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RESEARCH ARTICLE

Effect of *Thuja occidentalis* and its polysaccharide on cell-mediated immune responses and cytokine levels of metastatic tumor-bearing animals

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Abstract

Context: Tumor microenvironment induces an active immune tolerance and escapes immune surveillance. In order to achieve an effective antitumor immune response, appropriately activated immune cells should maintain their antitumor activity to overcome the immune suppressive tumor microenvironment.

Objectives: This study focuses on the effect of *Thuja occidentalis* L. (Cupressaceae) extract and its polysaccharide (TPS) on cell-mediated immune response (CMI) in metastasis bearing mice.

Materials and methods: Metastasis was induced by injecting B16F-10 melanoma cells in mice through the tail vein and effector mechanisms of CMI was studied by analyzing cytotoxic T-lymphocyte (CTL) activity, natural killer (NK) cell activity, antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent complement-mediated cytotoxicity (ACC). The effect of *T. occidentalis* and TPS on pro-inflammatory cytokines and tissue inhibitor matrix metalloproteinases (TIMP) levels were also analyzed.

Results and discussion: Administration of *T. occidentalis* and TPS enhanced the NK cell activity, ADCC and ACC much earlier than the control tumor-bearing animals. *T. occidentalis* and TPS were also found to decrease the elevated level of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, GM-CSF and tumor necrosis factor (TNF)-α in the serum of metastatic tumor-bearing animals. The level of antitumor factors such as IL-2 and TIMP was elevated by the treatment with *T. occidentalis* and TPS in the serum, which was lowered in the untreated tumor-bearing animals.

Conclusion: This study clearly suggests that *T. occidentalis* and TPS effectively stimulate cell-mediated immune system and decrease pro-inflammatory cytokines, thereby inhibiting metastasis of tumor cells.

Keywords: Cytokines, cytotoxic T-lymphocyte, metastasis, natural killer cells, TIMP

Introduction

Invasion and metastasis are hallmarks of malignancies and in a significant number of clinical cases, metastasis is the first sign of the malignant disease whereas the primary tumor remains undetectable, suggesting that dissemination of malignant cells from the primary site occurs when parental tumors are at microscopic sizes (Rouhi et al., 2010). Highly malignant tumor cells are often resistant to chemotherapeutic and cytoreductive anticancer drugs. Although various immune responses can be generated against tumor cells, the responses frequently are not sufficient to prevent tumor growth. One approach to cancer treatment is to augment or supplement the natural defense mechanisms (Haque & Baral, 2006).

The fate of the host–tumor interaction is considered to depend on the balance between the intrinsic aggressiveness of the tumor and the strength of the host–immune response. Cytotoxic T-lymphocyte (CTL) and natural killer (NK) cell activity play an important role in the immunological surveillance to neoplasia and metastasis (Cooper et al., 2001). NK cells comprise 5 to 20% of human peripheral blood lymphocytes, and are derived from $CD34^+$ hematopoietic progenitor cells. These cells mostly

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are believed to have an immunoregulatory role exerted through the secretion of cytokines and chemokines. These cells appear to function predominantly in direct cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC). NK cells can directly induce apoptosis via the perforin-granzyme pathway or by expressing death–receptor ligands on their cell surface. Low NK cell activity has been associated with poor prognosis in advanced cancer patients (Cho & Campana, 2009; Villegas et al., 2002). There is abundant evidence suggesting that lymphocytes also play a central role in the host response to tumors (Theano et al., 2002). Lymphocytes kill target cells including tumor cells by inducing them to undergo programmed cell death (apoptosis). Two effector pathways account for T-cell-mediated cytotoxicity, namely, the granule exocytosis pathway and Fas pathway. These two pathways mediate tumor cell killing *in vivo* by T-cells and account for cytotoxicity by CTLs against tumor cells (Kägi et al., 1996).

Cytokines are important mediators of immune responses and are found to stimulate immune cells (Kelly et al., 2002). But, there are several pro-inflammatory cytokines that are linked with carcinogenesis and tumor initiation, promotion and metastasis, which suggests that inflammation, is associated with cancer development. Numerous studies have indicated that tumor cells exhibit an elevation in the constitutive production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and granulocyte monocytes-colony stimulating factor (GM-CSF) (Lázár-Molnár et al., 2000). Tissue inhibitor matrix metalloproteinases (TIMP) is an endogenous protease inhibitors involved in extracellular matrix (ECM) remodeling. Many reports have shown that upregulation of TIMP inhibits tumor growth, invasion and metastasis (Albini et al., 1991).

