

T-2 Toxin Regulated *Ganoderma lucidum* Induced Cytokine Release

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Abstract: The water-soluble extract of *Ganoderma lucidum* (Reishi) has been used as immunomodulator to stimulate spleen cells proliferation and cytokine expression. It has also been shown that at some level of exposure, T-2 toxin typically act as immunosuppressive agent and can increase disease susceptibility. The aim of this study was to investigate the effect of T-2 toxin on cytokine production by *Ganoderma lucidum* (*G. lucidum*) treated-cells. Mice peritoneal macrophages and lymphoid T cells were prepared by usual manner and plated out at 1×10^6 or 1×10^4 cell/well respectively in RPMI 1640 supplemented with 10% FCS, 50 μg streptomycin and 50U penicillin. Cells were incubated with different concentrations of *G. lucidum* in the presence or absence of 1 ng mL^{-1} T-2 toxin at 37°C and 5% CO_2 for 48 h. Cell free medium was removed and used for cytokine assay by ELISA method. The results showed that T-2 toxin in the absence of *G. lucidum* enhanced IL-2, IFN- γ release compared with control group, but it reduced the production of other cytokines. *G. lucidum* enhanced the production of IL-1 β , TNF- α , IL-12, IL-2 and IFN- γ compared with control group, but reduced IL-4 and IL-10 release. T-2 toxin, up regulated the enhancement effect of *G. lucidum* on IFN- γ , IL-2 and TNF- α , but it down regulated its effect on the production of other cytokines. In conclusion our results indicate that T-2 toxin at 1 ng mL^{-1} may augment the immunomodulating effects of *G. lucidum* on cytokine release.

Key words: *Ganoderma lucidum*, T-2 toxin, IFN- γ , IL-2, IL-4, IL-10, IL-1 β , IL-12, TNF- α

INTRODUCTION

T-2 toxin is one of the mycotoxins of type a trichothecenes produced by several fungal genera including *Fusarium* species^[1]. This toxin has been shown to cause a variety of toxic effect in both animal and human experimental. These include inhibition of DNA, RNA and protein synthesis in several cellular systems^[2], membrane and lipid peroxidation^[3], hamatotoxicity^[4] and immunotoxicity^[5]. It has also been shown that mycotoxins suppressed phagocytic activity and increased susceptibility to bacterial infections in animal models^[6,7].

On the other hand, Trichothecenes are extremely toxic to leukocytes and other rapidly dividing cells. Since these mycotoxins can be acquired via food or air, they have the potential to cause human and animal immune dysfunction and disease^[8]. Leukopenia, granulopenia, exhaustion of bone marrow, progressive lymphocytosis and thymic apoptosis are the most important pathological symptoms^[9]. T-2 toxin has also been found to produce significant immunosuppression and suggested that effect of T-2 toxin might be related

to apoptosis of immune cells^[9]. On the other hand, depending on the dose, timing and route of exposure, mycotoxins can also have stimulatory effects on immune cells. It can affect both cell mediated and humoral immune compartments^[10,11]. Specific effects ascribed to the trichothecenes include suppressed mitogenic response in human T and B lymphocytes^[12]. Furthermore, mycotoxin has also been shown to super induce IL-2, IL-4 and IL-5 cytokine mRNA expression and production^[13]. Mycotoxin modulated kinetic of IL-2, IL-4, IL-6 production^[14] and also suppressed reovirus-induced INF- γ elevation in bronchial alveolar lavage fluid, but enhanced production of IL-6^[15,16].

Furthermore, *Ganoderma lucidum* (Reishi) is well known for its immuno-modulatory and anti tumor activities^[17,18]. It has also been reported that F3 fraction of Reishi polysaccharides activate the expression of IL-1, IL-6, IL-12, IFN- γ , TNF- α , GM-CSF and M-CSF of mice spleenocytes^[17]. Meanwhile, Previous studies have shown that *G. lucidum* activate macrophages (m Φ), T lymphocytes, NK cells and induction of cytokines such as TNF- α , interleukins and IFNs by human and mice immune cells^[19-22]. Some investigator,

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believe that *Ganoderma lucidum* acts as adjuvant so that it has also been used as a natural adjuvant for immunotherapy^[23]. The purpose of this study was to elucidate the effect of T-2 toxin on cytokine production by *G. lucidum* treated-cells.

