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Ganoderma lucidum dry extract supplementation modulates T lymphocyte function in older women

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Abstract

Ganoderma lucidum (a mushroom used in traditional Chinese medicine) compounds may attenuate aging-related physiological changes and restore normal immunity. However, studies on the physiological effects of *Ganoderma lucidum* dry extract food supplements are few. Therefore, here, we aimed to investigate the effects of *Ganoderma lucidum* dry extract food supplement on the lymphocyte function of older women. This was a double-blind clinical trial (n = 60) with a final 39 older volunteers, divided into two groups, *Ganoderma lucidum* (n = 23) and placebo (n = 16). The *Ganoderma lucidum* group received 2,000 mg/day of *Ganoderma lucidum* dry extract for 8 weeks. We used flow cytometry to determine the lymphocyte profile. $CD4^+$ lymphocyte gene expression was evaluated by real-time PCR. We observed that in the *Ganoderma lucidum* group, concanavalin A (ConA) stimulation increased lymphocyte proliferation. Further, we observed an increase in expression of FOXP3, TGF-β, IL-10, IL-6, RORɣ, GATA-3, and IFN-ɣ genes in the *Ganoderma lucidum* group. Furthermore, in the *Ganoderma lucidum* group, ionomycin and PMA stimulation led to decrease in $Th17^+$ cells and increase in $Th2^+$ cells. Thus, in older women, *Ganoderma lucidum* regulates T lymphocyte function leading to a predominant anti-inflammatory action but does not induce T lymphocyte proliferation through CD28 signaling pathway.

Keywords: Ling Zhi, immunomodulation, $CD4^+$ cells, older adults, Th17.

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Introduction

Immunosenescence is the change/decline in immune functions due to aging. It is characterized by a decrease in immune responses to new infections and vaccines, or immunological failure. Innate and adaptive immunity are compromised. Adaptive immunity is mediated by T and B lymphocytes. Natural aging leads to a decline in naive T cells production, which modifies peripheral T cells composition; further their phenotype and functions are altered[(1)].

Aging is also associated with inflammaging, an aging-related chronic, low-level, persistent pro-inflammatory state observed in the absence of acute infections. Proinflammatory factors found in inflammaging are mainly attributed to a combination of senescence-associated secretory phenotype, involving the release of cytokines and pro-inflammatory factors, such as IL-6, TNF-α, and IL-1β. Inflammaging is a risk factor for morbidity and mortality in chronic age-related diseases[(2)]. It is associated with severity and increased incidence of cardiovascular, neurodegenerative diseases, and cancers in older adults[(3,4,5)].

Immunosenescence is characterized by lymphocytes dysfunction[(4)] and reduced production of IL-2 by T lymphocytes[(4,6,7)]. Decrease in IL-2 is associated with a greater activation of Th17 cells[(5)], which secrete inflammatory cytokines, such as IL-17. Regulatory T lymphocytes (Treg), such as $CD4^+CD25^+Foxp3^+$ cells, act on other cellular subtypes of innate and adaptive immunity, promoting a immunosuppressive effect[(8)]. These cells release large amounts of IL-10, IL-35, and TGFβ, controlling cell activation[(9)]. A reduction in circulating IL-10 and an increase in IL-6 and TNF-α levels have been observed in elderly people with cardiovascular complications and type 2 diabetes mellitus[(3)].

Ganoderma lucidum, a popular mushroom used in traditional Chinese medicine for more than 2,000 years, has immunomodulatory capacity. It is also called *Ling Zhi* or *Reishi*. *Ganoderma lucidum* increases T and B lymphocytes, natural killer cells, and dendritic cells[(10)]. *Ganoderma lucidum* can exert antibacterial, antiviral, anti-fibrotic, and antitumor effects by modulating the immune response[(11,12)].

Ganoderma lucidum is rich in the bioactive polysaccharides (GLP). GLP cellular activities are determined by their molecular weights, conformations, solubility degree, and the organ of origin. Mushroom GLP differ in their composition, immunomodulatory activity, response potency, and action on immune cells, depending on which part of the mushroom they are isolated from (mycelium or spores)[(13)]. *Ganoderma lucidum* is rich in β-glucans (a

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type of polysaccharide) which can modulate immune cell functions, as reviewed by Chan *et al.*[14]. β-glucans modulate innate and adaptive responses through activation of TLR4[15], TLR2 and Dectin-1[16]. Intracellular NFκB is activated downstream of these membrane receptors, which stimulates immune system cells (such as monocytes, dendritic cells, and macrophages) and promotes activation of a wide variety of cytokines, including pro-inflammatory cytokines[15]. In addition, β-glucans also are involved in NLRP3 inflammasome activation leading to IL-1β release[17].

Exposure to isolated components of *Ganoderma lucidum* stimulates different leukocyte functions, mainly through cytokine production[18,19,20,21,22]. Importantly, Haak-Frendscho *et al.*[20] observed an increase in IL-2 expression in human mononuclear cells in the presence of the *Ganoderma lucidum* protein LZ-8, indicating that LZ-8 is involved in blocking the Th17 response. This effect has anti-aging therapeutic potential because, as previously described, during immunosenescence, IL-2 production by lymphocytes is reduced. Moreover, elderly people, who present high levels of IL-10 and low levels of IL-6, may live longer[23,24]. The T lymphocytes function, differentiation, and activation are impaired in the elderly, leading to immune imbalance. Therefore, *Ganoderma lucidum* supplements can be crucial in correcting the immunosenescence-associated immune lymphocyte imbalance.

Thus, *Ganoderma lucidum* may have an important role in mitigating immunosenescence. However, studies on the influence of whole *Ganoderma lucidum* extract on isolated lymphocytes, especially in the elderly population, are lacking. Thus, here, we aimed to investigate the effects of whole *Ganoderma lucidum* extract food supplement on the differentiation and function of circulating lymphocytes in elderly women.

Materials and methods

Study Design

The study was approved by the Ethics Committee of Cruzeiro do Sul University under protocol CEP 23275819.0.0000.8084, and the participants signed a free and informed consent form in compliance with the standards of the Ethics Committee in research of the Cruzeiro do Sul University.

The inclusion criteria were women aged between 60 and 80 years old and free from neurodegenerative diseases. The exclusion criteria were patients with a previous diagnosis of diseases that could influence the results of the study, such as severe heart disease, insulin-dependent diabetes mellitus, autoimmune diseases, viral infections (such as HIV and cytomegalovirus infections) and other infectious diseases (such as syphilis,

hepatitis). Volunteers chronically using anti-inflammatory and/or immunosuppressant drugs were also excluded from this study.