Indigenous drugs have been found to be useful in many disease conditions including cancer. Since immunity is suppressed in cancer and during cytoreductive therapy of cancer, use of indigenous drug in cancer therapy was found to be highly indicative. Several herbal agents may modify the relationship between host and tumor growth leading to therapeutic effects (Werner & Jollès, 1996). Northern white cedar, *Thuja occidentalis* L. (Cupressaceae), also called "arborvitae" or "tree of life" was first identified in the 16th century by the French explorer Cartier and was found effective in the treatment of weakness from scurry. Today it is used mainly in homeopathy as mother tincture or dilution and also being used to treat acute and chronic infections of the upper respiratory tract (Naser et al., 2005). Spleen cells isolated from the mice treated with *T. occidentalis* produced higher amounts of immunostimulatory cytokines IL-2 and interferon (INF)-γ *ex vivo* (Bodinet et al., 2002). *T. occidentalis* polysaccharide fraction (TPS) was shown to be an inducer of the $CD4^+$ fraction of the human peripheral blood T-cell subset (Offergeld et al., 1992). Based on the pharmacological activities of *T. occidentalis* and TPS, the present study designed to evaluate their effects on cell-mediated immune response (CMI) in mice models.

Materials and methods

Animals

C57BL/6 mice and BALB/c mice were purchased from National Institute of Nutrition, Hyderabad, Andhra Pradesh, India. The animals were housed in well-ventilated cages in air-controlled rooms. They were fed normal mouse chow (Sai Durga Feeds, Bangalore, Karnataka, India) and water was given *ad libitum*. All the animal experiments were carried out according to the rules and regulations of Institutional Animal Ethics Committee as well as Animal Ethics Committee, Government of India.

Cells

B16F-10, highly metastatic mouse melanoma cells, K-562 leukemia cells and EL-4 thymoma cells were procured from National Centre for Cell Sciences, Pune, Maharashtra, India. B16F-10 cells and EL-4 cells were maintained in culture using Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) (Biological Industries, Kibbutz Beit-Haemek, Israel). K-562 cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% FCS. Fresh sheep red blood cells were collected in Alsever's solution from local slaughterhouse and washed three times in normal saline.

Reagents and kits

DMEM and RPMI-1640 were purchased from Hi-media Laboratories, Mumbai, Maharashtra, India. $Na_2{}^{51}CrO_4$ was purchased from Board of Radiation and Isotope Technology, Mumbai, Maharashtra, India. Highly specific quantitative "Sandwich" enzyme-linked immunosorbent assay (ELISA) kits for mouse IL-1β, IL-6, TNF-α, GM-CSF and IL-2 were purchased from Pierce Biotechnology (Rockford, IL, USA) and the ELISA kit for TIMP was purchased from R&D System (Minneapolis, MN, USA). A cell to cDNA™ kit was purchased from Ambion Inc. (Austin,Texas, USA). All other reagents used were of analytical reagent grade.

Drug preparation

Aerial parts of the authenticated *T. occidentalis* were collected locally and dried at 45°C. A voucher specimen of the plant (CP-01) was kept in the herbarium of Amala Ayurvedic Hospital, Thrissur, Kerala, India. Dried leaf and small twig powder (100 g) was stirred overnight in 70% methanol, centrifuged at 7225*g* at 4°C and supernatant was collected. Methanol was removed in vacuum and the yield obtained was 15%. Phytochemical analysis of the extract was found to be positive for anthrone-polysaccharide test and showed the presence of flavonoids also. The extract was resuspended in 1% gum acacia to the desired concentration (200 mg/kg body weight).

To isolate the polysaccharide, we used the protocol of Chintalwar et al. (1999). In brief, the powdered plant material was pre-extracted using dichloromethane in Soxhlet apparatus to remove lipophilic substances. The residue was air-dried and extracted with 50% ethanol. The extract was precipitated using acetone. The proteins from this precipitate were removed by the treatment with trichloroacetic acid. The yield was about 0.3%. It was tested for the presence of polysaccharide by anthrone reagent. This compound, TPS was given to the animals at a concentration of 40 mg/kg body weight of animal. Non-toxic concentrations (5 μg/mL) of *T. occidentalis* and TPS (1 μg/mL) obtained by MTT (3-(4,5-dsimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used for *in vitro* studies (data not shown).

Effect of *T. occidentalis* and TPS on CMI in metastatic tumor-bearing animals

C57BL/6 mice (4–6 weeks old, male) were divided into three groups. Group I animals were prophylactically treated with five doses of *T. occidentalis* (200 mg/kg body weight, i.p) and group II with TPS (40 mg/kg body weight) for five consecutive days. Group III was kept as untreated control. After the fifth dose, metastasis was induced in all animals by injecting B16F-10 melanoma cells (1×10^6 cells/animal) through the lateral tail vein. At various time intervals, animals were sacrificed, spleen and blood was collected and processed. Spleen cells were used as effector cells for assaying the NK cell activity (Tsavaris et al., 2002) and ADCC by 4 h ^{51}Cr-release assay (Kim et al., 2000). Serum was used as anti-Ehrlich ascites carcinoma antibody for antibody-dependent complement-mediated cytotoxicity (ACC) by Trypan blue exclusion method (Singh et al., 1984).