MATERIALS AND METHODS

Cell preparation: Eight weeks old BALB/c male mice were maintained in a dust free bedding cages in the animal unit of Baqiyatallah University of Medical Sciences. The animals were anesthetized and subjected to 5 mL intra-peritoneal ice cold PBS. Then, the peritoneal cells were extracted immediately and kept at 2-8°C ice cold to avoid non-specific attachments before being washed at 4°C for three times (1500g for five minutes) with cold RPMI 1640 (Tissue culture medium with L-Glutamin, without phenol Red, Gibco Co.). Lymph node were also removed and lymphoid cells were extracted immediately^[24]. Cells were kept at 2-8°C ice cold before being washed at 4°C for three times (1500 g for 5 min) with cold RPMI 1640. After that, cells were passed through a siring containing special sponge to omit non T cells. The cells were re-suspended in RPMI 1640 supplemented with 10% FCS (Fatal Cow Serum- Gibco Co.), 50µg mL⁻¹ streptomycin and 50 U mL⁻¹ penicillin (Gibco Co.). Next, the cells were counted and finally, cell viability was checked by trypan blue (Sigma Co.) exclusion test. Sample of cell suspension was mixed with an equal volume of 0.4% (W/V) Trypan blue in PBS and incubated for 10 min. The cells failing to exclude the dye were counted and expressed as a percentage of the total cells present.

$$\text{Cell viability} = \frac{(\text{number of viable cells})}{(\text{sum of viable and dead cells})} \times 100^{[24]}$$

Cell culture: Peritoneal macrophages were plated out at 1×10^6 cells/well in 24 well plates (Nunclon @DELTA- Denmark) and allowed to adhere for 2 h (n = 6). Non-adherent cells were then removed by washing with warm PBS. Next, the adherent cells were cultured at 37°C at 5% CO₂ in air for 48h in 1 mL RPMI 1640, containing 10% FCS, 50 µg streptomycin and 50 U penicillin and different concentrations of *G. lucidum* (JHS natural products- Reishi extract with 12% polysaccharide and 6% triterpenes- USA) ranging from 5 µg to 160 µg mL⁻¹ in the presence or absence of 1ng mL⁻¹ T-2 toxin (Sigma Aldrich).

Lymphoid T cells were also plated out at 1×10^4 cells/well (96 well plate-Nunclon @DELTA- Denmark) in 0.1 mL RPMI 1640, supplemented with 10% FCS, 50 µg streptomycin and 50 U penicillin, 2.5 µg mL⁻¹

concanavalin-A and different concentrations of *G. lucidum* ranging from 5 µg-160µg mL⁻¹ in the presence or absence of 1 ng mL⁻¹ T-2 toxin (Sigma Aldrich). All plates (24 wells and 96 wells plates) were incubated at 5% CO₂, 37°C for 48 h. The selection of the concentration of T-2 toxin for the evaluation of the effect of this toxin on the cytokine production was based on cytotoxicity assays, whereby at concentrations below 10 ng mL⁻¹ was associated to noncytotoxic effect, 100 ng mL⁻¹ to partial cytotoxic effect and at concentrations over 100 ng mL⁻¹ to full cytotoxic effects.

Culture medium of both above mentioned tissue culture plates was subsequently removed from each well, placed into 1.5 mL micro centrifuge-tubes (Citolab, BRAND) and centrifuged at 13000 g for 10 min at room temperature. Finally, the supernatants from the centrifuged tubes were transferred to clean tubes and were stored at -70°C until they were analysed for cytokine assay using the ELISA kit (Bender Med System Company, USA).

Statistical analysis: Data expressed as the mean±S.E.M. An analysis of variance (ANOVA) was used to determinate differences between the control and test wells. When statistically significant differences (p<0.05) were found between the groups, unpaired t-test were used to determine the level of significant difference between the two groups.

