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Original Research Article

Anti-inflammatory activity of *Agaricus blazei* Murill extract in the spleen of mice fed a high-fat diet

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Abstract

Purpose: *To examine the effects of A. blazei Murill on the production of proinflammatory cytokine TNF-α and regulatory cytokine IL-10 in the spleen of mice fed a high-fat diet.*
Methods: *The study was conducted on 25 BALB/c male mice divided into five groups consisting of five mice in each group and utilized three A. blazei Murill extract doses: 100, 200 and 400 mg/kg. The mice were fed a high-fat diet (HFD) and given A. blazei Murill extract for 12 weeks. The relative number of $CD4^+TNF\text{-}\alpha^+$, $CD11b/c^+TNF\text{-}\alpha^+$, and $CD4^+CD25^+IL\text{-}10^+$ were measured using flow cytometry.*
Results: *Oral administration of A. blazei Murill extract (100 mg/kg) in mice fed a high-fat diet significantly decreased ($p < 0.05$) the level of TNF-α that produced by $CD4^+$ T cells and macrophages (1.64 %), compared with control. The 200 mg/kg dose decreased the level of $CD11b/c^+TNF\text{-}\alpha^+$ by 5.37 % ($p < 0.05$) compared to 100 mg/kg dose. The 400 mg/kg dose significantly enhanced regulatory cytokines IL-10 by 17.56 % ($p < 0.05$) in mice fed a high-fat diet.*
Conclusion: *This findings suggest that Agaricus blazei Murill can inhibits processes in HFD-induced mice by reducing TNF-α production and increasing the anti-inflammatory cytokine IL-10. These results provide new insight into the pharmacological actions of Agaricus blazei Murill as a medicinal food for potential therapy of atherosclerosis disease.*

Keywords: *Anti-inflammatory agent, Agaricus blazei Murill, Proinflammatory cytokine, TNF-α, IL-10*

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INTRODUCTION

Atherosclerosis is one of the factors that causes mortality arising from cardiovascular disease [1,2]. Atherosclerosis is a chronic inflammatory disease characterized by the formation of foam cells in initial atherosclerotic lesions which then become plaques. Low-density lipoprotein (LDL) in the intima of arteries is modified into oxidized LDL (OxLDL). The presence of OxLDL triggers an inflammatory response in endothelial cells, and then causes the release of several pro-

inflammatory cytokines, chemokines, and expression of adhesion molecules [2]. Pro-inflammatory molecules guide monocytes in the blood stream to the OxLDL site in the intima of arterial walls and lead in the development of atherosclerosis [3,4]. IFN-γ is one of the major pro-inflammatory cytokine that present in the atherosclerosis progression as it is capable of inducing the expression of genes expressed in macrophages [5]. One new potential therapeutic target is IFN-γ due to its key roles in atherosclerosis development. There are two strategies that have been developed that either target IFN-γ directly or inhibit its signaling pathways [6-8].

Targeting either IFN-γ directly or its signaling pathways in both *in vitro* and *in vivo* studies has shown that directed therapies have the potential of reducing atherosclerosis development [9]. TNF-α is also involved in the pathogenesis and progression of atherosclerosis [11,12]. In the later stages of atherosclerosis, vascular remodeling is accelerated by TNFα; wall thickness of the ascending aortic arch is increased at 11 weeks of age in ApoE KO mice, but only at 26 weeks in TNFα/ApoE double KO mice [12]. Mice deficient for both TNFα and ApoE exhibit a reduction of atherosclerotic lesion size [13,14]. Thus, there is evidence from experimental and clinical studies that IFN-γ and TNFα contributes to the development of early atherosclerosis [14].

Due to the increase of pro-inflammatory molecules in atherosclerosis disease, thus it need anti-inflammatory agent to reduce the progression of atherosclerosis. The traditional mushroom *Agaricus blazei* Murill has raised scientific interest because of evidence of its immunomodulatory effects. Here, we investigated the anti-inflammatory effect of *A. blazei* Murill extract in mice fed a high-fat diet.

EXPERIMENTAL

Mice

Normal Balb/c mice (7 - 8 weeks old) were obtained from Gadjah Mada University, Yogyakarta, Indonesia. The mice were maintained in a pathogen-free facility. The experimental protocol received ethical clearance from Research Ethics Committee, Medicinal Faculty, Brawijaya University (no. 335/EC/KEPK-S3/09/2016). All animal experiments were performed according to the Principles of Laboratory Animal Care (NIH publication No. 85-23) [32].