Effect of *T. occidentalis* and TPS on NK cell activity of metastatic tumor-bearing animals

NK cell activity was tested using spleen cells against the NK-sensitive K-562 tumor cells by the 4 h ^{51}Cr-release assay (Tsavaris et al., 2002). Spleen cells (effector cells) and ^{51}Cr labeled target cells (K-562) were added to 96-well round-bottom titer plates in the ratio of 100:1. Final volume was adjusted to 0.2 mL with RPMI-1640 supplemented with 10% FCS and incubated at 37°C in 5% CO_2 atmosphere for 4 h. Spontaneous and total release was determined by incubating the target cells in the absence and presence of 1N HCl respectively. Cultures were set up in triplicate. The plates were centrifuged, supernatant was collected and radioactivity was measured in a γ-ray spectrophotometer. Percentage of cell lysis was calculated as follows

$$\% \text{ Specific target cell lysis} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Total release} - \text{spontaneous release}} \times 100$$

Effect of *T. occidentalis* and TPS on ADCC of metastatic tumor-bearing animals

ADCC was determined by 4 h chromium release assay (Kim et al., 2000). Chromium labeled sheep erythrocytes (SRBC) was used as the target cells and the spleen cells from animals were used as effector cells. Anti-SRBC antibody raised in rabbits was used as the source of antibody in ADCC assay. The experiment was set up as above along with anti-SRBC antibody in 96-well round-bottom titer plate. The released chromium in the supernatant was counted in a γ-ray spectrophotometer and the percentage of cell lysis was calculated as above.

Effect of *T. occidentalis* and TPS on ACC of metastatic tumor-bearing animals

Serum collected from the experimental animals was heat inactivated at 56°C for 30 min then diluted with RPMI-1640 to get 1:1 dilution. Fresh rabbit serum diluted with RPMI-1640 was used as source of complement. The diluted serum was mixed with 0.1 mL of (1×10^4) and 50 μL of complement were added. Final volume was made up to 2 mL and incubated at 37°C for 3 h. Samples with cells alone, cells treated with complement and cells treated with antibody were kept as controls. The cytotoxicity was assessed by Trypan blue exclusion method (Singh et al., 1984). All the tests were done in triplicate.

Effect of *T. occidentalis* and TPS on CTL production *in vivo*

Alloimmunization was carried out by injecting the spleen (2×10^7) cells from C57BL/6 mice subcutaneously to normal, *T. occidentalis* and TPS-treated BALB/c mice. Winn's neutralization test was carried out according to the method of Kobayashi et al. (1992). Alloimmune spleen cells from BALB/c mice (effector cells) were mixed with complete medium containing 5×10^5 EL-4 cells (target cells). The cells were incubated for 1 h at 37°C in 5% CO_2 atmosphere and 0.2 mL of this mixture was injected intraperitoneally to eight groups of BALB/c mice. Group I animals received EL-4 cells alone. Group II animals received EL-4 cells incubated with normal spleen cell suspension, group III animals received EL-4 cells incubated with normal spleen cells and continued with 10 doses of *T. occidentalis* and group IV received EL-4 cells incubated with normal spleen cells and continued with 10 doses of TPS. Group V animals received EL-4 cells incubated with *T. occidentalis* treated spleen cells and group VI received EL-4 cells incubated with TPS-treated spleen cells. Group VII animals received EL-4 cells incubated with *T. occidentalis* treated spleen cells and continued the *T. occidentalis* administration for 10 days. Group VIII animals received EL-4 cells incubated with TPS-treated spleen cells and continued the TPS administration for 10 days. The animals were observed daily for 80 days after tumor inoculation. Increase in mean survival days of the treated group compared with the control animals were considered as the indication of CTL activity.

Effect of *T. occidentalis* and TPS on cytokine and TIMP level

Two groups of C57BL/6 mice were injected with highly metastatic B16F-10 melanoma cells (1×10^6 cells/animal) through the lateral tail vein. Group I animals were treated with extract of *T. occidentalis* (200 mg/kg body weight) and group II with TPS (40 mg/kg body weight) intraperitoneally. Group III was kept as untreated metastatic tumor-bearing controls. Three animals from each group were sacrificed at two different time points (7th h and 21 days after tumor induction). Blood was collected, serum separated and cytokines such as IL-1β, IL-6, TNF-α, GM-CSF, IL-2 and TIMP were assayed using respective ELISA kits.