RESULTS AND DISCUSSTION

The results showed, when T-2 toxin was added to cells without any *G. lucidum*, it significantly enhanced IFN-γ and IL-2 release (Fig. 1 and 2, Control wells, p<0.025), but reduced the production of remaining cytokines in the same conditions (Fig. 3-7). The results also show that *G. lucidum* enhanced IFN-γ, IL-2 release (Fig. 1-2), but reduced IL-4, IL-10 production (Fig. 3-4) by T cells in a concentration dependent manner. When, 1ng mL T-2 toxin was added to any concentration of *G. lucidum*, it augmented both stimulatory and inhibitory effects of *G. lucidum* on cytokine release by T cells (Fig. 1-4).

The results also showed, that *G. lucidum* enhanced IL-1β, TNF-α release in a concentration dependent manner (Fig. 5-7), but in case of IL-12, the highest enhancement was induced in response to 5 µg mL *G. lucidum* (Fig. 6). In contrast to the effect of T-2 toxin on cytokine release by Th₁ cells, here, it reduced IL-1β, IL-12 and TNF-α release by peritoneal macrophages comparing with control groups (Fig. 5-7). T-2 toxin augmented the stimulatory effect of *G. lucidum* on TNF-α release (Fig. 7), but reduced its

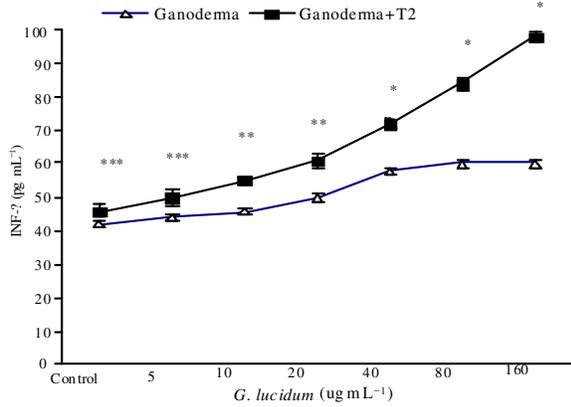


Fig. 1: Effect of different concentrations of *G. lucidum* on IFN- γ production by T cells in the presence or absence of 1 ng mL⁻¹ T-2 toxin

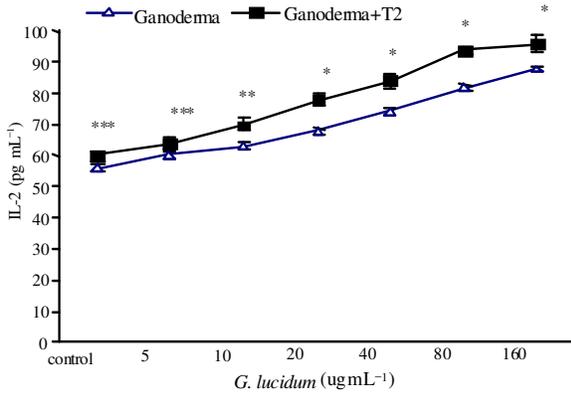


Fig. 2: Effect of different concentrations of *G. lucidum* on IL-2 production by T cells in the presence or absence of 1 ng mL⁻¹ T-2 toxin

effect on IL-1 β , IL-12 (Fig. 5 and 6) release by peritoneal macrophages. We have previously demonstrated the effect of T-2 toxin and *G. lucidum* on nitric oxide and cytokine release by peritoneal m Φ of mice^[22,25]. In this study, the results showed that T-2 toxin without any *G. lucidum* supplementation significantly enhanced IFN- γ and IL-2 release (Fig. 1 and 2, Control wells, p<0.025), but reduced the production of remaining cytokines in the same conditions (Fig. 3-7), demonstrating both enhancement and inhibitory effects of T-2 toxin on cytokine production depending on cell sources.

