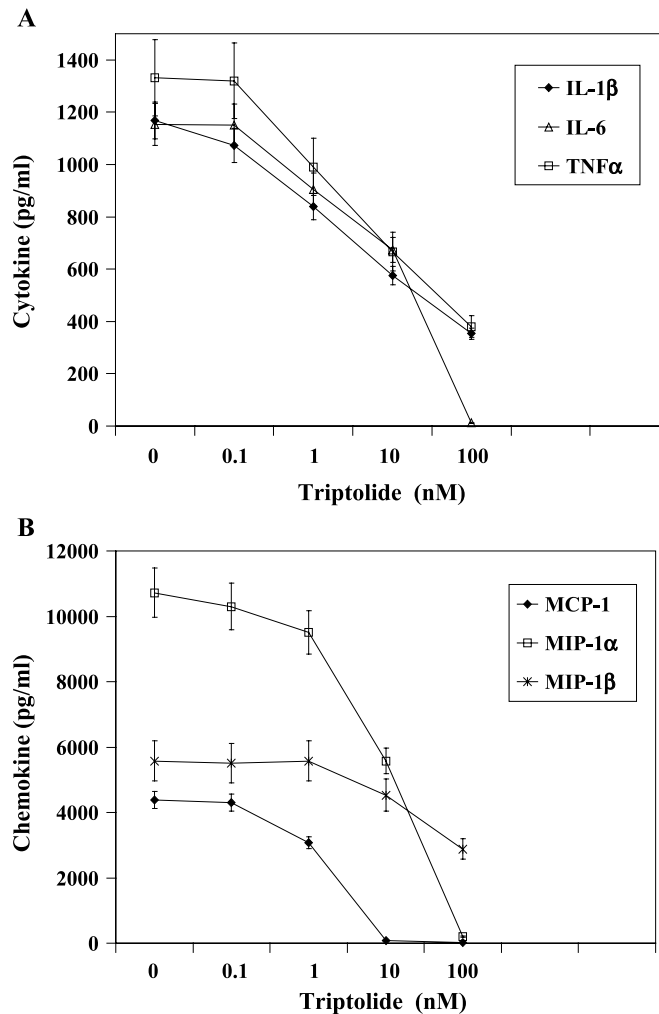


Figure 2: Inhibition of (A) IL-1 β , IL-6, and TNF α ; (B) MCP-1, MIP-1 α , and MIP-1 β production by PBMC stimulated with 5 ng/mL of LPS in the presence of various concentrations of triptolide. Values represent the mean $\pm$ SD of duplicate samples and results represent three experiments. Results are statistically significant ($p < .05$) between LPS and LPS plus triptolide samples at concentrations of 1 to 100 nM of triptolide for all except MIP-1 α and MIP-1 β . Results are statistically significant ($p < .05$) between LPS and LPS plus triptolide samples at 10 to 100 nM for MIP-1 α and MIP-1 β .

IL-1 β , IL-6, and TNF α production by LPS-stimulated PBMC dose-dependently, reducing IL-1 β , IL-6, and TNF α by 49%, 58%, and 50%, respectively, at 10 nM of triptolide (Fig. 2A and 2B). Higher concentrations of triptolide blocked the production of these cytokines and the chemokines, MIP-1 α and MIP-1 β , by LPS-activated cells more completely, whereas MCP-1 production was totally inhibited at 10 nM of triptolide. Triptolide did not affect the viability of

the cells over the concentration range used in these studies (1–30 nM), as confirmed by trypan blue dye exclusion test. However, at 100 nM triptolide, 20% of PBMC took up trypan blue stain after 48 hr.

Figure 3 compares the inhibition by 10 nM triptolide of cytokine and chemokine production by PBMC cultures stimulated with another staphylococcal exotoxin, SEB, with the effects of TSST-1 and LPS. The inhibition of SEB-stimulated cells was similar to that of TSST-1 suggesting that triptolide is an effective inhibitor of the superantigen-activated pathways.