Effect of *T. occidentalis* and TPS on the gene expression of pro-inflammatory cytokines

To determine the effect of *T. occidentalis* and TPS on IL-1β, IL-6, GM-CSF, and TNF-α gene expression, total RNA was subjected to cDNA synthesis using cells to cDNA™ II kit. B16F-10 cells (2×10^4 cells/well) were grown in 96-well titer plate and the cells were incubated in the presence and absence of *T. occidentalis* (5 μg/mL) and TPS (1 μg/mL) for 4 h at 37°C in 5% CO_2 in serum-free medium. Total RNA was extracted from B16F-10 cells and cDNA was synthesized, using moloney murine leukemia virus reverse transcriptase. Polymerase chain reaction (PCR) was performed with mouse inflammatory cytokine multiplex PCR kit. This kit has been designed to detect the expression of mouse GAPDH (reduced glyceraldehyde phosphate dehydrogenase), IL-1β, IL-6, GM-CSF and TNF-α gene. The PCR primers (Table 1) have similar Tm's and PCR products also generated from a positive control cDNA which was included in this kit. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

Statistical analysis

All data were represented as mean ± SD. Data were statistically analyzed using one-way analysis of variance (ANOVA) using Graph Pad Instat software package (GraphpadSoftware,SanDiego,CA, USA). If found significant, the groups were further analyzed by Bonferronni's multiple comparison test– $p < 0.05$ were considered as significant.

Results

Effect of *T. occidentalis* and TPS on NK cell activity of metastatic tumor-bearing animals

As shown in Figure 1, administration of *T. occidentalis* and TPS enhanced the NK cell activity significantly. A maximum NK cell-mediated lysis was observed after fifth day (53.3% cell lysis, 52.1% cell lysis, respectively) of tumor induction in *T. occidentalis* and TPS-treated group. The NK cell activity in untreated tumor-bearing animals was (20% cell lysis) observed only on 11th day. In normal animals, the maximum lysis observed was only 6%.

Effect of *T. occidentalis* and TPS on ADCC of metastatic tumor-bearing animals

The ADCC activity of *T. occidentalis* and TPS-treated animals was increased to 47.5 and 46.3% cell lysis on 7th day respectively (Figure 2), while it was only 13.9% cell lysis in control animals on the same day. The maximum ADCC activity in control animals (15.9% cell lysis) was observed only on 13th day after tumor induction.

Effect of *T. occidentalis* and TPS on ACC of metastatic tumor-bearing animals

The ACC activity was elevated in the *T. occidentalis* and TPS-treated metastatic tumor-bearing animals (Figure 3). In treated animals, the maximum ACC was observed on the 17th day. In *T. occidentalis* and TPS treated animals, the maximum of 30% and 27.1% cell lysis was observed on the 17th day respectively whereas in untreated tumor-bearing animals only 13.3% cell lysis was observed on the same day.

Effect of *T. occidentalis* and TPS on CTL production in vivo

The survival rate of animals transplanted with EL-4 after various treatments is given in Table 2 which indicates the generation of CTL. In the untreated tumor-bearing (EL-4 alone), the survival rate was only 26.8 ± 4.4 days whereas *T. occidentalis* and TPS fraction treated group showed an increase in survival rate to 46.9 ± 3.7 and 43.1 ± 3.7 days, respectively. When the animals were injected with normal spleen cells co-cultured with EL-4, the survival rate was 36.8 ± 4.2 days. But when EL-4 cells were incubated with *T. occidentalis* and TPS-treated spleen cells and the animals were continued with drug administration the survival rate was significantly increased up to 54.4 ± 5.8 and 50.2 ± 3.9 days, respectively (Table 2).

Effect of *T. occidentalis* and TPS on pro-inflammatory cytokines levels

Administration of *T. occidentalis* and TPS showed varying pattern of regulation of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α in the serum of metastasis-induced animals during the period of study. In control animals, the level of IL-1β and TNF-α in the serum was drastically elevated compared to normal level. Treatment with *T. occidentalis* or TPS effectively reduced the IL-1β and TNF-α level and reached near to normal level by 21st day after tumor induction. In the same way, the level of IL-6 was also drastically increased after tumor induction. But administration of *T. occidentalis* and TPS could effectively downregulate the elevated level of IL-6 by 21st day of tumor induction (Table 3).

Similarly, the level of GM-CSF in the serum was elevated after 7 days of tumor induction, compared to the normal levels. After the 21st day of tumor induction,

Table 1. Primer sequences.

Genes	Primer sequence		Product size
IL-6	Forward	5′-ATGAAGTTC CTCTCTGCAAGAGAC-3′	638bp
	Reverse	5′-CACTAGGT TTGCCGAGTAGATCTC-3′	
IL-1β	Forward	5′-ATGGCAACTG TTCC TGAACTCAACT-3′	563bp
	Reverse	5′-CAGGACAGGTATAGAT TCTTTCCTTT-3′	
TNF-α	Forward	5′-ACTCCCAGAAAAGCAAGCAA-3′	688bp
	Reverse	5′-T G GAAGACTCCTCCCAGGTA-3′	
GM-CSF	Forward	5′-TGTGGTCTACAGCCTCTCAGCAC 3′	368bp
	Reverse	5′-CAAAGGGGATATTCAG TCAGAAAGGT 3′	
GAPDH	Forward	5′-CGTCCCGTAGACAAAATGGT-3′	557bp
	Reverse	5′-CCTTCCACAATGCCAAAGTT-3′	

bp, base pairs; GAPDH, reduced glyceraldehyde phosphate dehydrogenase; GM-CSF, granulocyte monocyte-colony stimulating factor; IL, interleukin; TNF, tumor necrosis factor.