Our data presented here in, is supported by a study on human lymphocyte where 1 and 5 ng mL⁻¹ of T-2 toxin induced 50% inhibition of PHA-stimulated proliferation, but had no statistically significant effect on the viability of unstimulated lymphocytes^[26].

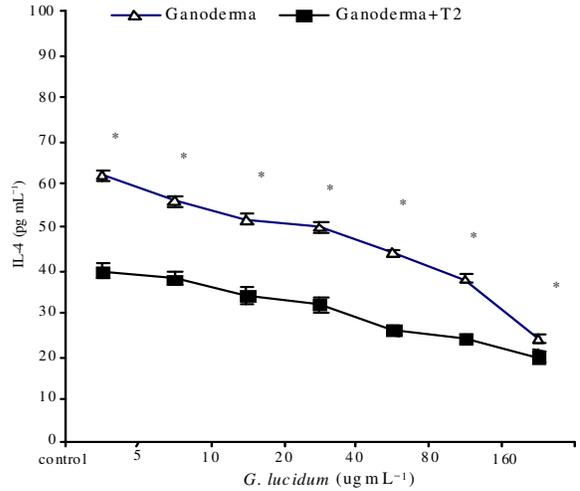


Fig. 3: Effect of different concentrations of *G. lucidum* on IL-4 production by T cells in the presence or absence of 1 ng mL⁻¹ T-2 toxin

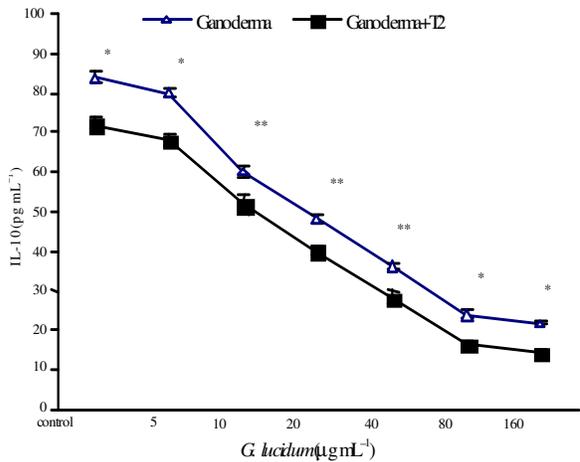


Fig. 4: Effect of different concentrations of *G. lucidum* on IL-10 production by T cells in the presence or absence of 1 ng mL⁻¹ T-2 toxin

It is of interest to note that similar studies on the effect of T-2 toxin on lymphocyte function have been demonstrated that modulation of the immune system is one of the major effects of T-2 toxin in animals and humans^[27]. T-2 toxin also augmented the stimulatory effect of *G. lucidum* on IL-2, IFN- γ , TNF- α release and its inhibitory effect on IL-4, IL-10 production. On the other hand, *G. lucidum* enhanced the production of IL-1 β , IL-12 by peritoneal macrophages, but T-toxin reduced the stimulatory effect of *G. lucidum* on the production of these two cytokines. Supporting our data presented here in, Jaradat *et al.*^[28] demonstrated that an

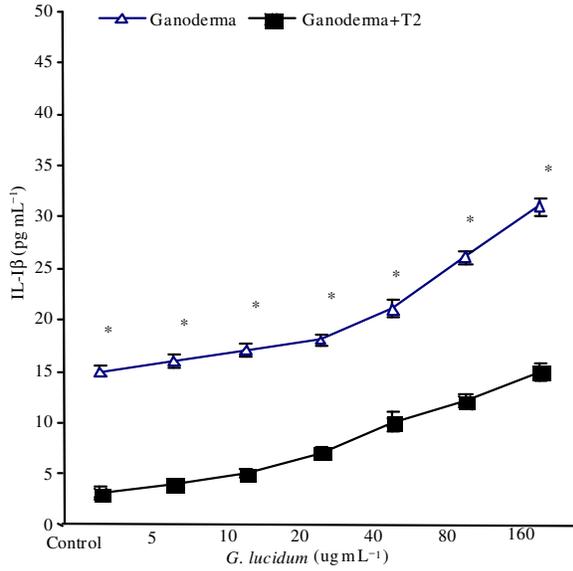


Fig. 5: Effect of different concentrations of *G. lucidum* on IL-1 β production by peritoneal macrophages in the presence or absence of 1 ng mL⁻¹ T-2 toxin