A total of 60 women enrolled for the 8-week long *Ganoderma lucidum* supplementation study. The sample size calculation was performed using the statistical software Epi Info 6.0. It was considered for the calculation a confidence interval of 95% and an α of 5%. It was determined that the sample should consist of 15 individuals in each group for the lymphocyte profile analysis. The volunteers were distributed into the two groups—*Ganoderma lucidum* group and Placebo group—in a paired manner based on age, medications (thyroxine, hypoglycemic agents, antihypertensive agents, lipid-lowering agents, antiplatelet agents and antidepressants), body composition, and level of physical activity (following the classification of the International Physical Activity Questionnaire). The study followed a double-blind protocol.

The Placebo group received 2,000 mg/day of placebo (corn starch) for 8 weeks; and *Ganoderma lucidum* group received 2,000 mg/day of *Ganoderma lucidum* for 8 weeks. We selected the *Ganoderma lucidum* dosing regimen based on previous studies[22,25,26]. No adverse reactions, side effects or toxicity reactions are reported in the literature for the doses used in this study. The doses reported in literature ranged from 20 mg/Kg/day to 8g/kg/day, reinforcing the safety and appropriateness of the doses used here.

The *Ganoderma lucidum* was sourced from the VITAJOY company in China. We used the crude dry extract of the *Ganoderma lucidum* sporophore, which consisted of 52.83% of β-glucans (Figure S1A and Table S1). The *Ganoderma lucidum* sporophore extract was obtained using water as the solvent in a ratio of 5:1. The dry extract obtained was utilized in the preparation of the capsules. Both groups were instructed to take 4 capsules (Placebo/ *Ganoderma lucidum* capsules) 1 h before lunch once a day for 8 weeks.

Twenty-one volunteers dropped out/were excluded by the end of the study period due to various reasons, including personal health problems, family health issues, or low adherence to study protocol. All volunteers were interviewed to identify possible health problems associated with dropping out. Finally, 39 volunteers completed the 8 weeks long study: the Placebo group (n = 16) and *Ganoderma lucidum* group (n = 23).

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Determination of total β-glucans and their molecular weight distribution in Ganoderma lucidum extract

To determine total β-glucans (52,83%), the Mushroom and yeast beta-glucan – assay kit (K-YBGL, Megazyme, Ireland) was used. To analyze the molecular weight distribution of *Ganoderma lucidum* polysaccharides, we used the High-performance Size Exclusion Chromatography with refractive index detection (HPSEC-RID), following the protocol of Prado *et al*.[27].

Sample collection

We collected 30 mL of blood from the antecubital vein into tubes containing ethylenediaminetetraacetic acid (EDTA, 1 mg/mL), after overnight fasting (8 h), before and after 8 weeks of supplementation. The blood was centrifuged at 400 × *g*, at 4°C, for 10 min. Plasma samples were separated and stored (−80°C) until further assays.

Urine was collected in a reserved place using a universal collector. The volunteers were instructed about mid-stream, aseptic urine collection and the first-catch urine was discarded.

Evaluation of Clinical and Hematological Parameters

Plasma glucose, total cholesterol, triglycerides, HDL cholesterol (high density lipoproteins), LDL cholesterol (low density lipoproteins), Gamma-Glutamyl Transferase (GGT), creatinine, lipase, Aspartate Aminotransferase (AST), and Alanine Aminotransferase (ALT) were performed by kinetic enzymatic method. Complete blood count analysis was performed by flow cytometry. The analyses are presented in the supplementary material (Figure S2 and S3).

The pre– and post– supplementation percentages of erythrocytes, leukocytes, lymphocytes, including hemoglobin concentration, and hematocrit percentage, were not significantly different between Placebo and *Ganoderma lucidum* groups. The analyses are presented in the supplementary material (Figure S4).

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Body Composition Assessment

Body mass index was calculated from the body weight and height measures and body composition by using bioimpedance measurement (Biodynamics Corporation, USA-A310), as described by Passos *et al.*[28].

Lymphocyte Analysis

Venous blood was collected and lymphocytes were isolated using Histopaque-1077 as described by Passos *et al.*[28]. Briefly, blood samples were diluted in phosphate buffer saline (PBS) (1:1), and the resulting suspension (3 mL) layered onto Histopaque-1077 (6 mL) and centrifuged at 400 × *g*, for 30 min, at room temperature. Peripheral blood mononuclear cells were collected from the interphase. The remaining erythrocytes were lysed in a solution containing 150 mM NH_4Cl, 10 mM $NaHCO_3$, and 0.1 mM EDTA, pH 7.4. Cells were washed once with PBS and were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin, pH 7.4, in sterile tissue culture flasks. To obtain a lymphocyte suspension with high purity (>98%), the cells were maintained at 37°C in a humidified atmosphere, with 5% CO_2 and 95% air, to allow the monocytes to adhere to the plates. The lymphocyte suspension was centrifuged at 400 × *g*, for 10 min, and then the supernatant was discarded. Cells were resuspended in PBS, counted in Neubauer chamber and the specific number of cells was separated for the analyses.

For the determination of polarization of naïve $CD4^+$ into T helper (Th) cells exhibiting Th1, Th2 and Th17 profiles, lymphocytes were incubated with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL) and ionomycin (Ionomycin) (1μg/mL) in complete RPMI 1640 medium for 6 hours. Subsequently, cells were washed with PBS containing 1% bovine serum albumin and labeled with PERCP-CY5.5 anti-CD4 (341654 – Becton Dickinson, CA, EUA). Cells were fixed (1% formaldehyde) and treated with permeabilizing agent (Becton Dickinson) followed by incubation with anti-IL-17A PE (560486 Becton Dickinson, CA, EUA), anti- IFN-ɣ FITC (554551 – Becton Dickinson, CA, EUA) and anti-IL4 APC (554486 – Becton Dickinson, CA, EUA) antibodies. Twenty thousand $CD4^+$ positive gated events were acquired. The data are presented as percentage of the total $CD4^+$ cells. Histograms were analyzed using BD-Accuri Software (Becton Dickinson, CA, EUA).

$CD28^+$ expression on the lymphocyte surface was also measured through flow cytometry. The cell suspension (1×10^6 cells) was incubated with PerCP-Cy5.5- anti-CD28 (560685 Becton Dickinson, CA, EUA) at room temperature for 30 min in the dark. Negative

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control cells were incubated with the labeled IgG antibody. After this period, cells were washed twice with PBS and analyzed using a BD-Accuri flow cytometer (Becton Dickinson). Histograms were analyzed using the BD C6 Sampler Accuri Software by determining mean fluorescence intensity.

