

THE authors reported recently that endotoxaemia mediated elevated levels of tumour necrosis factor (TNF- α) and interleukin-1 α (IL-1 α) were involved in the pathophysiology of acute heat stroke patients. Pentoxifylline (PTX) is known to modulate neutrophil functions. In the present study the effects of PTX on lipopolysaccharide (LPS) and cytokine induced T-cell and macrophage (ϕ M) activation, and on natural killer (NK) cell and lymphokine activated killer (LAK) cell mediated cytotoxicity were examined. Finally, the effect of PTX on the expression of adhesion molecules (LFA-1, Mac-1 and ICAM-1), and cytokine (IL-1 α , IL-2, TNF- α , IL-6 and IFN- γ) production and their surface receptor expression in response to LPS activation was investigated. PTX free cultures served as a control. Results revealed that PTX can down-regulate all the above-mentioned immunological parameters in a dose-dependent manner. These findings might have far reaching clinical implications.

Key words: Adhesion molecules, Cytokines, Lymphokine activated killer cells, Natural killer cells, Pentoxifylline

Mechanism of pentoxifylline mediated down-regulation of killer lineage cell functions*

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Introduction

Cytokines and adhesion molecules play an important role in normal immune regulation,^{1,2} haematopoieses³⁻⁵ and inflammatory responses.⁶ Imbalances in the production of cytokines, particularly those affecting inflammation response, can have profound effects on immunoregulation and may contribute to pathogenesis of numerous diseases.^{6,7} High physiological levels of cytokines and enhanced expression and production of soluble adhesion molecules have been implicated in a number of clinical situations.⁶ Recently, considerable interest has also been focused on the adhesion molecules,⁸ particularly for their role in pathogenesis during inflammatory processes. Cytokines are known to activate^{4,9} a wide variety of killer lineage cells, induce cytokine production and adhesion molecules expression. PTX has been shown^{10,11} to modulate neutrophil and monocyte functions. In the present study, to understand the mechanism and immuno-modulatory potential, the effects of PTX on the activation of peripheral blood mononuclear cells (PBMC), killer cells function, production of cytokines, surface receptor expression of cytokines and adhesion molecules was examined.

Materials and methods

Pentoxifylline: Lot 61H0058 was purchased from Sigma (St Louis, MO, USA) and a stock solution

of 1 mg/ml was prepared in sterile physiological saline (Abbott Laboratories, IL, USA). Further working dilutions were made in RPMI-1640 tissue culture medium (Gibco, Grand Island, NY, USA).

Lipopolysaccharide: Serotype 026.86 was purchased from Sigma, USA. Stock solution and working dilutions were prepared in RPMI-1640 medium just before use in various experiments.

Cytokines: Recombinant human IL-6 was purchased from Genzyme Corp. (Boston, MA, USA), recombinant human IL-2 (Batch No. C151597-02, R0330) was kindly provided as a gift from Hoffman-La Roche, Switzerland. All the cytokines were reconstituted in RPMI-1640 and were diluted further immediately before use.⁴

Collection and preparation of peripheral blood mononuclear cells: Samples (20–30 ml) of whole blood from healthy individuals of either sex were collected into heparinized tubes. PBMC were isolated by Ficoll-Hypaque fractionation, cell count and viability was carried out using the Trypan blue dye (0.02%) exclusion method. PBMC were finally suspended into RPMI-1640 medium supplemented with 25 mM HEPES and glutamine, streptomycin 100 μ g/ml, fungizone 25 μ g/ml, penicillin 100 U/ml and 10% AB human serum.

Activation of monocytes and PBMC: Monocytes were isolated as described previously.^{12,13} Six replicate cultures of either ϕ M (98% as judged by non-specific esterases staining) or PBMC were set

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up for 72 h in complete RPMI-1640 medium at 37°C, 5% CO₂ as follows:

- (a) PBMC (1 × 10⁶/ml) + IL-2 100 U or IL-6 100 U
- (b) ϕ M (1 × 10⁶ ml) alone or + LPS 10 μ g/ml
- (c) PBMC alone or + LPS 10 μ g/ml
- (d) PBMC + LPS + PTX (10–250 ng/well/250 μ l)

Effects of PTX on γ IL-2, IL-6 and LPS induced proliferation of PBMC was measured by [³H]TdR uptake in culture (a) after 72 h. Culture (b) containing ϕ M was used in antibody dependent cytotoxicity (ADCC) type killer assays. Fresh PBMC alone or after co-culturing with γ IL-2 1000 U/1 × 10⁶ PBMC/ml for 3 days were used as effector cells for NK and LAK cell assays respectively. PBMC from cultures (c and d) were used to examine surface receptor expression for various cytokines and adhesion molecules, whereas supernatant from these cultures was used to quantitate various cytokines. Unstimulated cultures of PBMC and ϕ M alone or with PTX were used as a control.