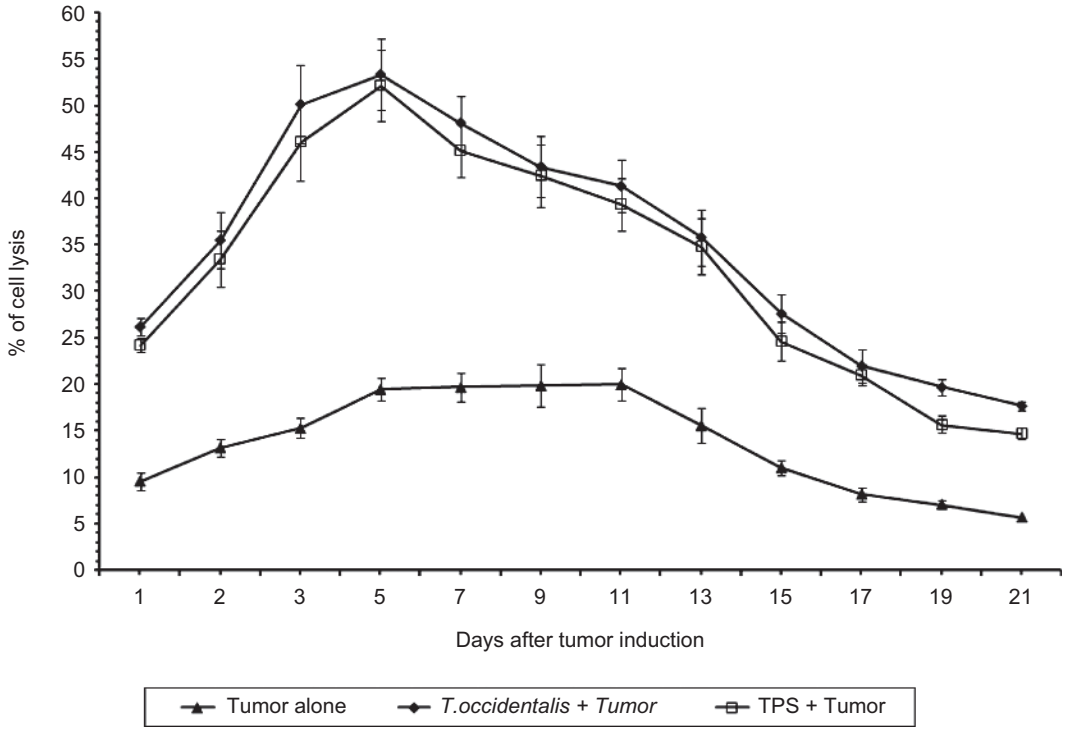

Figure 1. Effect of *Thuja occidentalis* and *T. occidentalis* L. (Cupressaceae) polysaccharide (TPS) on natural killer cell activity in metastasis tumor-bearing animals. Mice were treated with *T. occidentalis* or TPS and spleen cells were obtained. The spleen cells were mixed with chromium labeled K-562 cells and % cell lysis was determined by 4-h chromium release assay ($n = 3$/point).

GM-CSF levels reached the normal value in *T. occidentalis* and TPS administered group (Table 3).

Effect of *T. occidentalis* and TPS on the IL-2 and TIMP levels

IL-2 and TIMP are endogenous antitumor factors. The decrease in the levels of IL-2 and TIMP after the induction of tumor indicated the tumor progression in untreated group. But in *T. occidentalis* and TPS administered group, IL-2 and TIMP levels were increased after 21 days of metastasis induction (Table 3).

Effect of *T. occidentalis* and TPS on the pro-inflammatory cytokine gene expression

The effects test compound on the pro-inflammatory cytokine production can also be studied by analyzing their mRNA expression. Highly elevated expression of pro-inflammatory cytokines such as IL-1β, IL-6, GM-CSF and TNF-α has been observed in the metastatic B16F-10 melanoma cells. *T. occidentalis* and TPS treatment significantly inhibited the elevated expression of these pro-inflammatory cytokines (Figure 4).