Experimental design

The mushroom extract was produced by Agaricus Sido Makmur Sentosa (ASIMAS) Ltd., Lawang, Indonesia. Determination of *A. blazei* Murill extract doses were based on the previous study of 200 mg/kg [33]. *A. blazei* Murill extract was administered in mice by oral gavage once a day for 12 weeks. Random experiments were conducted with 5 replications in 5 groups: the control group, the high fat diet group, the high fat diet group followed by *Agaricus blazei* murill extracts administration at 3 different doses: 100, 200, and 400 mg/kg. All mice were first fed with standard diets for 1 week for acclimatization before the study. For control group, the mice were fed with a standard diet consisting of PARS (water, protein, fat, fiber, ash, CA, phosphor, antibiotic, coccidiostat) 66.6 % and wheat flour 33.4 %. For high-fat diet (HFD) treatments, the mice were orally injected with 10 g of high-fat diet (HFD) fed once a day for 12 weeks. HFD feed was consisting of a standard diet (PARS 66.6 % and wheat flour 33.4 %) and HFD feed {duck yolk (5 %), goat fat (10 %), coconut oil (1 %), cholic acid 0.03 g and pig oil 1.07 g).

Lymphocyte isolation and flow cytometry analysis

The mice were sectioned on week 12. The mice spleen were isolated and separated by gentle pipetting. The spleen suspension in propylene was added with Phosphate-buffered saline (PBS) up to 10 mL and then centrifuged at 1500 rpm, 4 ^{o}C for 5 min. The supernatant was removed and the pellet was resuspended with 1 mL of sterile PBS, which was co-incubated with monoclonal antibodies: FITC-conjugated rat anti-mouse CD4, phycoerythrin (PE) anti-mouse CD-25 and PE-Cy5 conjugated anti-mouse CD11b for 15 min. Antibodies for intracellular staining was PE/Cy5 anti-mouse TNF-α and PE/Cy5 anti-mouse IL-10. Antibodies were purchased from BioLegend, Inc (San Diego, CA). For intracellular staining, 50 µL cytofix-cytosperm was added to the pellet and incubated for 20 min at 4 °C. Then 500 µL washperm was added and centrifuged at 2500 rpm at 4 °C, for 5 min. The pellet was resuspended using 50 µL of antibodies in sterile PBS. Next, the pellet was re-suspended in 500 µL PBS and accessed via a BD FACS Calibur™ flow cytometer (BD Biosciences, San Jose, CA, USA). The data was then processed using the BD Cell Quest Pro™ software.

Statistical analysis

Data were analyzed using SPPS 16.0 for Windows. One-way ANOVA was used to assess

the statistical difference between the treatments. $P < 0.05$ was defined as statistically significant. Significant treatment effect was further analyzed for between-treatment differences with Tukey HSD Test.

RESULTS

Effect of *A. blazei* on the level of TNF-α production by $CD4^+$ T cells

Pro-inflammatory cytokine play a role in various chronic inflammatory disease such as atherosclerosis. This study showed that consumption of high fat promoted the production of TNF-α in mice. The relative number of TNF-α by $CD4^+$ T cells in the normal mice group was significantly higher ($p < 0.05$) after high-fat diet treatment (5.64 % vs 9.23 % (Figure 1 a).