ϕ M, NK and LAK cell mediated killer cell assay: Using K562 cells (erythroleukaemia cells, NK sensitive target) or Daudi cells (Burkitt lymphoma, NK resistant target) as described previously^{14–16} in a ⁵¹Cr release killer assay (ϕ M 18 h assay using K562 target cells), or (NK and LAK cells in a 4 h assay), specific cytotoxicity was measured at various effector:target ratios (100:1, 50:1, 25:1, 12.5:1) for ϕ M, NK or LAK cells. Spontaneous and maximum ⁵¹Cr releases were also set up. Results were computed as follows:

% specific cytotoxicity

$$= \frac{{}^{51}\text{Cr CPM exp} - {}^{51}\text{Cr CPM spontaneous}}{{}^{51}\text{Cr CPM max} - {}^{51}\text{Cr CPM spontaneous}} \times 100$$

Cytokine quantitation: Highly sensitive ELISA kits from Endogen (Boston, MA, USA), Genzyme Corp. and R & D System (USA) were used to quantify IL-1 α , IL-2, TNF- α , IL-6 and IFN- γ from the supernatant collected from PBMC culture under various conditions as described in the methodology. Manufacturer's guidelines were followed to measure these cytokines.^{4,17} Supernatant from PBMC cultured without LPS and PTX were considered negative controls, whereas supernatant from PBMC plus LPS were considered positive controls.

Cytokine surface receptor assay: Using fluorokine kits (British Biotechnology, R & D System, UK) surface receptors for IL-1 α , IL-2 and TNF- α were measured on resting or activated (with LPS) PBMC with or without co-culturing with PTX as described earlier in the methodology. Manufac-

turer's guidelines were followed to measure the receptor of the cytokine using fluorescence microscopy (Zeiss, Germany) or FACS (Flow Cytometry). Results were expressed as a percentage of positive cells compared with unactivated PBMC.

Expression of adhesion molecules LFA-1, Mac-1 and ICAM-1: Adhesion molecules (LFA-1, Mac-1 and ICAM-1) on LPS activated PBMC (with or without PTX) were measured by indirect labelling. PBMC were first incubated with anti-LFA-1 (1:20 dilution), anti-Mac-1 (1:25 dilution) or anti-ICAM-1 (1:50 dilution, Bender & Co., Vienna, Austria) monoclonal antibody for 1 h at room temperature. After washing secondary labelling was carried out using goat anti-mouse FITC antibody (1:25 dilution) for 45 min. Negative controls were set up by excluding primary antibody. PBMC was analysed with FACScan (Becton and Dickinson, USA) and fluorescence microscopy (Zeiss, Germany). Results were expressed as percentage of positive cells.

Statistical evaluation: The results were expressed as the mean \pm the standard error (S.E.). The difference between mean values was considered significant at $p \leq 0.05$. This was determined with Student's *t*-test when comparing two sets of means. A two-way analysis of variance was employed for comparing percentage of specific cytotoxicity under two different conditions, inclusive of all effector:target ratios.

Results

Effects of PTX on and cytokine induced activation of PBMC: PBMC activation either with LPS or cytokines, that is IL-2 and IL-6, was down-regulated in a dose-dependent manner when PTX was added to the cultures at the initiation of the cultures (Fig. 1) indicating suppressive effect on the pre-effector level. Addition of PTX to unstimulated PBMC had no significant effect on [³H]TdR incorporation. Once the PBMC had achieved their maximum activations, additions of PTX was somewhat less effective (results not shown). A maximum suppression was achieved at 250 ng/well/250 μ l of PTX concentration ($p < 0.002$ for IL-2; $p < 0.023$ for IL-6 and $p < 0.005$ for LPS).

