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ORIGINAL CONTRIBUTION

Pro-inflammatory effects of the mushroom *Agaricus blazei* and its consequences on atherosclerosis development

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Abstract
Purpose Extracts of the mushroom *Agaricus blazei* (*A. blazei*) have been described as possessing immunomodulatory and potentially cancer-protective activities. However, these effects of *A. blazei* as a functional food have not been fully investigated in vivo.
Methods Using apolipoprotein E-deficient ($ApoE^{-/-}$) mice, an experimental model of atherosclerosis, we evaluated the effects of 6 or 12 weeks of *A. blazei* supplementation on the activation of immune cells in the spleen and blood and on the development of atherosclerosis.
Results Food intake, weight gain, blood lipid profile, and glycemia were similar between the groups. To evaluate leukocyte homing and activation, mice were injected with ^{99m}Tc-radiolabeled leukocytes, which showed enhanced leukocyte migration to the spleen and heart of *A. blazei*-supplemented animals. Analysis of the spleen showed higher levels of activation of neutrophils, NKT cells, and monocytes as well as increased production of TNF-α and IFN-γ. Circulating NKT cells and monocytes were also more activated in the supplemented group. Atherosclerotic lesion areas were larger in the aorta of supplemented mice and exhibited increased numbers of macrophages and neutrophils and a thinner fibrous cap. *A. blazei*-induced transcriptional upregulation of molecules linked to macrophage activation (CD36, TLR4), neutrophil chemotaxy (CXCL1), leukocyte adhesion (VCAM-1), and plaque vulnerability (MMP9) were seen after 12 weeks of supplementation.
Conclusions This is the first in vivo study showing that the immunostimulatory effect of *A. blazei* has proatherogenic repercussions. *A. blazei* enhances local and systemic inflammation, upregulating pro-inflammatory molecules, and enhancing leukocyte homing to atherosclerosis sites without affecting the lipoprotein profile.

Keywords *A. blazei* · Diet · Atherosclerosis · Inflammation · $ApoE^{-/-}$ mice

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Background

Extracts from the mushroom *Agaricus blazei* (*A. blazei*) have been reported to be medicinal [1] and have been studied for their immunostimulatory effects [2–5]. Among these effects are the induction of nitric oxide (NO) secretion in murine macrophages [5] and transcriptional upregulation of cytokines such as IL-8, IL-6, IL1-β, IL2, and TNF-α [6, 7]. These effects can be ascribed to the high concentration of proteoglycans and β-glucans, important

bioactive agents, in *A. blazei* extracts [6]. Moreover, *A. blazei* can stimulate dendritic cell maturation and expression of co-stimulatory molecules, such as CD40, CD80, and CD86, as well as MHC-II [8, 9]. Oral administration of *A. blazei* extracts activates natural killer (NK) cells and cytotoxic T lymphocytes in BALB/c mice [10, 11]. Although this immunostimulation could be beneficial in some diseases, it could potentially accelerate tissue damage in others, such as in atherosclerosis. In atherosclerotic lesions, signals trigged by lipid deposition in the intimae of arteries perpetuate inflammation and induce oxidative stress, accelerating atherogenesis [12, 13]. Therefore, *A. blazei* supplementation, although potentially beneficial for cancer prevention, could accelerate atherosclerosis progression as a consequence of its pro-inflammatory properties. Because there are no studies evaluating the effects of dietary supplementation with the powdered dry fruiting body of *A. blazei*, we investigated the role of supplementation with *A. blazei* (5%) on spleen cell activation and especially on atherogenesis development.

Materials and methods

Macronutrient composition of *A. blazei*

Analysis of protein, lipids, fibers, and ash content of *A. blazei* was performed according to the Association of Official Analytical Chemists (AOAC) guidelines [14].

Animals and diet

ApoE-deficient mice ($ApoE^{-/-}$), a well-known model of experimental atherosclerosis, were used in this study. Animals were distributed into a *Control* group ($n = 30$) fed on a standard diet AIN-93M [15] and an *A. blazei* group ($n = 30$) fed the same diet but supplemented with 5% of the *A. blazei* powdered dry fruiting body. Mouse body weight and food intake were evaluated weekly. After 6 experimental weeks, a subgroup of animals was euthanized after 12-h fasting and anesthesia to collect blood, heart, liver, kidney, cecum, aorta, and spleen for posterior analyses. The remaining mice were kept under the same diet and euthanized under the same conditions in the 12th experimental week. The animal protocol was approved by the Animal Care Committee of the Universidade Federal de Minas Gerais (CETEA/UFMG #157/2007).