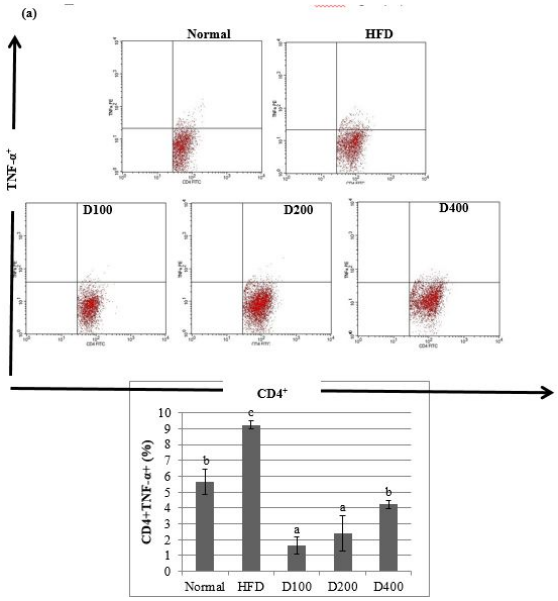

Figure 1: The effects of *A. blazei* extract TNF-α production in mice fed a high-fat diet (HFD). (a) Relative number of TNF-α ($CD4^+TNF\text{-}\alpha^+$) after 12 weeks of *A. blazei* treatment. (b) $CD4^+TNF\text{-}\alpha^+$ after administration of *A. blazei.* Data are mean ± SD (n = 5). Normal: control mice (non-HFD mice); HFD: High-Fat Diet (HFD) mice; D100: HFD mice accompanied by oral gavage of low dose of *A. blazei* extract (100 mg/kg), D200: HFD mice accompanied by oral gavage of normal dose of *A. blazei* extract (200 mg/kg), D400: HFD mice accompanied by oral gavage of high dose of *A. blazei* extract (400 mg/kg).

The overproduction of TNF-α in mice fed a high-fat diet is caused by the accumulation of oxidized LDL in arteries which triggers the immune cells such as machrophages and dendritic cells. The triggered immune cells produce proinflammatory cytokines such as IL-6, TNF-α and IFN-γ, indicators of inflammatory response. From Fig 1b, *A. blazei* extract was able to reduce the relative number of this cytokines significantly compared to the normal group. The relative number of $CD4^+TNF\text{-}\alpha^+$ was decreased significantly ($p < 0.05$) in *Agaricus blazei* Murill D1 (1.64 %) and D2 (2.40 %). In D3 treatment of *A. blazei* Murill extract, the number of TNF-α was similar to the normal mice. This study have revealed that all doses of this mushroom extract were effective to reduce the inflammatory cytokine in mice fed a high-fat diet.

Effect of *A. blazei* extract on the level of TNF-α production by macrophages

High consumption of fat was induced the production of pro-inflammatory cytokines TNF-α in mice. The relative number of TNF-α produced by macrophages was raised until 10.21 % compared to normal mice (6.90 %). This finding suggests that the overproduction of TNF-α have been implicated in the initiation and progression of atherosclerosis mice model.

Highly specific anti-inflammatory agents can precisely block the activity of cytokines generated during the inflammatory progression in atherosclerosis mice model. In this study, the extract of *Agaricus blazei* Murill that have the potential activity as an inflammatory agent in high fat diet-induced mice were used. Administration of this mushroom extract was significantly blocked the TNF-α production in HFD-mice (Figure 2 b). The relative number of TNF-α was significantly decrease in D200 (200 mg/kg) of *Agaricus blazei* Murill (5.37 %) (Fig 2a) compared with other dose (D100: 12.51 %; D400: 13.91 %). This results suggests that dose of 200 mg/kg was optimal dose to decrease the production of TNF-α produced by macrophages.

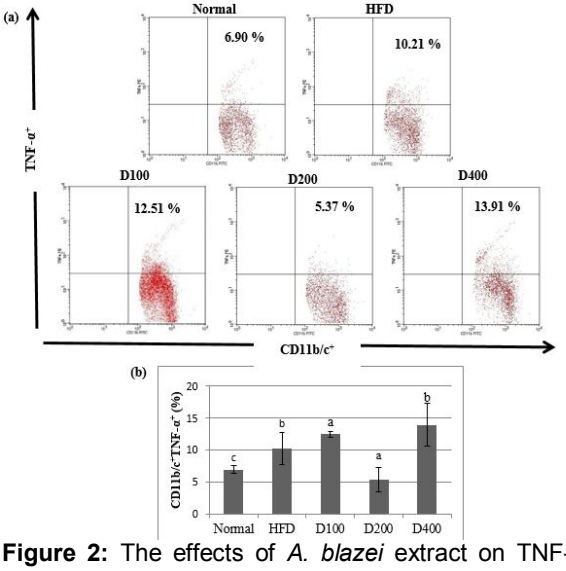

Figure 2: The effects of *A. blazei* extract on TNF-α production by macrophages in mice fed a high-fat diet (HFD). (a). The relative number of TNF-α

(CD11b/c$^+$TNF-α$^+$) in mice fed a high-fat diet after 12 weeks of *A. blazei* treatment. (b). The percentage of CD11b/c$^+$TNF-α$^+$ after administration of *A. blazei* extract in mice fed a high-fat diet. Data are mean ± SD values of 5 mice in each group. Normal: control mice (non-HFD mice); HFD: High-Fat Diet (HFD) mice; D100: HFD mice accompanied by oral gavage of low dose of *A. blazei* extract (100 mg/kg), D200: HFD mice accompanied by oral gavage of normal dose of *A. blazei* extract (200 mg/kg), D400: HFD mice accompanied by oral gavage of high dose of *A. blazei* extract (400 mg/kg)