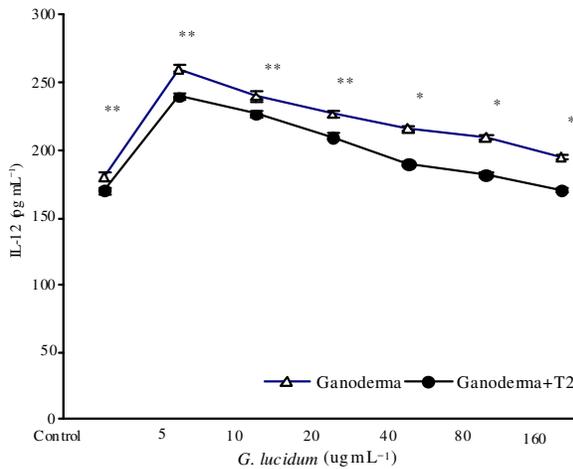


Fig. 6: Effect of different concentrations of *G. lucidum* on IL-12 production by peritoneal macrophages in the presence or absence of 1 ng mL⁻¹ T-2 toxin

acute exposure of animals or human to T-2 toxin resulted in severe damage to actively dividing cells in tissues such as bone marrow, lymph nodes, spleen, thymus and intestinal mucosa. This is also in agreement to other studies that reported the exposure to T-2 toxin either enhanced or suppressed B and T lymphocyte mitogen proliferation in a dose dependent manner^[29,30].

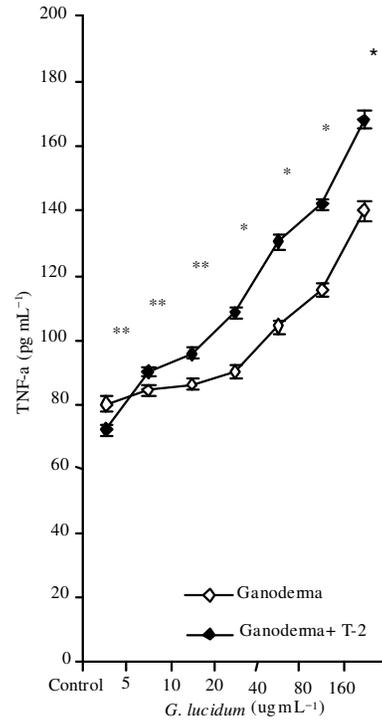


Fig. 7: Effect of different concentrations of *G. lucidum* on TNF- α production by peritoneal macrophages in the presence or absence of 1 ng mL⁻¹ T-2 toxin

Therefore, it is not surprising if T-2 toxin, depending on dose, time of administration and cell origin can induce different effects on immune cell function^[31-34]. In contrast to our results in which T-2 toxin reduced IL-1 production by peritoneal m Φ , a significant increase of IL-1 β and IL-6 in supernatants of chondrocytes cultivated for 24 h with T-2 toxin at 8 ng mL⁻¹ after PMA stimulation is reported previously^[31]. Present results also are in favour of Hymery *et al.*^[35] who demonstrated that trichothecenes have adverse effects on dendritic cells maturation process. Therefore, Effect of T-2 toxin on peritoneal macrophages and lymphoid T cells of mice observed in this study in some parts is different that those described in the literature^[36,37], which could be contributed to the dose and condition used in this study. Thus, it seems that the effects of T-2 toxin and *G. lucidum* on cell function are different in single usage or using them in combination form. On the other hand, this effect of *G. lucidum* might be related to its adjuvant property, as Chan *et al.*^[23] reported *G. lucidum* mycelium and spore extracts acts as natural adjuvant for immunotherapy.

CONCLUSION

Based on data presented here in and the finding of others, it is possible to suggest that T-2 toxin may modulate the effect of *G. lucidum* on immune function.

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