Effects of PTX on the ϕ M mediated cytotoxicity: The activated macrophages mediated cytotoxicity was down-regulated in a dose-dependent manner (Fig. 2) only when PTX was added to the cultures at the initiation of cultures. Maximum suppression in ϕ M cytotoxicity was only observed at 72 h, results of 24 h and 48 h cytotoxicity assays in the presence of PTX (data not shown) did not show significant suppression. Addition of PTX (100 ng/well/250 μ l) to similar cultures after 48 h or

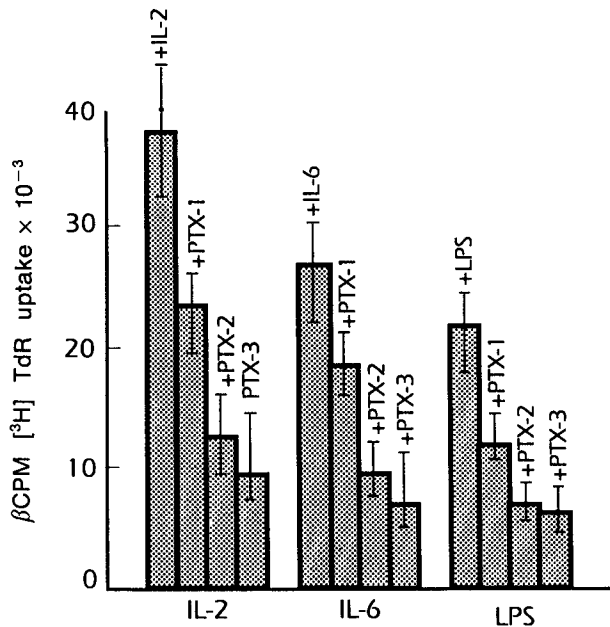


FIG. 1. Effects of PTX (PTX-1 = 50 ng, PTX-2 = 100 ng, PTX-3 = 250 ng) on IL-2, IL-6 and LPS induced proliferation of PBMC. PTX was introduced into the cultures at 0 h. Addition of PTX (250 ng) had no significant effect on the [³H]TdR incorporation of unstimulated (control) PBMC (data not shown). The results are mean \pm S.E. of three different experiments.

72 h was less effective, whereas a minor (5–7%) suppression was observed when PTX was added at 24 h to LPS stimulated cultures of ϕ M. A high significant suppression ($p < 0.005$) at a 100:1 effector:target ratio was observed in the presence of PTX ≥ 100 ng/well when added at 0 h to the cultures.

Effects of PTX on NK and LAK cell function: Addition of PTX (10–250 ng/well/250 μ l) to both NK and LAK cell assays at the initiation of cultures resulted in a dose-dependent suppression (Fig. 3). A highly significant suppression $p < 0.001$ was noted for both NK and LAK cells at 250 ng PTX/well or (1 μ g/ml) concentration. Addition of similar dose of PTX during LAK cells assay, however, was not effective (result not shown).

Effects of PTX on cytokine production and their receptor expression: PBMC activated with LPS for 48 to 72 h with or without PTX when analysed for the surface receptor expression of IL-1 α , IL-2, TNF- α and the cytokine production of IL-1 α , IL-2, TNF- α , IL-6 and IFN- γ in the supernatant of these cultures indicated (Figs 4 and 5) a highly significant ($p < 0.001$) suppression of cytokine production and their surface receptor expression in the presence of ($p < 0.05$) PTX. Surface receptor expression and production of IL-6 was only noticed at the higher concentration of PTX (0.4 μ g/ml).

Effects of PTX on adhesion molecule expression: Activated PBMC when analysed for the expression of LFA-1,