Evaluation of gene expression

The mRNA expressions of IL-35, TGF-β, GATA-3, T-bet, FOXP3, RORɣ, β2M, IL-10, IL-6, IFN-γ, TNF-α, IL-2, and IL-17 genes were evaluated using real-time PCR. Total RNA was extracted from 1×10^7 lymphocytes under basal conditions using the TRIzol reagent following the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA) with DNase treatment. RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) by measuring the absorbance at 260 and 280 nm. The purified RNA (1 µg) was transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA).

The real-time polymerase chain reaction (qPCR) assays were carried out in duplicate in the *QuantStudio™ 3* Real-Time PCR (Thermo Fisher Scientific, Waltham, MA, USA) by using the *Power SYBR™ Green PCR Master* (Thermo Fisher Scientific, Waltham, MA, USA), accordingly, to manufacture specifications. The sequences of the specific primers for the analysis of IL-35, TGF-β, GATA-3, T-bet, FOXP3, RORɣ, β2M, IL-10, IL-6, IFN-γ, TNF-α, IL-2, and IL-17 gene expression were designed using information from the *GeneBank* public database of the National Center for Biotechnology Information. These sequences are described in Table S2.

The relative quantification value of each target gene was expressed using the Ct comparative method with the $2^{-\Delta\Delta Ct}$ formula. The β2M reference gene was chosen after *GeNorm™* analysis[29,30,31].

Statistical analysis

Quantitative variables were presented as mean ± standard error of the mean (SEM) The normality of the data distribution was determined by the Shapiro-Wilk normality test. Comparisons between two groups was tested using repeated-measures analysis of variance (Two-Way ANOVA). In case of significant differences, post-hoc analyses were conducted using Sidak post-test adjustment. A p-value ≤ 0.05 was used as the criterion for statistical significance. All analyses were carried out using *GraphPad Prism* software, version 8.0.

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Results

Sample characterization

The anthropometric parameters and body composition before and after supplementation with *Ganoderma lucidum* or Placebo are shown in Table 1. There was no difference after supplementation between the *Ganoderma lucidum* and placebo groups for all the evaluated parameters.

[Table 1 here]

Evaluation of CD28^{+} expression and lymphocyte proliferation

When stimulated with Concanavalin A (ConA), in vitro, lymphocytes from the *Ganoderma lucidum* group demonstrated a heightened proliferative capacity compared to those from the Placebo group. Notably, this difference was evident solely in the post-supplementation analysis (Figure 1A). Interestingly, no discernible distinctions were observed between the analyses conducted before and after the supplementation period for any of the groups studied (Placebo or *Ganoderma lucidum*).

Pre- and post-supplementation CD28^{+} expression, in response to ConA stimulation, was similar in both groups (Figure 1B and 1C).

Evaluation of gene expression

Post-supplementation IL-6, TNF-α and IFN-γ gene expressions in the *Ganoderma lucidum* group was higher compared to that in pre-supplementation period. Post-supplementation IL-10 and IFN-ɣ gene expressions, in the *Ganoderma lucidum* group were higher compared to that in the Placebo group. However, post-supplementation IL-17 and IL-2 gene expression, were increased in *Ganoderma lucidum* and Placebo groups compared to their pre-supplementation expression levels. Moreover, post-supplementation IL-17 and IL-2 gene expression in *Ganoderma lucidum* group was not significantly different from that in Placebo group. This indicates that the post-supplementation IL-17 and IL-2 gene expression changes are not caused by *Ganoderma lucidum* supplementation. Further, post-supplementation IL-35 gene expression, was not significantly different between the two groups (Figure 2).

TGFβ expression is associated with T-cell proliferation inhibition and maturation of regulatory T cells (Tregs). TGFβ expression is increased in the *Ganoderma lucidum* group. FOXP3 transcription factor gene expression, which is activated during the process of Treg

differentiation, was also increased in the *Ganoderma lucidum* group. Thus, post-supplementation TGFβ and FOXP3 expressions in *Ganoderma lucidum* group were higher compared to that in Placebo group. Post-supplementation expression of T-bet transcription factor gene was increased in both *Ganoderma lucidum* and Placebo groups (above). Post-supplementation GATA-3 and RORɣ gene expression, were increased only in the *Ganoderma lucidum* group (Figure 3).

The post-supplementation IL-10/TNF-α ratio, was increased in the *Ganoderma lucidum* group (Figure 4A). The post-supplementation IL-10/IL-17 ratio was reduced in both groups (Figure 4B). The post-supplementation IL-10/IFN-ɣ ratio was not significantly different between *Ganoderma lucidum* and Placebo groups (Figure 4C).

Evaluation of T lymphocyte subpopulations

The *Ganoderma lucidum* group exhibited lower mean fluorescence intensity related to IFN-ɣ (Figure 5A) in comparison to Placebo group, as assessed through flow cytometry, only in post-supplementation period. These findings suggest that *Ganoderma lucidum* has the potential to inhibit Th1 cell activity. However, pre- and post-supplementation Th1 percentage was similar in *Ganoderma lucidum* group; further, post-supplementation Th1 percentage was similar in *Ganoderma lucidum* and Placebo groups (Figure 5 D). Therefore, the *Ganoderma lucidum* supplementation did not affect lymphocyte polarization. Pre- and post-supplementation mean fluorescence intensity associated with IL-4 (Figure 5 B) was lower in *Ganoderma lucidum group* compared to that in the Placebo group. Moreover, post-supplementation Th2 cells percentage in *Ganoderma lucidum* group was higher compared to that in Placebo group (Figure 5 E). Pre- and post-supplementation average mean fluorescence intensity associated with of IL-17 were not significantly different between *Ganoderma lucidum* and Placebo groups (Figure 5C). Post-supplementation Th17 percentage in the *Ganoderma lucidum* group was lower compared to that in the Placebo group (Figure 5F).

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Discussion

Here, we examined the biological activity of the crude polysaccharide from *Ganoderma lucidum*. Almost 52.83% of the crude dry extract of the mushroom sporophore, studied here, were β-glucans (see supplementary information). Thus, the results obtained in this study can be attributed to the presence of high β-glucans content (52.83%) in the extract. These results corroborate earlier studies, which also attributed their results to the polysaccharides and proteins present in the mushroom(19,22,32). Notably, our results indicate that the effects of *Ganoderma lucidum* extract observed in our study were not associated with body composition parameters.

In our investigation, we employed key mitogens to evaluate lymphocyte activity. Ionomycin, is a calcium ionophore that facilitates calcium mobilization across the cell membrane, initiating the opening of calcium-dependent channels. The influx of calcium activates enzymes (calmodulin, calcineurin, and serine/threonine phosphatase), leading to the dephosphorylation of serine residues on NFAT (nuclear factor of activated T cells) proteins. NFAT dephosphorylation induces a conformational change in NFAT, promoting its translocation into the nucleus. Nuclear NFAT, in turn, stimulates the expression of genes encoding Th1 profile cytokines (IL-2, IFN-γ, and TNF-α) and Th2 profile cytokines (IL-4, IL-5). In contrast, the mitogen PMA (phorbol myristate acetate) acts as an agonist through intracellular activation via DAG (diacylglycerol), leading to the release of inflammatory cytokines. Therefore, we used these two (PMA/ ionomycin) mitogens to verify the influence of *Ganoderma lucidum* extract on the intracellular pathways stimulated by them (21,32).