Discussion

Progressive tumor growth in human and animal models is frequently accompanied by a concomitant immune suppression and the downregulation of cytotoxic cells, such as T-lymphocytes and NK cells regardless of tumor location and etiology (Bosch et al., 2002; Miyahira et al., 2003). One explanation for the evasion of host defenses by tumors is the production of soluble factors affecting the function of host cells involved in immunity. In this respect, different tumor derived factors may affect the

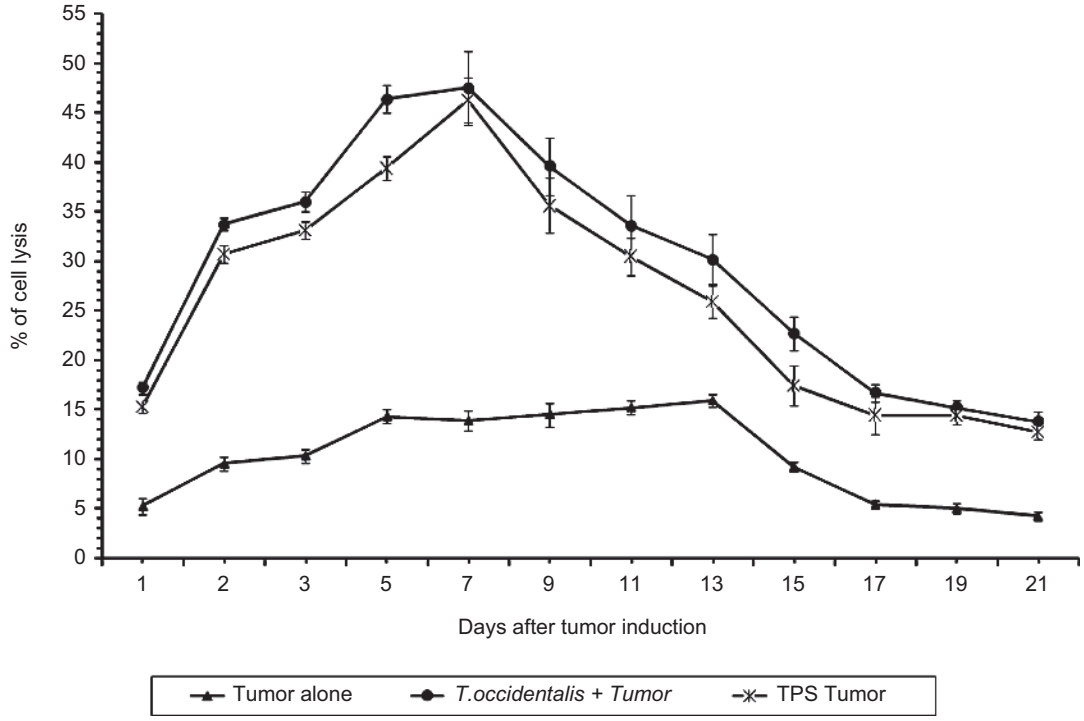

Figure 2. Effect of *Thuja occidentalis* and *T. occidentalis* L. (Cupressaceae) polysaccharide (TPS) on antibody-dependent cell-mediated cytotoxicity in metastasis tumor-bearing animals. Mice were treated with *T. occidentalis* or TPS and spleen cells were obtained. The spleen cells were incubated with chromium labeled sheep erythrocytes and the % cell lysis was determined by 4-h chromium release assay ($n = 3$/point).