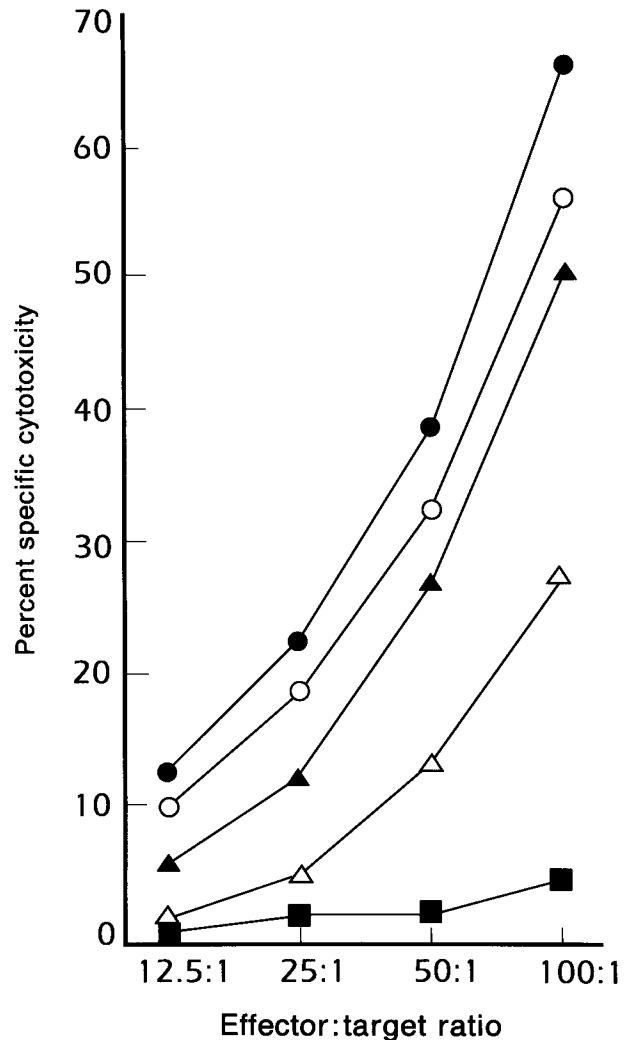


FIG. 2. Effects of various concentrations of PTX on ϕ M mediated 18 h cytotoxicity assay (measured against K562 target). Each point represents the mean of triplicates with less than 5% variation. The results are representative of one experiment from a total of three. Addition of PTX to unstimulated ϕ M had no significant effect (data not shown). ●, LPS activated ϕ M; ○, +PTX 10 ng; ▲, +PTX 50 ng; △, +PTX 100 ng; ■, unactivated ϕ M.

MAC-1, ICAM-1 revealed a dose-dependent suppression of LFA-1 and MAC-1 adhesion molecules whereas ICAM-1 expression was modulated only at higher concentrations of PTX (≥ 100 ng/ 10^6 PBMC/well) (Fig. 6).

Discussion

Polymorphonuclear neutrophils (PMN) participate significantly during certain pathological states to induce a variety of cellular injuries.¹⁸ Activation of PMN may lead to free oxygen radical production, enhance cytotoxicity via proteolytic enzymes and lead to increased adhesion to endothelial-cell lining.^{19–21} Several cytokines are known to activate a wide variety of killer lineage cells, induce surface receptor expression, induce the production of adhesion molecules and lead to production of other

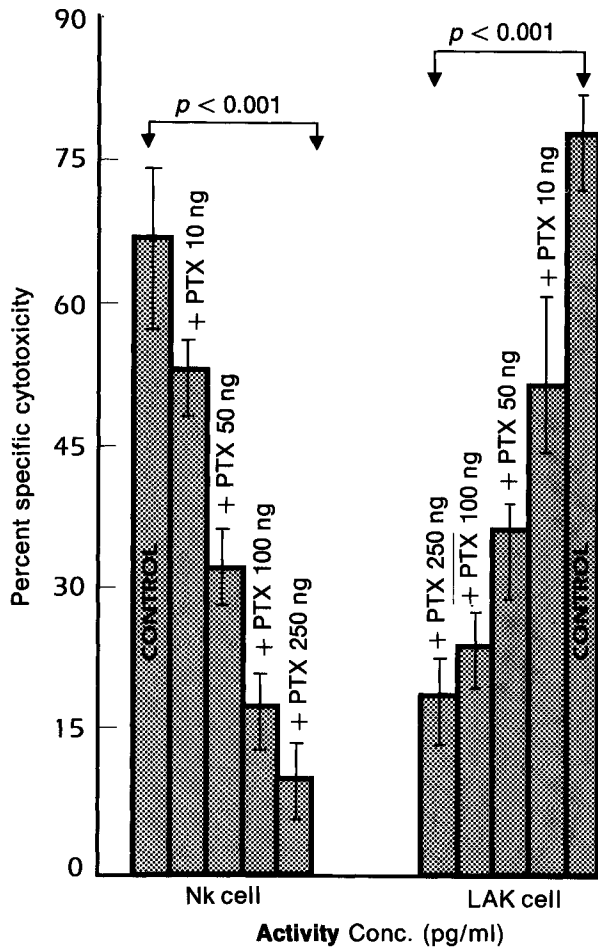


FIG. 3. Modulation of NK and LAK cell function by various concentrations of PTX. The results are the mean \pm S.E. of three different experiments.