Blood, liver, and cecal samples

Hepatic and cecal lipids ($n = 6$/group) were extracted as previously described [16]. Serum triacylglycerol, total and HDL cholesterol ($n = 10$/group), and glycemia ($n = 6$/group) were determined using enzymatic kits (Doles, Brazil). The non-HDL fraction of cholesterol was calculated as the difference between total cholesterol and HDL cholesterol ($n = 10$) [17].

Lipid peroxidation measured by the thiobarbituric acid reactive substances (TBARS) analysis

Lipid peroxidation (TBARS) was measured in the liver and kidney of 5 animals as described by Wallin et al. [18]. Results are expressed from the calculation under the concentration curve of malondialdehyde (MDA) per microgram of protein measured by Lowry method [19].

Detection of inflammatory foci by ^{99m}Tc-HMPAO leukocytes

The protocol for detecting inflammatory foci in mice using labelled leukocytes was performed in 7 animals per group, as previously described [20, 21]. Briefly, hexamethylpropyleneamine oxime (HMPAO) was reconstituted using 2.0 ml of sterile saline and fractionated into four vials containing 0.5 ml each. Next, 0.5 ml of 1,480 MBq of sodium pertecnate ($Na^{99m}TcO4$) was added to each vial, resulting in *^{99m}Tc-HMPAO*. An aliquot of 100 µL of ^{99mTc}-HMPAO was used to label the leukocytes isolated from thirty C57/BL6 mice. The preparation was incubated at 37 °C for 15 min and centrifuged for 5 min at 400*g*. The supernatant was aspirated and discarded, leaving the labelled leukocyte and resuspended with PBS. A volume of 100 µl was injected intravenously at the beginning of 6th experimental week in animals from each group. The animals were killed 2 h after the injection, and the spleen, the heart, and the thoracic and abdominal aortas were removed for measurement of radioactivity using an automatic scintillation apparatus (ANSR-Abbot, USA). Recovered radioactivity was calculated from the standard radiation dose.

Histological analysis

The analysis was performed in 10 samples per group. Specimens were fixed in 10% paraformaldehyde and processed in paraffin as previously described [22, 23]. Briefly, each consecutive 10 µm thick section for 300 µm of the aortic valve length was stained with hematoxylin and eosin [24]. Collagen was visualized using Gomori trichrome stain [25]. The total lesion area was calculated as the sum of lesion areas obtained from 10 selected sections separated by 30 µm. Collagen content presented as the percentage of the lesion area stained by Gomori trichrome stain. Results were analyzed using the image analyzer Image-Pro Plus software (Mediacybernetic, USA). Aortas were

separated from the adventitia and stained with Sudan IV before measuring the lesion area, using the same image analyzer.

Determination of *N*-acetyl-beta-D-glucosaminidase (NAG) activity

Macrophage infiltration was quantified in 5 animals per group by measuring the *N*-acetyl-beta-D-glucosaminidase (NAG) activity, as previously described [26], and the absorbance was measured at 400 nm. The NAG activity was expressed as change in OD per gram of wet tissue [27].

Determination of myeloperoxidase (MPO) activity

The extent of neutrophil accumulation in the spleen and aorta was evaluated in 5 animals per group through myeloperoxidase activity, as previously described [28]. Results are expressed as the absorbance at 450 nm per 100 mg of wet tissue.

Blood count

The blood cells from 10 animals per group were diluted in Turk's solution and counted using a hemocytometer at the 6th experimental week [29].