Pre- and post-supplementation lymphocytes proliferations in the presence of the ConA mitogen, were not significantly different in the *Ganoderma lucidum* group. However, it is noteworthy that post-supplementation proliferation seen in lymphocytes isolated from the *Ganoderma lucidum* was higher than that seen in lymphocytes isolated from the Placebo group (see Figure 1A).

ConA, a mitogen, stimulates the TCR (T-cell receptor) signaling pathway, triggering the activation of proteins such as RAS, MAPK, and NFAT1. This cascade ultimately leads to an increase in IL-2 production(20,32). Remarkably, our findings indicate that IL-2 gene expression was elevated following supplementation with *Ganoderma lucidum*, suggesting a potential regulatory mechanism mediated by this cytokine. This observed increase in lymphocyte proliferation and the associated upregulation of IL-2 gene expression

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in *Ganoderma lucidum* post-supplementation may signify a positive impact of *Ganoderma lucidum* on the TCR signaling pathway, contributing to enhanced immune response and regulatory mechanisms.

The higher lymphocytic proliferative capacity in the *Ganoderma lucidum* group is not associated with $CD28^+$ surface expression. Therefore, our data suggest that *Ganoderma lucidum* may regulate other co-stimulatory molecules involved in lymphocytic proliferation. Chan *et al.*[33] also observed that *Ganoderma lucidum* mycelium extract increases the proliferation of human mononuclear cells, indicating that specific compounds in *Ganoderma lucidum* mushroom exert an immunomodulatory effect.

We found that expression of the RORγ transcription factor gene was upregulated in the *Ganoderma lucidum* group. When activated, RORγ promotes the differentiation of lymphocytes into cells expressing the Th17 profile. Interestingly, although IL-17 gene expression increased in the *Ganoderma lucidum* group, there was a notable reduction in the percentage of IL-17 positive cells. These findings suggest that the elevated expression of RORγ in the *Ganoderma lucidum* group may be insufficient for the positive regulation of IL-17 secretion. Notably, IL-17 is typically secreted in the presence of high levels of TGF-β and IL-6. This synergistic action tends to favor the differentiation toward the Th17 profile.

Here, we observed an increase in the expression of IL-6, TGF-β, and RORγ genes, with a more pronounced increase in *TGF-β* expression. These elevated expressions, in concert, could potentially lead to an increase in IL-17 secretion[34,35]. However, we found a negative regulation of the Th17 lymphocyte profile, despite the heightened expressions of TGF-β, IL-6, and RORγ. This negative regulation is attributed to the increased expression of the IL-10 gene. Thus, our findings indicate that the increased IL-10 gene expression dampens creation of cells with Th17 lymphocyte profile, even when TGF-β, IL-6, and RORγ expressions are significantly upregulated.

We found that GATA-3 expression was upregulated. GATA-3 is a transcription factor associated with creation of cells with Th2 profile (IL-4, IL-5, IL-10, and IL-13)[36,37]. We found that IL-10 gene expression (Treg) negatively regulates the differentiation of lymphocytes into cells with Th2 profile in *Ganoderma lucidum* group.

We found that post-supplementation FOXP3, TGF-β, and IL-10 mRNA expression were upregulated in the *Ganoderma lucidum* group compared to that in the Placebo. Note worthily, the Th1 cell percentage was reduced in *Ganoderma lucidum* group. This suggests that the increased IL-10 expression negatively regulates IFN-ɣ secretion.

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Increase in TGF-β expression, and concomitant low IL-6 expression, favors the acquisition of Treg profile by lymphocytes. The FOXP3 transcription factor expression is subsequently increased[35]. TGF-β expression is related to inhibition of T effector cell proliferation and to Treg cell development. In addition, Treg also act by secreting the cytokines IL-10 and IL-35.

Importantly, post-supplementation IL-10/TNF-α expression ratio was higher in lymphocytes of *Ganoderma lucidum* group compared to that in lymphocytes of Placebo group. The IL-10/TNF-α and IL-10/IL-17 ratios provide valuable insights into the balance of immune regulatory and inflammatory responses. For instance, IL-10/TNF-α ratio provides insights into the balance between anti-inflammatory and proinflammatory signals. A higher ratio indicates a more predominant anti-inflammatory response, suggesting existence of a regulatory mechanism that controls excessive inflammation. Monitoring these ratios can be useful in assessing the effectiveness of anti-inflammatory treatments. For example, in conditions where TNF-α inhibitors are used, an increase in the IL-10/TNF-α ratio might indicate a therapeutic response.

Here, *Ganoderma lucidum* extract supplementation did not affect expression of IL-35, a cytokine known for its inhibitory properties. However, our data strongly indicate that *Ganoderma lucidum* extract regulates TGF-β and IL-10 gene expressions. Noteworthily, studies investigating the influence of *Ganoderma lucidum* compounds on humans, especially its effects on Treg and their associated transcription factors are lacking.

Notably, Hsu *et al.*[34] showed that the isolated LZ-8 protein from *Ganoderma lucidum* (>98% pure) expands Treg population in both, in vivo (BALB animal model) and in vitro (Jurkat cells) experiments. Agreeingly, our results indicate that supplementing food with *Ganoderma lucidum* extract (2,000 mg/day) containing 52.83% β-glucan content may induce lymphocyte to differentiate into Treg.

The increased expression of TNF-α, IFN-γ, and T-bet, observed in our study, suggests a positive correlation between the *Ganoderma lucidum* stimulus and the functioning of Th1 cells. Intriguingly, our data indicate that, despite the increased expression of specific genes associated with Th1 cells, the percentage of Th1 cells in the *Ganoderma lucidum* group was reduced compared to that in Placebo group.

Furthermore, the increased expression of IL-10 and TGF-β genes, in our study, indicate that, these proteins negatively regulate the secretion of Th1 profile cytokines. This implies that, despite the upregulation of specific genes, the overall impact on Th1 cell secretion may be tempered by the inhibitory influence of IL-10 and TGF-β. Notably, Meng *et*

al.[11] demonstrated that the TGF-β reduction negatively controls the cardiac fibrosis activity, highlighting the pleiotropic activity of this growth factor. These insights contribute to the idea that *Ganoderma lucidum* influences a complex regulatory network that affect Th1 cell function.