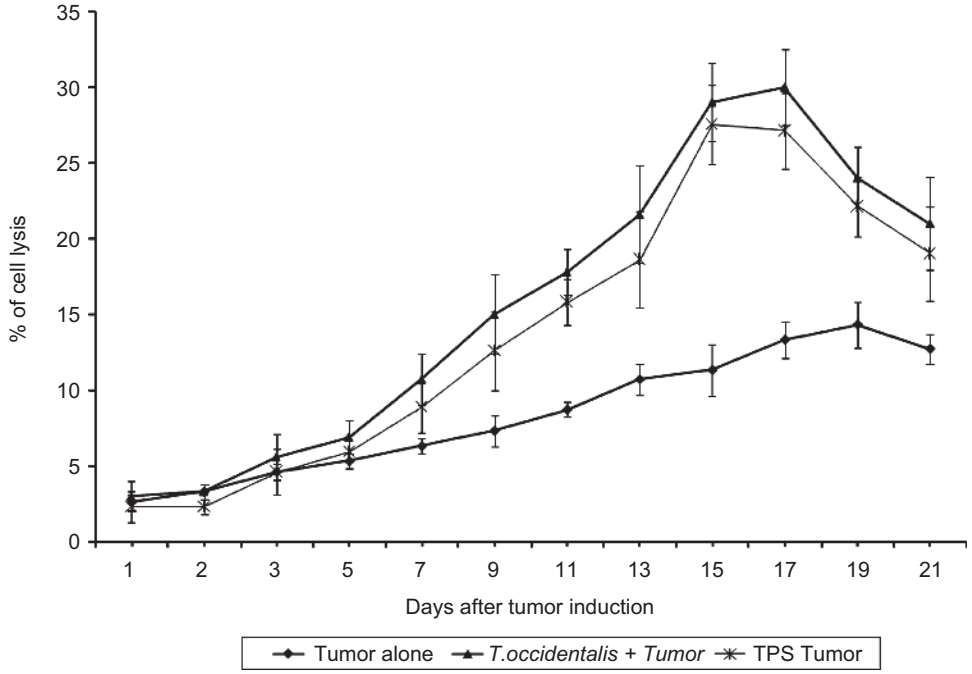

Figure 3. Effect of *Thuja occidentalis* and *T. occidentalis* L. (Cupressaceae) polysaccharide (TPS) on antibody-dependent complement-mediated cytotoxicity in metastasis tumor-bearing animals. Mice were treated with *T. occidentalis* or TPS and blood was obtained. Sera were incubated with fresh rabbit serum (complement) and B16F-10 cells. The cytotoxicity was assessed by Trypan blue exclusion method ($n = 3$/point).

function of lymphocytes, macrophages and NK cells, or may enhance the expansion of cells with downregulatory properties (Lopez et al., 1991). In addition, the production of factors in abnormal amounts by tumor-bearing host may affect normal cytokine network and cause a deleterious imbalance of the immune system. Inactivation of immune responses involving T-lymphocytes and NK cells may be mediated partly by a downregulation of IL-2

production (Malaguarnera et al., 2001). The enhancement of host–immune response has been recognized as a possible means of inhibiting tumor growth without harming the host. Therefore, it is very important to investigate novel antitumor substances with improving immunity potential. Immunomodulation through natural or synthetic substances may be considered an alternative for the prevention and cure of neoplastic diseases. (Xu et al., 2009).

In this experiment, administration of *T. occidentalis* and TPS after B16F-10 melanoma cell induction increased the CMI by promoting the NK cell activity, ADCC, ACC and IL-2 production. The enhanced CMIs and increased production of NK cells is the major protective measures of the immune system during metastasis. The presence of humoral immunity is not consistently correlated with increased tumor resistance in the host. But antibodies can mediate cell destruction either via binding with complement or by acting as an opsonin to facilitate phagocytosis by macrophages or other phagocytic cell bearing Fc receptors and ADCC. ADCC is the co-operative interaction of humoral and cell-mediated immune effector mechanism. The expression of NK cell activity and ADCC activity at an early stage of tumor may lead to the exitration of tumor mass by enhancing the immunological status. *T. occidentalis* and TPS were found to enhance ADCC and ACC activity in normal and tumor-bearing animals and the maximum activity was observed much early compared to control animals.

A number of growth factors and cytokines secreted by the tumor cells or by the surrounding local stroma have important roles in tumor progression and metastasis (Tanaka et al., 2005). Pro-inflammatory cytokines and chemokines are involved in promoting tumorigenesis by facilitating tumor proliferation and metastasis. Numerous studies have indicated that tumor cells exhibit an elevation in the constitutive production of pro-inflammatory cytokines namely TNF-α, IL-1β, IL-6 and GM-CSF (Budhu & Wang, 2006; Chuang et al., 2008). The serum levels of these cytokines are significantly elevated in patients with various types of cancers like renal cell carcinoma, colorectal carcinoma, hepatocellular carcinoma (Chuang et al., 2008, Budhu & Wang, 2006). In our study, treatment with *T. occidentalis* or TPS downregulated the production of these pro-inflammatory cytokine levels in mice. We also found that *T. occidentalis* and TPS could regulate the gene expression of pro-inflammatory cytokines in tumor cells.

Cytokines also regulate both cellular and humoral immune responses by affecting immune cell proliferation, differentiation and functions (Asano et al., 1997). IL-2 plays a central role in the vertebrate immune response. The IL-2 family members are classically considered to be T-cell growth factors, but they act on other lineage as well (Ozaki & Leonard, 2002). It is of clinical value for stimulating the natural immunity by stimulating NK cell and CTL production (Neville et al., 2001). Treatment with *T. occidentalis* and TPS has stimulated the level of IL-2 production, when compared to the tumor-bearing control animals. Many reports have shown that upregulation of TIMPs inhibits tumor growth, invasion and metastasis (Jiang et al., 2002). TIMP inhibits the activities of metalloproteinases which leads to reduced ECM remodeling

Table 2. Effect of *T. occidentalis* and TPS on CTL generation (*in vivo*).

	Survival rate of animals		
Treatment	Control	*T. occidentalis*	TPS
EL-4 alone	26.8 ± 4.4		
EL-4 + normal spleen	36.8 ± 4.2		
EL-4 + normal spleen + compound		46.9 ± 3.7[a]	43.1 ± 3.7[ns]
EL-4 + treated spleen		43.5 ± 4.0	40.3 ± 5.1[ns]
EL-4 + treated spleen + compound		54.4 ± 5.8[aΨ]	50.2 ± 3.9[aΨ]

Alloimmunization was carried out by injecting the spleen (2×10^7) cells from C57BL/6 mice to normal, *Thuja occidentalis* and TPS-treated BALB/C mice. Winns neutralization test was carried out according to the method. The cells were injected intraperitoneally to treated and untreated mice. The animals were observed daily for 80 days after tumor inoculation. [a]$p < 0.001$, [b]$p < 0.01$ compared to EL-4 + normal spleen. [Ψ]$p < 0.01$ compared to EL-4 + treated spleen + compound.