RNA extraction and RT-PCR

The mRNA levels were estimated by quantitative real-time polymerase chain reaction (PCR) at the 12th experimental weeks in 6 aortas of each group. First, total RNA was extracted from fresh samples of the aortic root, isolated as described in the histological analysis section, using TRIzol® reagent according to the manufacturer's protocol. Reverse transcription was performed using 2 μg of total RNA, 200 U of reverse transcriptase, RT buffer 5X (2.5 μL), 10 mM dNTPs (1.8 μL), RNAsin 10,000 U (0.2 μL), and oligo dT 15 50 μM (1.0 μL). The temperature settings for this reaction were 70 °C for 5 min, ice for 2 min, and back to the thermocycler set at 42 °C for 60 min, 70 °C for 15 min, and 4 °C for the final step. The resultant cDNA was used for real-time PCR as described below. The specific primers were designed using Primer Express software and synthesized by IDT. Real-time PCR was carried out on a StepOne sequence detection system (Applied Biosystems) using *Power* SYBR Green PCR Master Mix (Applied Biosystems). The relative levels of gene expression were determined using the ΔΔ Cycle threshold method as described by the manufacturer, in which data for each sample is normalized to 18S expression and data are shown as relative expression. The sequences of the employed primers are as follows: 18S—Forward: CGT TCC ACC AAC TAA GAA CG, 18S—Reverse: CTC AAC ACG GGA AAC CTC AC; VCAM—Forward: 5′-CCC CAA GGA TCC AGA GAT TCA-3′, VCAM—Reverse: 5′-ACT TGA CCG TGA CCG GCT T-3′; iNOS—Forward: AGC ACT TTG GGT GAC CAC CAG GA, iNOS—Reverse: AGC TAA GTA TTA GAG CGG CG GCA; MMP9—Forward: GGG CCG CTC CTA CTC TGC CT, MMP9—Reverse: TCG CGT CCA CTC GGG TAG GG; CXCL1 Forward: TGT CCC CAA GTA ACG GAG AAA, CXCL1 Reverse: TGT CAG AAG CCA GCG TTC AC CD36 Forward: GTA CAG ATT TGT TCT TCC AGC CAA T CCL2 Forward: AGG AAG ATC TCA GTG CAG AG : CCL2—Reverse: AGT CTT CGG AGT TTG CCT TTG, CD36—Reverse: TCA GTG CAG AAA CAA TGG TTG TC, SRA1—Forward: GGG AGA CAG AGG GCT TAC TGG, SRA1—Reverse: TTG TCC AAA GTG AGC TCT CTT G, SRA2—Forward: GGG AGA CAG AGG GCT TAC TGG A, SRA2—Reverse: ATG TTC AGG GAG TTA TAC TGA TC, TLR4—Forward: GGC TCC TGG CTA GGA CTC TGA, TLR4—Reverse: TCT GAT CCA TGC ATT GGT AGG T.

Flow cytometry

At the 6th and 12th experimental weeks, spleen cells and circulating leukocytes were isolated from 7 mice of each group, resuspended in PBS, and incubated for 30 min with various combinations of the following fluorochrome-conjugated anti-mouse antibodies. The antibodies used were IgG FITC and IgG PE as isotype controls, anti-CD49bPan-FITC, anti-NK1.1 FITC, anti-CD4-FITC, anti-CD44-FITC, anti-CD11b-FITC, anti-CD8-PE, anti-CD19-PE, anti-CD86-PE, anti-CD3-PE, anti-CD44-PE, anti-CXCR2-PE, anti-F4/80-Cy5, anti-Ly-6G/Ly-6C (Gr-1)-Alexa 488 (Pharmingen and BioLegend, San Diego, CA). The preparations were fixed, processed using a FACScan flow cytometer, and analyzed using FlowJo®software (Tree Star, Inc.).

Cytokine assessment

TNF-α and IFN-γ concentrations were detected by ELISA in the spleen homogenate from 5 animals per group at the 6th experimental week using commercial kits (R&D Systems, Minneapolis, MN).

Statistical analysis

The Kolmogorov–Smirnov test to normal distribution and the Student's *t* test (parametric) were used to compare *AIN-93M* and *A. blazei* groups. A level of significance set at p value < 0.05 was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego,

CA, USA. Results are shown as the mean ± SEM. It should be noted that the data presented here might be limited by small sample sizes due to ethical reasons in animal experimentation.