Besides polysaccharides, *Ganoderma lucidum* contains numerous bioactive molecules, such as ganoderic acids. Specifically, ganoderic acid A, has demonstrated efficacy in inhibiting STAT3 phosphorylation at a concentration of 60 μM. STAT3 phosphorylation plays a pivotal role in cytokine secretion, influencing the delicate balance between Th1 and Th2 responses[38]. The ganoderic acid A inhibition of STAT3 phosphorylation may be correlated with the observed alteration in cytokine secretion, suggesting a potential role for *Ganoderma lucidum* in modulating the Th1/Th2 balance.

It is plausible that ganoderic acids present in the *Ganoderma lucidum* extract contributed to the increased gene expression of Treg lymphocyte markers, including IL-10, FOXP3, and TGF-β, as indicated by our findings. Thus, our study sheds light on the molecular mechanisms through which *Ganoderma lucidum*, specifically its ganoderic acid content, may exert immunomodulatory effects, potentially influencing Treg activity and maintaining immune homeostasis.

Here, we employed key mitogens, Ionomycin and PMA, to evaluate lymphocyte activity. The percentage of T lymphocytes exhibiting a Th1 profile in response to PMA and Ionomycin stimulation, were not significantly different between the pre- and post-supplemented *Ganoderma lucidum* groups and among the post-supplemented groups. Intriguingly, however, the post-supplementation mean fluorescence intensity for IFN-γ (in Th1 lymphocytes stimulated by Ionomycin and PMA) in the *Ganoderma lucidum* group, was significantly lower compared to that in the Placebo group.

Our observations, indicate that, when exposed to these two stimuli (Ionomycin and PMA), *Ganoderma lucidum* supplementation induces a decrease in the function of Th1 cells, specifically in terms of IFN-γ production. Noteworthily, this reduction in Th1 function did not correspond with a decrease in the percentage of Th1 cells. Therefore, it appears that *Ganoderma lucidum* might suppress the activity of Th1 cells, specifically in the context of IFN-γ secretion, without altering the overall proportion of these cells.

Upon stimulation with Ionomycin and PMA, the lymphocytes from the *Ganoderma lucidum* group registered an increase in the percentage of Th2 profile cells, compared to those from the Placebo group. However, the *Ganoderma lucidum* group

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displayed a reduced mean fluorescence intensity of IL-4 expression in CD4$^+$IL-4$^+$ cells compared to the Placebo group. Thus, in the presence of mitogens, in addition to increasing the percentage of Th2 cells, *Ganoderma lucidum* may suppress in Th2 cell activity, rendering them less active.

Interestingly, and consistent with other studies, here, we found that *Ganoderma lucidum* does not significantly influence IL-4, a key cytokine associated with the Th2 profile. This nuanced impact on Th2 activity underscores the complex and context-dependent nature of *Ganoderma lucidum's* immunomodulatory effects, suggesting a differential influence on specific aspects of T-cell function within the Th2 profile[21,32,35].

The present study has a limitation related to the selection of the elderly study subjects. A noteworthy factor contributing to this limitation is the prevalent use of multiple medications within the group, including thyroxin, hypoglycemic agents, antihypertensives, lipid-lowering agents, antiplatelet agents, and antidepressants. This medication-related complexity arises due to the inherent challenges in recruiting elderly volunteers who are not on any medications, a common scenario in this age group.

Conclusion

In summary, our study demonstrates that the supplementing food with 2,000 mg/day of whole *Ganoderma lucidum* extract for eight weeks positively regulates the expression of genes associated with activating Th1, Th2, and Th17 cell profiles. Notably, this regulation is accompanied by a decrease in the percentage of Th17 cells (as illustrated in Figure 6). This observed modulation likely occurs through the upregulation of genes associated with regulatory T lymphocytes, including FOXP3, IL-10, and TGFβ.

The shift toward a Th2 profile, as indicated by the increased cellular percentage of IL4$^+$ cells in the presence of Ionomycin and PMA, suggests a predominance of an anti-inflammatory response. Noteworthily, *Ganoderma lucidum* did not activate lymphocyte proliferation through the CD28$^+$ expression pathway in the presence of concanavalin A. This may imply that *Ganoderma lucidum* exerts its modulation through another co-stimulatory molecule, as depicted in Figure 6.

In future investigations, it will be crucial to explore the influence of *Ganoderma lucidum* on T lymphocyte proliferation signaling pathways, including the examination of CD40L protein, STATS (1,3, and 5), NFκB, and CD28$^+$ expression. We recommend conducting studies with extended the supplementation periods (beyond eight weeks) to comprehensively understand the temporal dynamics and sustained effects of *Ganoderma*

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lucidum on these signaling pathways.

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Conflict of Interest

The authors declare that they have no conflicts of interests.

Author contributions

Patricia Nancy Iser-Bem (Professor), contributed with investigation, methodology, and original draft. Alecrim, A.L (Ph.D.) and Lobato, T.B, (Ph.D. student) contributed to the investigation, analysis of the real-time PCR, and editing. Santos-Oliveira, L.C (Ph.D.) and Diniz, V.L.S (Ph.D. student) contributed with data curation of the lymphocyte profile and CBA analysis. Passos, M.E, Professor, helped with methodology and biochemical analysis. Manoel, R.; Correa, I.S.; Poma, S.O; de Almeida, M.M.; Dias, B.B. (Ph.D. students) and Gritte, R.B; (Professor), Silva, E.B (Ph.D.), contributed with blood collection and treatment of samples. Levada-Pires, A.C.; Masi, L.N.; Hatanaka, E.; Hirabara, S.M.; Pithon-Curi, T.C.; (researchers), contributed with financial support and supervision. Fabi, J.P (researcher) helped with the analysis of β-glucans and HPSEC-RID, data curation, funding acquisition and editing. Curi, R.; Gorjao, R.(researchers) contributed with investigation, funding acquisition, data curation, review, and editing.

Abbreviations: LZ-8: Ling Zhi-8, Th: T helper, Treg: regulatory T cells, CD: cluster of differentiation, STAT: signal transducers and activators of transcription, ConA: Concanavalin A, PMA: phorbol myristate acetate, DAG: diacylglycerol, NFAT: nuclear factor of activated t-cells, TCR: T cell receptor, MAPK: mitogen-activated protein kinase, NFκB: nuclear factor kappa B, IL: Interleukin, TGF-β: transforming growth factor beta, GATA-3: GATA-binding protein 3, T-bet: T-box transcription factor, FOXP3: Forkhead box P3 , RORɣ: retinoic acid receptor-related orphan receptor gamma, β2M: Beta-2 microglobulin, IFN-γ: Interferon

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gamma, TNF-α: tumor necrosis factor alpha, GLP: *Ganoderma lucidum* polysaccharides, NLRP3: NOD-like receptor family, pyrin domain containing 3, HIV: Human Immunodeficiency Virus, EDTA: Ethylenediaminetetraacetic acid, HDL cholesterol: High-density lipoprotein cholesterol, LDL cholesterol: Low-density lipoprotein cholesterol, AST: Aspartate aminotransferase, GGT: Gamma-glutamyl transferase, ALT: Alanine aminotransferase.