Table 3. Effect of *Thuja occidentalis* and TPS on pro-inflammatory cytokine profile of metastasis-induced animal.

	Normal	Control		*T. occidentalis*		TPS	
Cytokine	Day 0	Day 7	Day 21	Day 7	Day 21	Day 7	Day 21
IL-1β	16 ± 3.5	44.8 ± 4.9	59.4 ± 5.7	38.9 ± 2.5[b]	20.3 ± 2.7[a]	39.4 ± 3.1[b]	23.9 ± 2.6[a]
TNF-α	20 ± 1.8	241.8 ± 20.7	317.2 ± 26.7	134.4 ± 12.4[a]	31.1 ± 2.3[a]	149.4 ± 17.1[a]	42.4 ± 3.6[a]
IL-6	35.3 ± 6.5	353.4 ± 39.1	593 ± 47.3	73.9 ± 6.5[a]	255.3 ± 29.7[a]	92.9 ± 7.3[a]	273.4 ± 29.8[a]
GM-CSF	18 ± 0.94	38.38 ± 2.7	20.8 ± 1.5	30.7 ± 2.9[b]	16.6 ± 0.5[a]	34.1 ± 4.7[ns]	19.3 ± 1.5[ns]
IL-2	23 ± 2.7	22.4 ± 2.0	20.9 ± 2.4	38.0 ± 2.7[a]	36.1 ± 3.0[a]	29.8 ± 2.4[a]	28.5 ± 2.8[b]
TIMP	652 ± 46.8	514 ± 69.8	468 ± 13.6	656 ± 41.3[b]	701 ± 93.4[a]	593.1 ± 21.7[b]	623.4 ± 82.1[b]

All the values are mean ± SD. Blood was collected from the metastasis-induced animals by injecting B16F-10 melanoma cell after seventh and twenty-first day. Serum was separated by centrifugation and cytokine levels were estimated by ELISA method. [a]$p < 0.001$, [b]$p < 0.01$ compared to respective control.

ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte monocyte-colony stimulating factor; IL, interleukin; TIMP, tissue inhibitor matrix metalloproteinases; TNF, tumor necrosis factor.

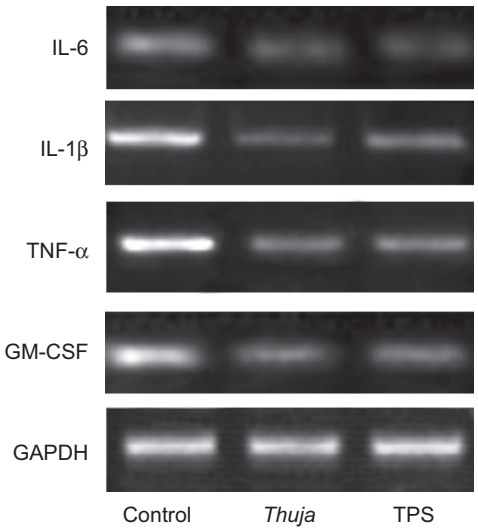

Figure 4. Effect of *Thuja occidentalis* and TPS on pro-inflammatory gene expression. B16F-10 cells were incubated in the presence and absence of *T. occidentalis* or TPS for 4 h at 37°C in 5% CO_2 in serum free medium. Total RNA was extracted from B16F-10 cells and cDNA was synthesized and used for the amplification of pro-inflammatory cytokines, namely, IL-1β, IL-6, GM-CSF, and TNF-α genes. Mouse GAPDH (reduced glyceraldehyde phosphate dehydrogenase) was used as housekeeping gene. Polymerase chain reaction products were analyzed by agarose gel electrophoresis ($n = 3$).

and suppression of endothelial cell migration and invasion (Gomez et al., 1997). Our results also shows that *T. occidentalis* and TPS could downmodulate TIMP levels giving a positive sign for tumor regression.

In conclusion, *T. occidentalis* and TPS could enhance immune response against tumor-bearing mice by activating NK cells and antibody-dependent cellular immune responses. It could decrease the elevated level of pro-inflammatory cytokines in B16F-10 melanoma cell. Moreover, it could also enhance the level of IL-2 which can augment the natural immunity by stimulating NK cells. Furthermore, endogenous tumor inhibitor TIMP level was also increased by the treatment with these compounds. These results revealed the immune stimulatory effect of *T. occidentalis* and TPS on metastatic tumor-bearing animals.

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Declaration of interest

The authors report no declarations of interest.

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