Results

Agaricus blazei was chemically and microbiologically analyzed and did not show any contaminants, as certificated by the manufacturer (Kenkoo Cogumelo, Brazil). The macronutrient composition and their contribution to the diet are presented in Table 1.

The total food intake and final body weight were similar between the groups (Table 2). *A. blazei* supplementation did not influence glycemia, blood lipoprotein concentration, hepatic lipids and cholesterol concentration, or the cecal lipid and 3-alpha-OH sterol content (Table 2).

Oxidative stress was assessed in the kidney and liver. The products of lipid peroxidation, as assessed by TBARS, were reduced in the kidney but not in the liver of the *A. blazei* group (Table 2).

To confirm the reported immunostimulatory effects of *A. blazei*, animals were injected with Tecnecium^{99m}-labelled leukocytes on the 6th experimental week to study leukocyte homing. The results showed that leukocyte migration to the heart and aorta and spleen of *A. blazei*-supplemented animals was increased compared to control animals (Fig. 1a).

In the spleen, the immune cell populations were assessed using flow cytometry. The results did not indicate differences in the frequency or in the total numbers of B cells, $CD8^+$ T cells (data not show), macrophages (Fig. 2b), NK cells between groups (Fig. 2c), and $CD4^+$ T cells (Fig. 2e). However, phagocytic/monocytes cells (Fig. 2a), NKT cells (Fig. 2d), and activated CD4+T cells (Fig. 2f) were increased in supplemented mice. Following cell activation, the pro-inflammatory cytokines IFN-γ and TNF-α were also higher in the *A. blazei* group (Fig. 1b).

The atherosclerosic lesion area in the aortic valve was about 60% larger in the *A. blazei* group compared to the control group (Fig. 3a). Control mouse lesions were characterized by foam cell infiltration with rare areas of fibrosis and necrosis (Fig. 3c). In the *A. blazei* group, the lesion areas were larger and presented with a fibrous cap and cholesterol crystal deposition, suggesting a more advanced lesion (Fig. 3d). Deposition of collagen, a major component of the fibrous cap, was reduced by 30% in the *A. blazei* group (Fig. 3b, f) compared to control group (Fig. 3e). In agreement with these findings, the lesion area in the aorta was increased by 55% in the group receiving *A. blazei* supplementation (Fig. 4a). NAG and MPO activity, indicative of macrophage and neutrophil infiltration, respectively, was higher in the aorta of the *A. blazei* group (Fig. 4b, c).

Following the initial round of analysis, we kept the animals on their respective diets for an additional 6 weeks to observe if the pro-inflammatory and proatherogenic effects of *A. blazei* supplementation were sustained in more advanced lesions. The results showed that the lesion area was both more advanced and larger in the *A. blazei* group

Table 1 Macronutrient composition (g/100 g) of the control and powdered dry *Agaricus blazei* Murril diet given to ApoE$^{-/-}$ mice for 12 weeks

Nutrient	Control (AIN-93M) diet	*A. blazei* diet
Casein	20.0	20.0
Methionine	0.3	0.3
Sucrose	10.0	10.0
Cellulose	5.0	5.0
Soybean oil	7.0	7.0
Mineral mix	3.5	3.5
Vitamin mix	1.0	1.0
Choline bitartrate	0.2	0.2
Corn starch	53.0	48.0
Powdered dry *A. blazei* Murril	–	5.0

Composition (g/100 g) of powdered dry *A. blazei*: humidity, 9.8; lipids, 1.35; protein, 39.3; fiber, 10.3; minerals, 6.50; carbohydrate, 32.75

Table 2 Parameters in ApoE$^{-/-}$ mice fed a control (AIN93-M) or *Agaricus blazei*-supplemented diet (5%) for 6 weeks