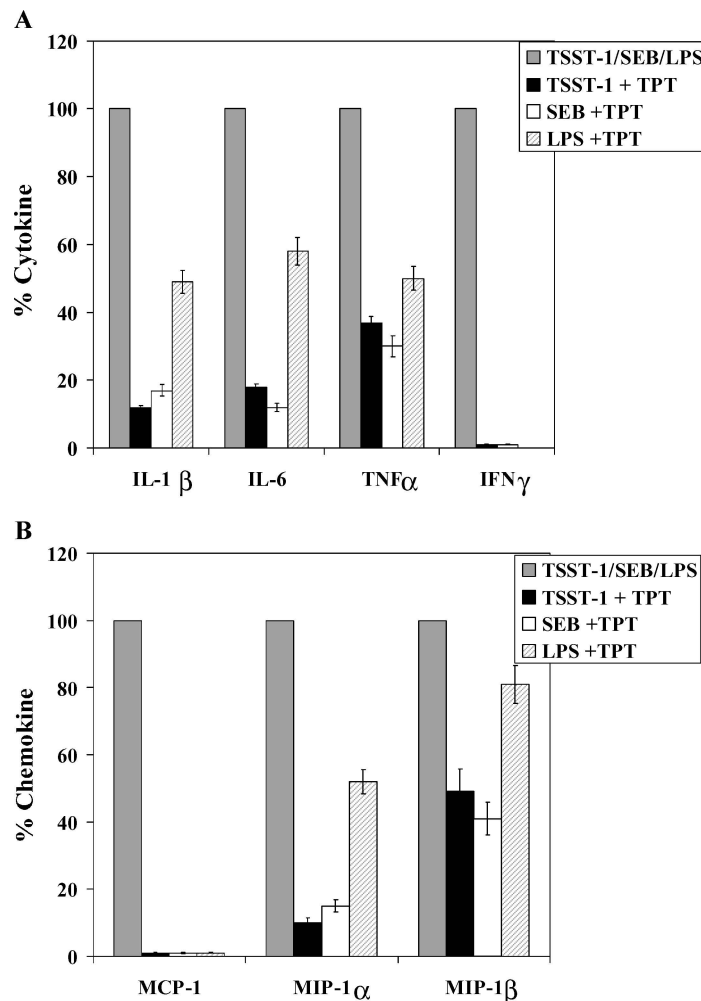


Figure 3: Inhibition of (A) IL-1 β , IL-6, TNF α ; and (B) MCP-1, MIP-1 α , and MIP-1 β production by PBMC stimulated with TSST-1 (200 ng/mL), SEB (200 ng/mL), or LPS (5 ng/mL) in the presence of 10 nM of triptolide. Values represent the mean $\pm$ SD of PBMC cultures from 6 blood donors. Results are statistically significant ($p < .05$) between stimulant (TSST-1, SEB, or LPS) and stimulant plus triptolide samples.

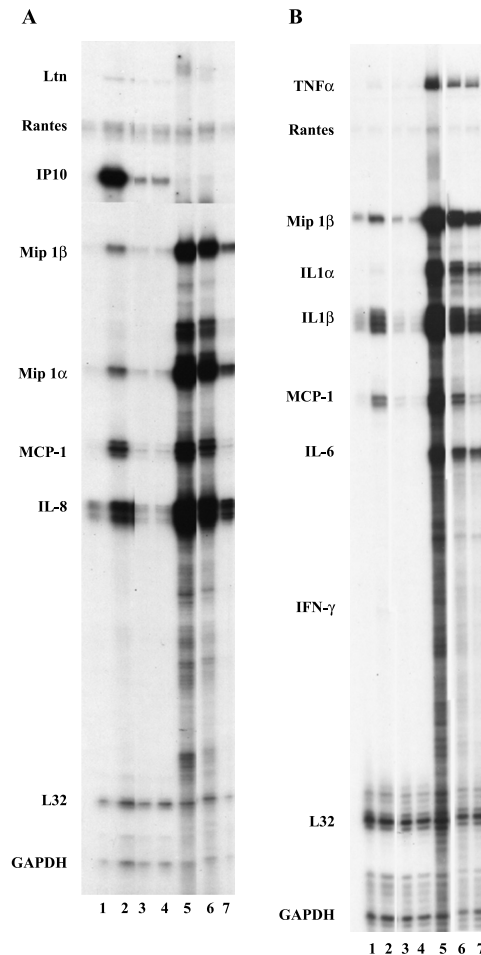


Figure 4 (A) and (B): Cytokine and chemokine mRNA analysis. Total RNA was extracted from human PBMC treated for 4 hr with 200 ng/mL of TSST-1 or 5 ng/mL of LPS in the presence or absence of triptolide. Multiprobe RNase protection analysis was performed as described in Materials and Methods using 5 µg of total RNA per lane. Lanes 1, 2, 3, and 4 represent cells in medium alone, TSST-1-stimulated cells, TSST-1-stimulated cells plus 5 nM of triptolide, and TSST-1-stimulated cells plus 10 nM of triptolide, respectively. Lanes 5, 6, and 7 represent LPS-stimulated cells, LPS-stimulated cells plus 10 nM of triptolide, and LPS-stimulated cells plus 30 nM of triptolide. Data shown are representative of experiments repeated three or more times. The cytokine or chemokine tested is shown to the left of each RPA; Ltn (lymphotoxin), Rantes (CCL5), IP10 (CXCL10), MIP-1β (CCL4), MIP-1α (CCL3), MCP-1 (CCL2), IL-8 (CXCL8), TNFα, IL-1α and IFNγ.