cytokines.^{4,5} PTX mediated modulation of neutrophil functions has been reported in previous studies.^{11,22,23} The authors¹⁷ and others^{24,25} recently showed that endotoxin induced cytokines i.e. IL-1 α TNF- α and IL-6 are implicated in the pathophysiology of acute heat stroke patients. Imbalance in cytokine or their soluble receptor production, killer cell activation and adhesion molecule expression has also been implicated in a number of diseases.^{26,27}

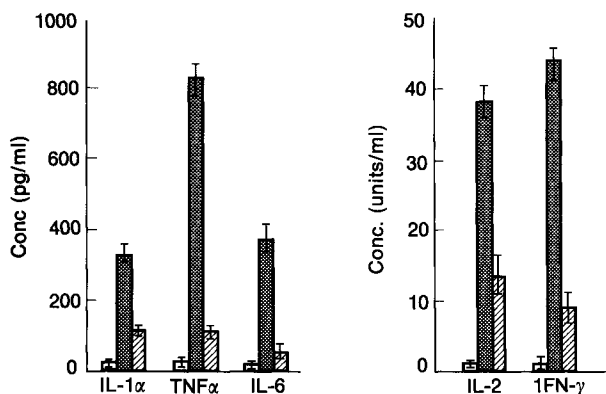


FIG. 4. Effects of PTX on *in vitro* cytokine production. The results are the mean \pm S.E. of three different experiments. \square , Control; \blacksquare , LPS; \boxtimes , PTX 100 ng/ml.

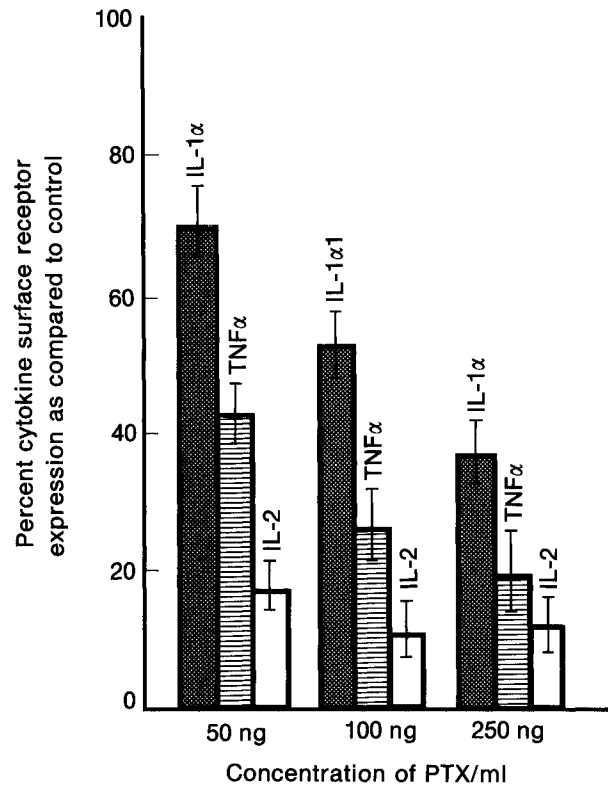


FIG. 5. Effects of PTX on the surface receptor expression on various cytokines. The results are the mean \pm S.E. of three different experiments.

The aim of the present study was to analyse to what degree PTX interferes with various immune cell functions that are thought to play roles in a variety of normal and abnormal inflammatory states.

The results of this study clearly demonstrate that PTX can block activation and subsequent generation of killer lineage cells in a dose-dependent manner. LPS induced nonspecific activation of PBMC or cytokine mediated cytotoxicity, as measured either by using [³H]TdR uptake or cytotoxicity assay, was significantly suppressed, when PTX was added at the initiation of PBMC