Parameter	Control	*A. blazei*
Food intake (g/mouse/day)[a]	4.52 ± 0.19	4.64 ± 0.18
Final body weight (g)	24.4 ± 0.91	24.37 ± 0.85
Blood total cholesterol (mmol/L)	4.99 ± 0.37	5.47 ± 0.38
HDL cholesterol (mmol/L)	0.74 ± 0.07	0.63 ± 0.10
Non-HDL cholesterol (mmol/L)	4.25 ± 0.48	4.84 ± 0.49
Blood triacylglycerols (mmol/L)	1.57 ± 0.17	1.40 ± 0.44
Glycemia (mmol/L)	9.47 ± 1.62	9.21 ± 0.47
Liver lipids (mg/dL)	87.1 ± 17.2	89.6 ± 17.3
Liver cholesterol (mg/g)	17.0 ± 0.89	17.4 ± 0.40
Cecal lipids (mg/g)	54.5 ± 5.2	62.3 ± 6.6
Cecal 3-alpha-OH sterols (mg/g)	14.5 ± 0.3	14.4 ± 0.3
Liver TBARS (μmol MDA/g protein)	0.13 ± 0.01	0.16 ± 0.01
Kidney TBARS (μmol MDA/g protein)	0.77 ± 0.05	0.54 ± 0.03*

Data are mean ± SE

[a] Food intake was evaluated during the 6 experimental weeks

* Statistically different ($p < 0.05$). $N = 5$–6 per group except for food intake and body weight ($n = 30$); and blood triacylglycerols, cholesterol total, and fractions ($n = 10$)

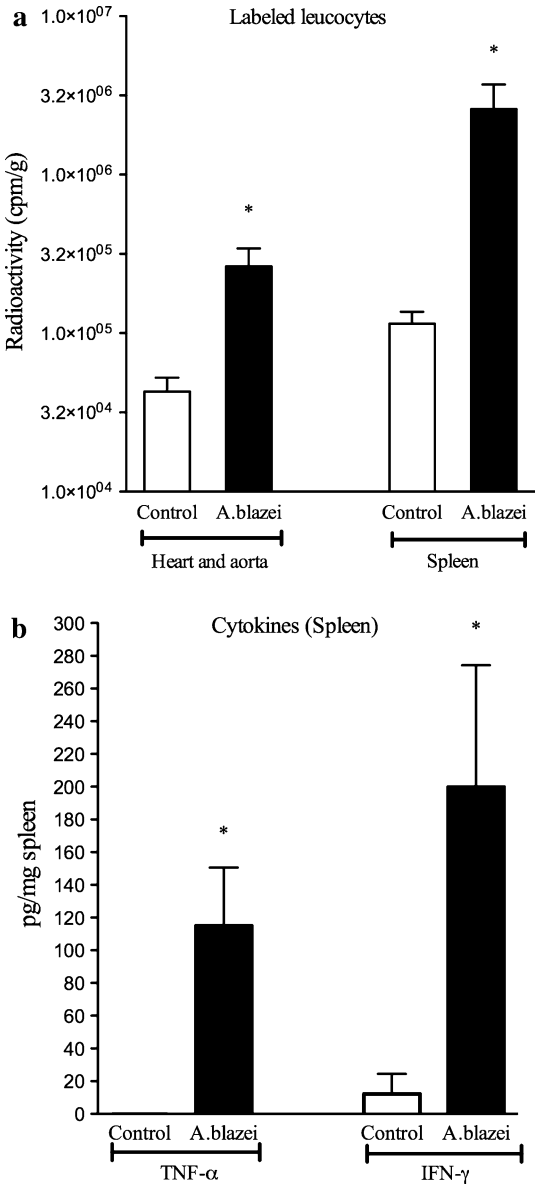

Fig. 1 Immunostimulatory effect of *A. blazei*: ^{99m}Tc-HMPAO leukocyte captation in the spleen and heart ($n = 7$) (**a**) TNF-α and IFN-γ production in spleen ($n = 5$) (**b**) of ApoE$^{-/-}$ mice fed a control diet or an *A. blazei*-rich diet (5%) for 6 weeks. *Bars* represent *average* and *vertical lines* SE. Student's *t* test $^{*}p < 0.05$

compared to the control (Fig. 3a). The frequency of blood monocytes, activated monocytes, neutrophils, and NKT cells were also increased in the blood of *A. blazei*-supplemented animals (Fig. 5a–d). Moreover, the expression of several molecules involved in atherosclerosis development such as molecules linked to macrophage activation (CD36, SRA2, TLR4), neutrophil chemotaxy (CXCL1), leukocyte adhesion (VCAM-1), and plaque vulnerability (MMP9) were also increased in the aorta of *A. blazei*-supplemented mice (Fig. 6a–d).