The effects of *A. blazei* on IL-10 production

Our next step was to analyze the modulation of anti-inflammatory cytokines IL-10 production induced by *A. blazei* extract. We observed that *Agaricus blazei* Murill extract was lead the production of anti-inflammatory cytokines IL-10 in HFD-induced mice. In the Fig 2b, the relative number of CD4$^+$CD25$^+$IL-10$^+$ were significantly decreased in mice after high fat diet treatment for 12 weeks compared to normal mice (18.50 % vs 24.65 %). The decrease of atherosclerosis progression by anti-inflammatory cytokines is crucial for homeostatic. *Agaricus blazei* Murill treatment in the high dose (17.56 %) was significantly increased the production of IL-10 compared to other doses (D100: 12.38 %; D200: 13.41 %) (Fig 3a). It was shown that only a high dose was optimal dose to increase the anti-inflammatory cytokines of IL-10.

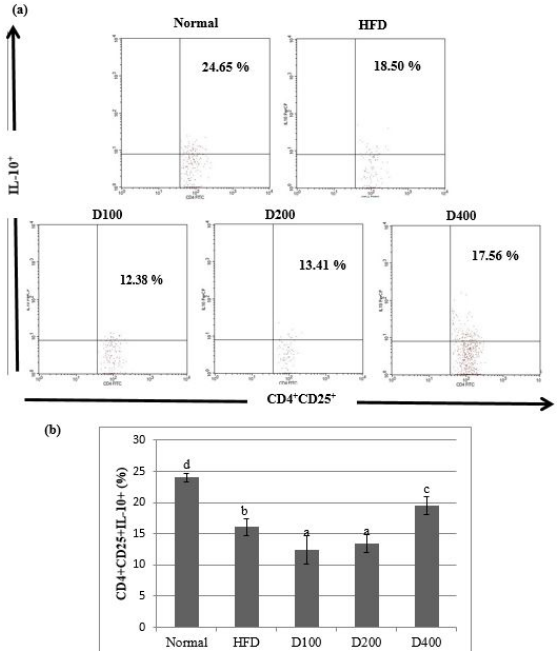

Figure 3: Effect of A. blazei extract on IL-10 production in mice fed a high-fat diet (HFD). (a) Relative number of CD4$^+$CD25$^+$IL-10$^+$ in mice after 12 weeks of *A. blazei* treatment. (b) IL-10 after administration of A. blazei extract in mice. Data are mean ± SD (n = 5). Control: normal mice (non-HFD mice); HFD: High-Fat Diet mice; D100: HFD mice accompanied by oral gavage of low dose of *A. blazei* extract (100 mg/kg), D200: HFD mice accompanied by oral gavage of normal dose of *A. blazei* extract (200 mg/kg), D400: HFD mice followed by oral gavage of high dose of *A. blazei* extract (400 mg/kg)

DISCUSSION

Inflammatory processes are elaborate in all stages of atherosclerosis disease. Cytokine-mediated pro-inflammatory responses are contributed to the atherosclerosis. Tumor Necrosis Factor-α (TNF-α) is a vital mediator of inflammatory reactions and cell death [15]. Inflammation and cell death are main processes in atherosclerotic lesion and modulated by TNF-α [16,17]. This study supports the fact that TNF-α reflects ongoing inflammation in atherosclerosis, TNF production was increased in the spleen of mice with high fat diet compared with normal mice. The overproduction of TNF-α is correlated with necrosis in atherosclerotic lesion, is in line with the role of this cytokines in modulating inflammatory process and cell death [18-20]. Macrophages and T-lymphocytes are the most prominent cells that secrete various pro- or anti-atherogenic cytokines that influence to development and plaque stability [21].

Macrophage is the most vital source of cytokine production in atherosclerotic lesions. This cell produces pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-12, IL-15, IL-18, as well as the anti-inflammatory cytokines IL-10 and TGF-β. Many reports have been well documented that pro-inflammatory cytokines can promote the development of atherosclerosis while anti-inflammatory cytokines like TGF-β and IL-10 could act as anti-atherogenic effect [22]. The central role of this cytokines makes it a promising therapeutic target for reducing the progression of atherosclerosis. Some researchers have shown that the function of pro-inflammatory cytokines during atherosclerosis development and plaque stability[11]. Anti-inflammatory agents are important for balancing the level of this cytokines in the body.