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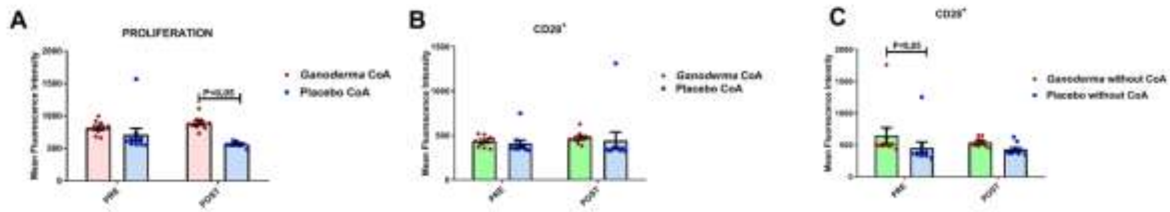

Figure 1. Effect of *Ganoderma lucidum* on the Proliferative capacity of lymphocytes stimulated in vitro with Concanavalin A and expression of CD28^{+} on the surface of human lymphocytes. The percentage of CD28^{+} cells and the proliferative capacity of stimulated lymphocytes in vitro were evaluated before (Pre) and after two months of supplementation with *Ganoderma lucidum* crude extract or Placebo. For evaluating the expression of CD28^{+}, the lymphocytes were incubated with anti-human CD28-PercP-Cy5 antibody (1:20). The analysis was performed by the percentage of CD4^{+} cells, positive for CD28^{+}, using the *"BD C-Sampler Software*." For the proliferation assay, lymphocytes were incubated with BRDU for 60 hours, in the presence of Concanavalin A (ConA). Subsequently, the anti-BRDU antibody staining with APC was performed and evaluated by mean fluorescence intensity using the "*BD-Accuri C6 Sampler Software*" (Values are presented as mean ± SEM; n = 10 for the *Ganoderma lucidum* group and for the placebo group. PRE = before supplementation; POST = after 8 weeks of supplementation).

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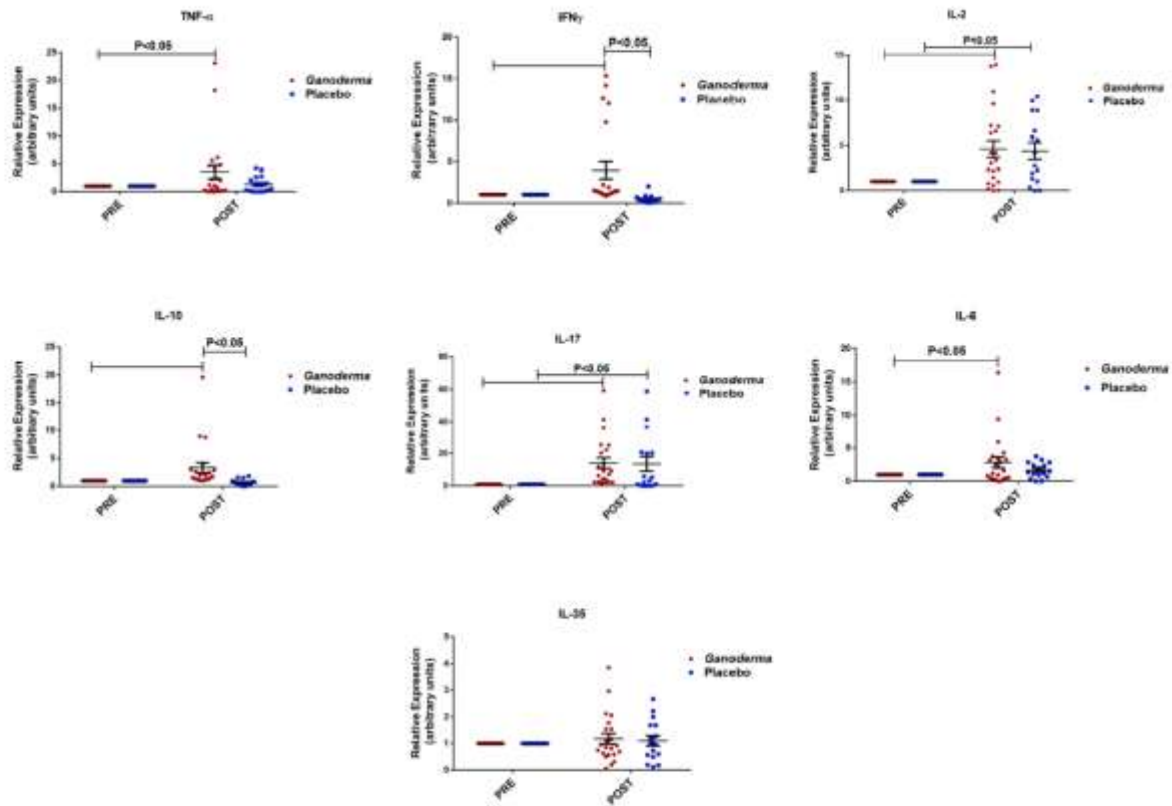

Figure 2. Evaluation of TNF-α, IFN-ɣ, IL-2, IL-17, IL-6, and IL-35 mRNA expression in lymphocytes. The analysis of IL-2, IL-6, IL-10, and IL-35 mRNA expression was performed using real-time PCR ($2^{-\Delta\Delta CT}$ analysis). The values were expressed before (Pre) and after (Post) eight weeks of supplementation for Placebo and *Ganoderma lucidum* groups. *β2M* gene was used as an internal control to normalize the results (Values are presented as mean ± SEM; *n* = 22 for the *Ganoderma lucidum* group and *n* = 16 for the Placebo group. PRE = before supplementation; POST = after 8 weeks of supplementation).