Discussion

Agaricus blazei is widely used in Oriental countries, both as an edible mushroom and as natural therapy for the prevention and treatment for cancer [1]. In the present study, we showed that the powdered dry fruiting body of *A. blazei*, given as an oral supplement, exerts immunostimulatory effects primarily in the spleen, activating CD4^{+} T cells, NKT cells, and phagocytes/monocytes and enhancing the production of pro-inflammatory cytokines.

Some reports have shown that *A. blazei* supplementation is beneficial for the treatment for dyslipidemia and other cardiovascular risk factors, assuming a protective role for this mushroom against atherosclerosis formation. However, to the best of our knowledge, this is the first study evaluating the effects of *A. blazei* not only on treatment for risk factors but also directly on atherosclerosis formation.

^{99m}Tc-labelled leukocyte administration has been used in clinical practice to localize sites of inflammation, such as intra-abdominal abscesses [30] and ulcerative colitis [31]. In our experiments, we found more intense ^{99m}Tc-labelled leukocyte retention in the heart and spleen of *A. blazei*-supplemented ApoE$^{-/-}$ mice, highlighting the pro-inflammatory nature of this mushroom and its possible influence on atherosclerosis formation.

In the spleen, *A. blazei*-supplemented animals exhibited higher radioactivity, associated with increases in the number or frequency of activated CD4^{+} T cells, phagocytes/monocytes, and NKT cells as well as higher levels of IFN-γ and TNF-α. In addition to the spleen, the frequency of monocytes, activated monocytes, NK/NKT cells, and neutrophils were also increased in the blood of *A. blazei*-supplemented mice. In agreement with our results, other studies showed that *A. blazei* targets cells associated with the innate immune response, such as monocytes, NK cells, and NKT cells [2–4, 10]. However, this is the first study that shows directly the effect of *A. blazei* in neutrophils activation. These effects could be mediated via proteoglycans [14] and β-glucans [14], which are potent stimulators of macrophages and polymorphonuclear cells (PMN), inducing the release of pro-inflammatory cytokines and sustaining inflammation.

The pro-inflammatory effects of *A. blazei* supplementation exacerbated atherosclerosis development, likely by enhancing both local and systemic inflammation. Other factors linked to atherogenesis, such as lipid excretion, lipid content of cholesterol, and blood lipoprotein and glycemia, were not influenced by supplementation. Because peripheral circulating monocytes circulate for 1–3 days before entering tissues, where they differentiate into mature resident macrophages, *A. blazei* could potentially activate immature monocytes and promote the Th1 response in tissue such as the aortic valve [10, 32].