In this study, macrophages is labelled with antibody of CD11b/c. Complement receptors CR3 (also called Mac-1 and CD11b/CD18) and CR4 (also referred to as CD11c/CD18 and p150,95) are transmembrane glycoproteins that belong to the β2 integrin family. They are expressed on the surface of neutrophils, monocytes, macrophages, and NK cells [23]. Antibody of CD11b/c that used in this study appears to recognize a common epitope shared between CD11b and CD11c (integrin α_M and α_X chains). CD11b is implicated in various adhesive

interactions of monocytes, macrophages and granulocytes. Macrophages lack CD11c but possess CD11b, also known as Mac-1 [24].

Regulatory T cells are classified into two groups: naturally regulatory T cells and induced regulatory T cells. Naturally regulatory T cells are indicated by $CD4^+CD25^+$ T cells. Some studies have suggested that the effector function of $CD4^+CD25^+$ regulatory T cells is correlated with the contribution of suppressive cytokines, such as TGF-β and IL-10 [25]. T regulatory cells are the main importance for immune tolerance and malfunction of T cell population can lead to increased suppression effect. $CD4^+CD25^+$ regulatory T cells are reported to produce IL-10, TGF-β, and IL-4 to stop T cells activation [26]. IL-10 is an effective anti-inflammatory cytokine for atherosclerosis protection. IL-10 is produced mostly by the macrophages and Th2 subsets. IL-10 exerts its atheroprotective effect on plaque progression, as well as the different stages of atherosclerosis by influencing the local inflammatory process within the atherosclerotic lesion [1]. Decreasing levels of IL-10 may lead to augmented MMP activity which may in turn promote plaque instability to cause acute cardiovascular events in certain individuals [27-31]. Targeting both suppressive cytokines is important for some inflammatory disease. The findings of this study showed that IL-10 produced by T regulatory cells is important to decrease the progression of atherosclerosis mice model. The quantitative changes of IL-10 is a promising target for atherosclerosis disease therapy using *A. blazei*.

Agaricus blazei Murill is rich in β-glucans, which are a class of bioactive polysaccharides with strong immunomodulating properties, found in the cell wall of the mushroom. The β-glucans are potent stimulators of macrophages, monocytes and NK cells. The effects are mediated via lectin-binding site for β-glucan in complement receptor 3 (CR3) (CD11b/18), Toll like receptor 2 (TLR 2) and dectin-1. Macrophage contains particular membrane receptors that might bind polysaccharides and glycoprotein as Toll-like receptor 4 (TLR4), CD14, complement receptor 3 (CR3), scavenger receptor, dectin-1, and mannose receptor. The binding of these receptors activates NF-κB, which controls the expression of genes in activated macrophages [30].

Another study have reported that the therapeutic effect of *Agaricus* genus against *Leishmania amazonensis*. The treated mice produced significantly higher levels of IFN-γ and nitric oxide (NO) and lower levels of IL-4 and IL-10 in the spleen and lymph node cell cultures than controls. *Agaricus* sp. presented a 60 % reduction in the inflammation of leismaniasis infected mice. *In vitro* study reported that *Agaricus blazei* Murill stimulation was reduced the releasing of Th2 cytokine IL-4 and also found reduced IL-2 and IFN-γ levels, IL-12 and IFN-γ-mediated NK cell activation [31]. When measuring different cytokines in human serum after 12 days intake of the *Agaricus blazei* extract, there was a significant reduction in proinflammatory cytokines from Th1 and Th2 [20]. This indicates a general anti-inflammatory effect of this mushroom, which agrees with its current antheroprotective effect.

CONCLUSION

The findings of the present study suggest that *Agaricus blazei* Murill extract inhibits inflammatory processes induced by HFD in mice by reducing the production of proinflammatory cytokines (TNF-α).

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by Dicky K. Tontowiputro, Djanggan Sargowo, Askandar Tjokroprawiro, and Muhaimin Rifa'i and all liabilities pertaining to claims relating to the contents of this article will be borne by the authors. DKT and MR designed the study, DKT, DS, and AT analyzed the data, and DKT and MR revised the manuscript. All authors read and approved the manuscript for publication.

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