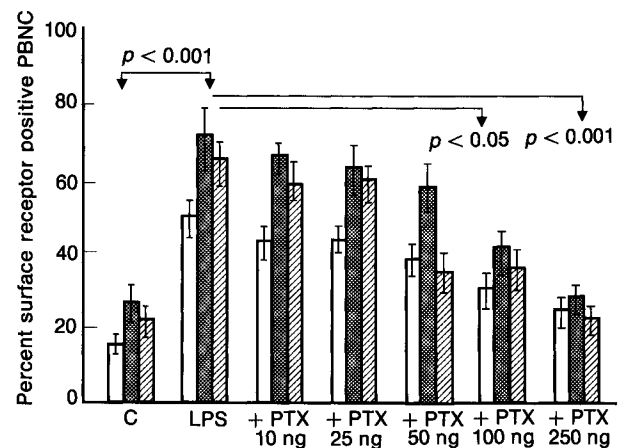


FIG. 6. Effects of PTX on the LPS induced surface adhesion molecules expression on PBMC on day 3. The results are the mean \pm S.E. of three different experiments. \square , LFA-1; \blacksquare , Mac-1; \boxtimes , ICAM1-1.

proliferation or killer cell assay. The significant suppression in mixed lymphocyte culture (MLR) and subsequent generation of allo-specific cytotoxic T-lymphocyte (CTL) killer cells (data not shown) was only achieved at a high concentration of PTX ($\geq 20 \mu\text{g/ml}$), when PTX was introduced into the culture at 0 h. Once the allo-specific CTL cytotoxic killer cells were generated, addition of PTX even at 10–20 $\mu\text{g/ml}$ during the CTL assay was less effective. An early report²⁸ on a phase I–II human trial using PTX for the prevention of transplant related toxicity following bone marrow transplant (BMT), showed significant suppression in the level of circulating TNF- α and was associated with the reduction in morbidity and mortality in patients undergoing BMT. LPS induced cytokine (IL-1 α , IL-2, IL-6, TNF- α and IFN- γ) and surface receptor expression of IL- α , IL-2 and TNF- α were significantly affected only when PTX was added at the beginning of the culture, indicating that once the maximum activation of PBMC had been achieved, PTX was no longer able to suppress the production of these cytokines. However, a recent study²⁹ showing a differential effect of PTX on TNF- α and IL-6 production *in vivo* in response to OKT3 monoclonal antibody in transplant patients clearly demonstrated a different mechanism of PTX action, depending on the cellular origin of these cytokines and the availability of accessory cells which can modulate the antigen presentation during induction of cytokine production. In the present study the effect of PTX on LPS stimulated PBMC, not on the pure macrophages population was measured. The amount of cytokines produced is the total amount secreted by T-lymphocytes and macrophages together, so a selective effect of PTX on either population of cells producing cytokines cannot be differentiated.

The expression of adhesion molecules, LFA-1, Mac-1 and ICAM-1, on PBMC was significantly affected by PTX at higher concentrations only. Modulation of various adhesion molecules on human endothelial cells activated with various cytokines was less affected using similar concentrations of PTX (R. S. Parhar and S. Al-Sedairy, manuscript in preparation) indicating that regulation of endothelial cell surface adhesion molecule expression could be different to that of PBMC. IL-1 α , IL-2, TNF- α , IL-6 and IFN- γ are implicated in the pathogenesis of several clinical settings including arthritis, septic shock, hyperthermia and organ transplantation. A high level of soluble ICAM-1 adhesion receptor has been reported in malignant melanoma patients.³⁰ The ability of PTX to suppress surface receptor expression of adhesion molecules merits further study to evaluate its role in malignant diseases. PTX has recently been shown to inhibit mRNA expression of TNF- α ²⁹ in PBMC

and TNF- α and IL-1 β in HL-60 leukaemia cells.³¹ Though the precise inhibitory mechanism of PTX at the molecular level is still elusive, its ability to down-regulate the cytokine surface receptor expression and cytokine production appears to be the key mechanism in down-regulating the activation of leukocytes macrophages, NK and LAK cell mediated cytotoxicity. Since the surface receptor expression of IL-1 α , IL-2, TNF- α , IFN- γ and adhesion molecules on killer lineage cells is related to cell activation and cytotoxic state, it is speculated that the mechanism of the PTX effect may be explained by its ability to down-regulate and suppress the mRNA of inflammatory cytokines which subsequently induce adhesion molecule expression and their production. The ability of PTX to down-regulate, modulate and suppress killer lineage cell function, cytokine and adhesion molecule expression and production, respectively, warrant studies in various clinical settings to evaluate its therapeutic benefits.

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