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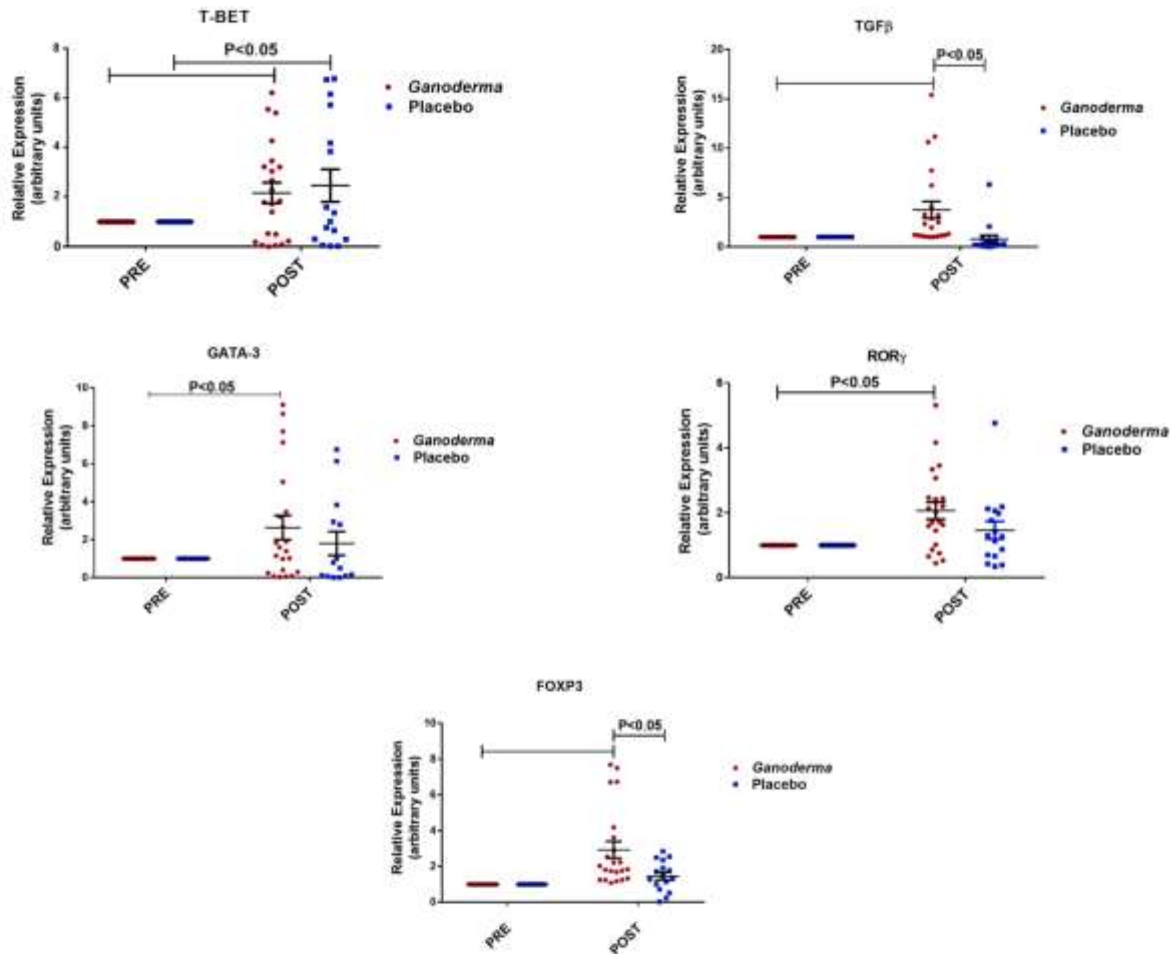

Figure 3. Evaluation of T-bet, TGFβ, GATA-3, RORɣ, and FOXP3 mRNA expression in lymphocytes. The analysis of mRNA expression was performed by real-time PCR by using $2^{-\Delta\Delta CT}$ analysis. The values were expressed before (Pre) and after (Post) eight weeks of supplementation for Placebo and *Ganoderma lucidum* groups. *β2M* gene was used as an internal control to normalize the results [Values are presented as mean ± SEM; *n* = 22 for the *Ganoderma lucidum* group and *n* = 16 for the Placebo group; *n* = 22 for the *Ganoderma lucidum* group and *n* = 14 for the Placebo group (GATA-3). PRE= before supplementation; POST=after 8 weeks of supplementation]

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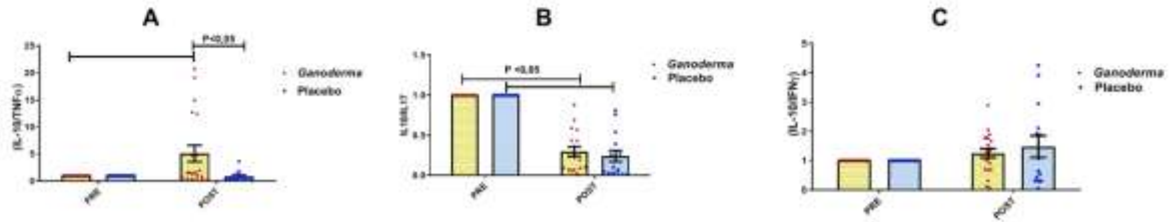

Figure 4. IL-10/TNF-α (A); IL-10/IL-17 (B); AND IL-10/ IFN-ɣ (C); AND mRNA expression ratios in lymphocytes. The results were obtained before (Pre) and after (Post) eight weeks of supplementation for the placebo and *Ganoderma lucidum* groups (Values are presented as mean ± SEM and the exclusion performed by the Rout method. For IL-10/TNF-α analysis, n = 20 for the *Ganoderma lucidum* group and n = 12 for the placebo group. For IL-10/ IFN-ɣ analysis, n = 21 for the *Ganoderma lucidum* group and n = 14 for the placebo group. For IL-10/IL-17 analysis, n = 18 for the *Ganoderma lucidum* group and n = 15 for the placebo group. PRE = before supplementation; POST = after 8 weeks of supplementation).

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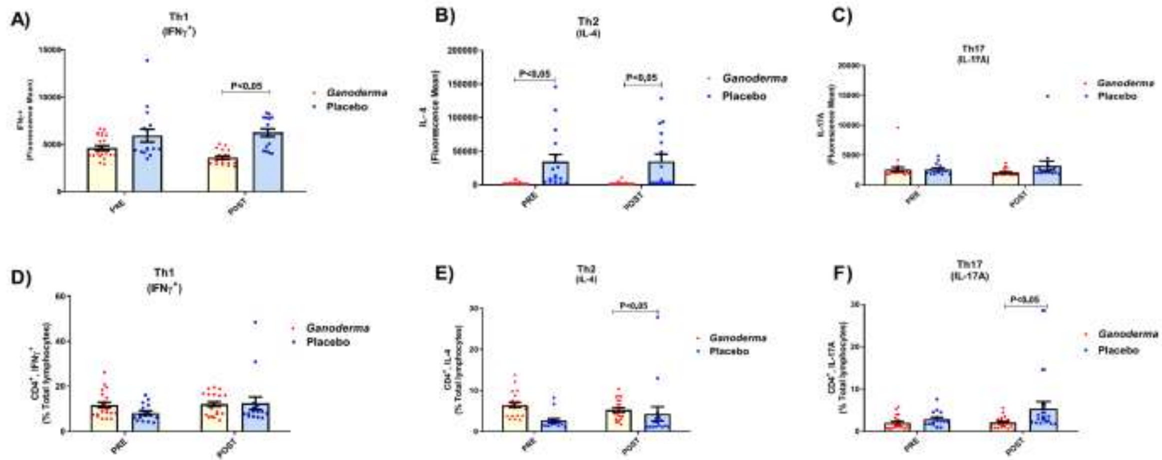