Triptolide Inhibited TSST-1- and LPS-Induced Cytokine and Chemokine mRNA Expression

We sought to determine the mechanism of inhibition of superantigen and LPS-induced cytokines and chemokines by triptolide at the molecular level. Total RNA was extracted from stimulated cells 4 hr after triptolide

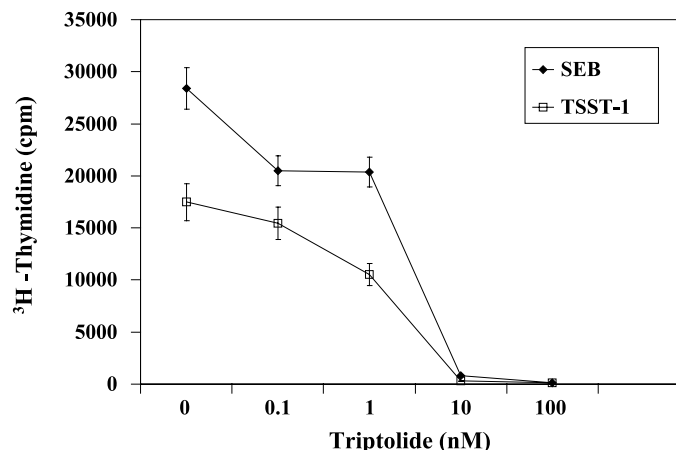


Figure 5: Inhibition of T-cell proliferation in PBMC stimulated with 200 ng/mL of TSST-1 or SEB in the presence of various concentrations of triptolide. Values represent the mean $\pm$ SD of triplicate samples and results represent three experiments. Results are statistically significant ($p < .05$) between stimulant (TSST-1 or SEB) and stimulant plus triptolide samples.

treatment and gene expression was measured with a Multiprobe RNase protection assay. L32 rRNA and GAPDH RNA were used as internal standards for RNA measurements. Figure 4A and 4B show that 5 and 10 nM of triptolide blocked TSST-1-mediated increases in the RNA for $\text{TNF}\alpha$, IL-1 β , IL-8, $\text{IFN}\gamma$, IP-10, MIP-1 α , MIP-1 β , and MCP-1. At this dose of triptolide, LPS-induced expression of most of the RNA examined was partially blocked. A higher concentration of triptolide (30 nM) further reduced the LPS-mediated mRNA expression of $\text{TNF}\alpha$, IL-1 β , IL-8, $\text{IFN}\gamma$, IP-10, MIP-1 α , MIP-1 β , and MCP-1.

Triptolide Inhibited Superantigen-Induced T-Cell Proliferation

Because superantigen polyclonally activates T cells, the effect of triptolide on SE-induced T-cell proliferation was next investigated. Figure 5 shows that triptolide is a potent inhibitor: reducing SEB- and TSST-1-stimulated T-cell proliferation in a dose-dependent manner and achieving 98% inhibition at 10 nM of triptolide.

DISCUSSION

Shock caused by bacterial products from both Gram-positive and Gram-negative bacteria is a serious clinical problem and inhibition of any single cytokine by a specific cytokine antibody or receptor antagonist often does not

result in successful treatment and recovery. Anti-inflammatory and immunosuppressive therapeutics represent a potentially useful treatment independent of the inciting agents by targeting common downstream signaling pathways affecting multiple cytokines and chemokines. The results presented here indicate that triptolide suppressed the induction of proinflammatory cytokines and chemokines by TSST-1-, SEB-, and LPS-stimulated human mononuclear cells. The production of these mediators by monocytes/macrophages and T cells in response to superantigens and LPS initiates leukocyte activation and migration, contributing directly to inflammation and tissue injury associated with shock. T-cell proliferation also was blocked by triptolide.

Previous studies showed that triptolide inhibited transcriptional activation through NF κ B^[28] and suppressed TNF α production by LPS-stimulated macrophages^[23] and IL-2 production by PHA-activated T cells.^[28] Our results extend these observations by showing inhibition of multiple inflammatory mediators in staphylococcal exotoxin-activated PBMC at both transcriptional and protein level. Genes for proinflammatory mediators IL-1 and TNF α contain DNA binding sequences for the transcriptional factor NF- κ B, and triptolide was shown to inhibit the activation of IL-2 transcriptional factors.^[28]

Attenuated T-cell activation with decreased elaboration of key proinflammatory cytokines by triptolide suggests triptolide may prove useful in treating superantigen-induced shock. Multiple clinical trials of extracts of TWHF in rheumatoid patients indicate that triptolide is the active component responsible for the immunosuppressive effects (reviewed in Ref. [15]). Oral and intraperitoneal administration of triptolide in both mice and rats at doses of up to 0.25 mg/Kg for prolonged periods of 3 to 4 weeks produce no lethal effects,^[16,17,35,36] although infertility is a known side effect.^[37]

Our studies showed that triptolide suppresses a broad range of cytokine production induced by superantigens and LPS, suggesting that triptolide targets several intracellular signaling pathways. One prominent pathway is the transcriptional activation of NF- κ B that regulates the expression of inflammatory cytokines, cyclooxygenase 2, and cell adhesion molecules. This interference of NF- κ B activation by triptolide likely accounts for its potent immunosuppressive effects. In conclusion, due to the broad spectrum of cytokines antagonized, and based on its beneficial therapeutic effects in autoimmune diseases, triptolide may prove useful as a therapeutic for the treatment of toxic shock.

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