Figure 5. Cytokine expression in lymphocyte and percentage of Th1 (CD4$^+$, IFNƔ $^+$), Th2 (CD4$^+$, IL-4$^+$), and Th17 (CD4$^+$, IL-17$^+$) cells. Lymphocytes were stimulated with PMA and ionomycin in both analyses, before and after *Ganoderma lucidum and* placebo supplementation. The mean fluorescence intensity for IFN-ɣ (Th1) (A); IL-4 (Th2) (B); and IL-17 (Th17) (C); positive cells and the percentage of Th1 (D); Th2 (E); and Th17 (F); cells in lymphocytes stimulated with PMA (PMA 5 µg / mL) and Ionomycin (1 µg / mL) were evaluated before (Pre) and after (Post) eight weeks of supplementation with Placebo and *Ganoderma lucidum* extract (Values are presented as mean ± SEM; *n* = 20 for the *Ganoderma lucidum* group and *n* = 16 for the placebo group. PRE = before supplementation; POST = after 8 weeks of supplementation).

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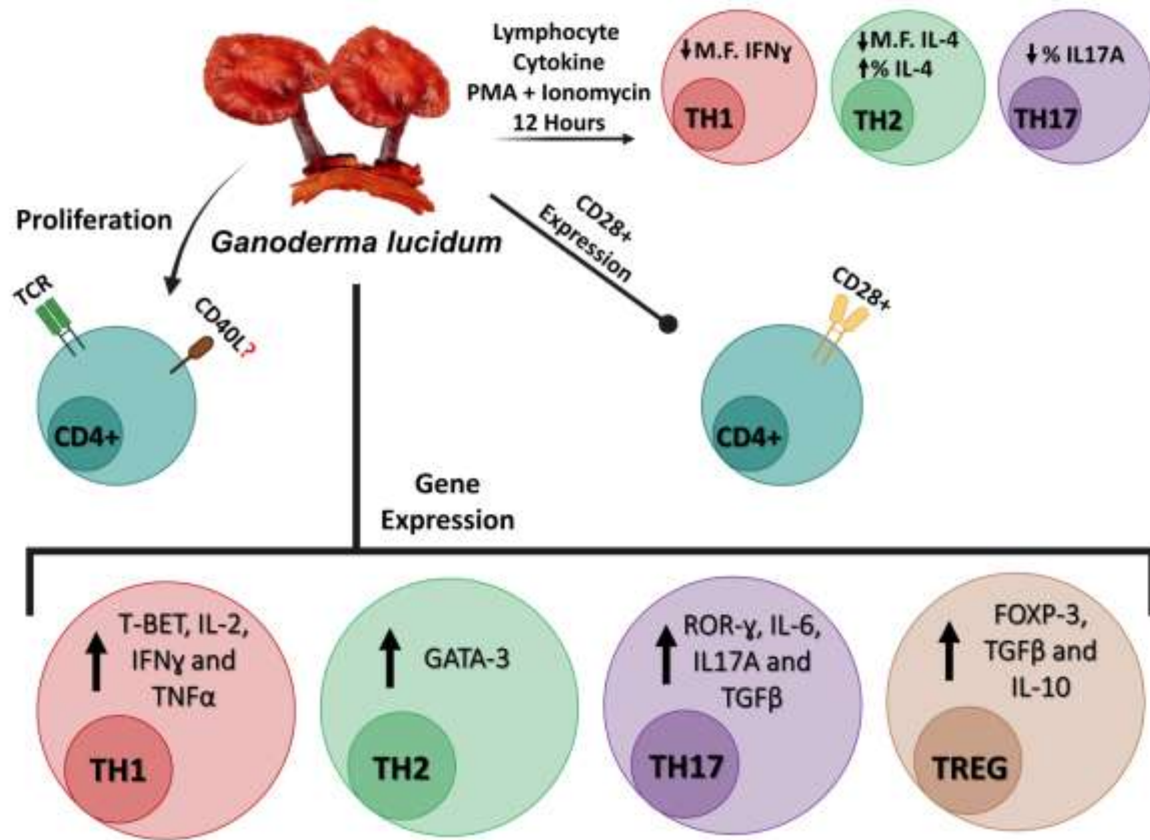

Figure 6. Summary of *Ganoderma lucidum* modulation of T helper lymphocytes. *Ganoderma lucidum* can regulate STATs and NFκB, leading to increased expression of Treg lymphocytes (IL-10, FoxP3 and TGFβ). In the presence of ionomycin and PMA (12-hour culture), it promoted a reduction in the percentage of $Th17^+$ and an increase in $Th2^+$ cells, with a reduction in the mean fluorescence intensity for IFN-ɣ and IL-4. *Ganoderma lucidum* also stimulates the proliferative capacity in the presence of ConA (60 h culture), but without influencing the $CD28^+$ expression (PRE = before supplementation; POST = after 8 weeks of supplementation).

Table 1 - Characterization of the study volunteers

Group	*Ganoderma lucidum*	*Ganoderma lucidum*	Placebo	Placebo
	Before	After	Before	After
Data	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Age (years)	68.0 ± 5.8	68.2 ± 5.8	66.4 ± 5.9	66.4 ± 5.9
Weight (Kg)	62.5 ± 13.2	61.6± 11.6	64.1 ± 14.7	64.8 ± 14.4
Height (cm)	154.1 ± 5.3	152.6 ± 4.8	155.2 ± 5.4	154.9 ± 5.4
Body mass index (Kg/m^2)	26.4 ± 5.5	26.6 ± 5.5	26.5 ± 4.9	26.8 ± 4.6
Body fat (%)	37.1 ± 8.0	36.4 ± 4.5	36.4 ± 6.2	36.1 ± 6.0
Lean mass (Kg)	39.8 ± 5.8	39.1 ± 6.0	40.9 ± 8.0	41.4 ± 9.1
Waist circumference (cm)	85.8 ± 12.1	84.9 ± 11.9	87.4 ± 12.8	86.0 ± 13.1
Hip circumference (cm)	98.3 ± 10.2	94.4± 14.8	96.8 ± 9.7	94.3 ± 12.0
Waist-to-hip ratio	0.9 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.1

SD: Standard deviation

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