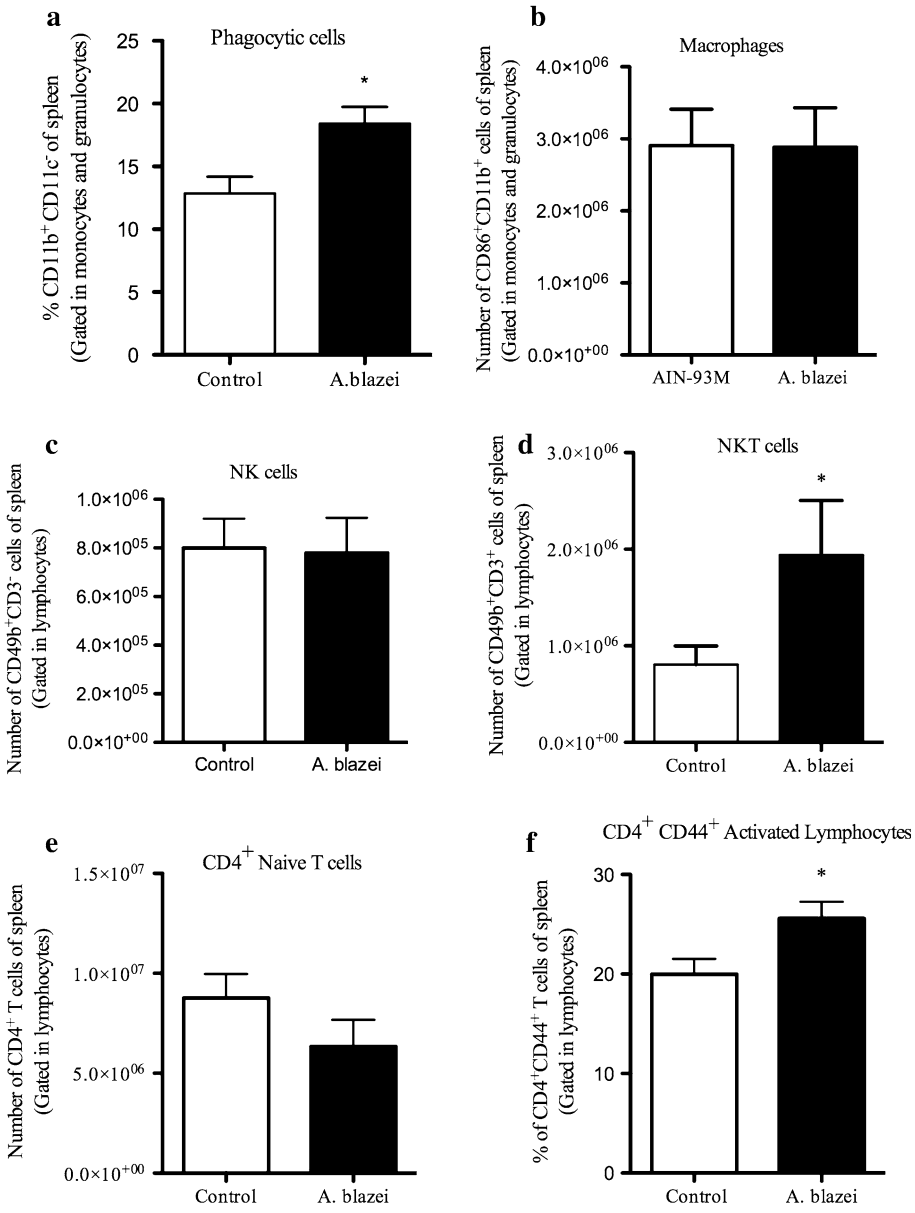

Fig. 2 Immune cell populations in the spleen: Phagocytes (**a**), Macrophages (**b**), NK cells (**c**), NKT cells (**d**), $CD4^+$ Naive T cells (**e**), $CD4^+$ $CD44^+$ high activated Lymphocytes (**f**) in the spleen of $ApoE^{-/-}$ mice fed a control diet or an *A. blazei*-rich diet (5%) for 6 weeks. Data were assessed using flow cytometry ($n = 7$). *Bars* represent the *average,* and *vertical lines* represent the SE. Student's *t* test $*p < 0.05$

Although the hypolipidemic effects of β-glucan [8] and fibers [33] isolated from Agaricus species have been previously described, we did not detect any differences in cholesterolemia and lipoprotein fractions. This finding could be due to our use of the whole fruit body instead of concentrated selected components of *A. blazei*, as were used in previous studies.

Oxidative stress is another important risk factor associated with the severity and progression of atherosclerosis, because it increases systemic levels of minimally oxidized LDL (oxLDL) and releases various pro-inflammatory cytokines and growth factors [34]. We investigated oxidative stress in two organs that are metabolically actives: the liver and kidneys. Although aqueous and alcoholic extracts of *A. blazei* have shown antioxidant activity in vitro [35], in our in vivo model, this effect was detected only in the kidney, an organ with a minor participation in atherosclerosis development. Antioxidant activity was not observed in the liver, the main organ related to lipoprotein and cholesterol metabolism. We assumed that, although an antioxidant activity could be detected after *A. blazei* intake, it is not sufficient to reduce lipid oxidation in the liver.

In our study, the *A. blazei*-supplemented group presented with larger lesion areas in the aortic valve, which were complicated by a thin fibrous cap and necrotic areas, suggesting a plaque in the advance stages compared to the intermediated lesions seen in control ones [36]. It is well known that inflammation is one of the bases of atherosclerosis [37, 38]. Because other important factors, such as lipid profile and oxidative stress, were not influenced by *A. blazei*

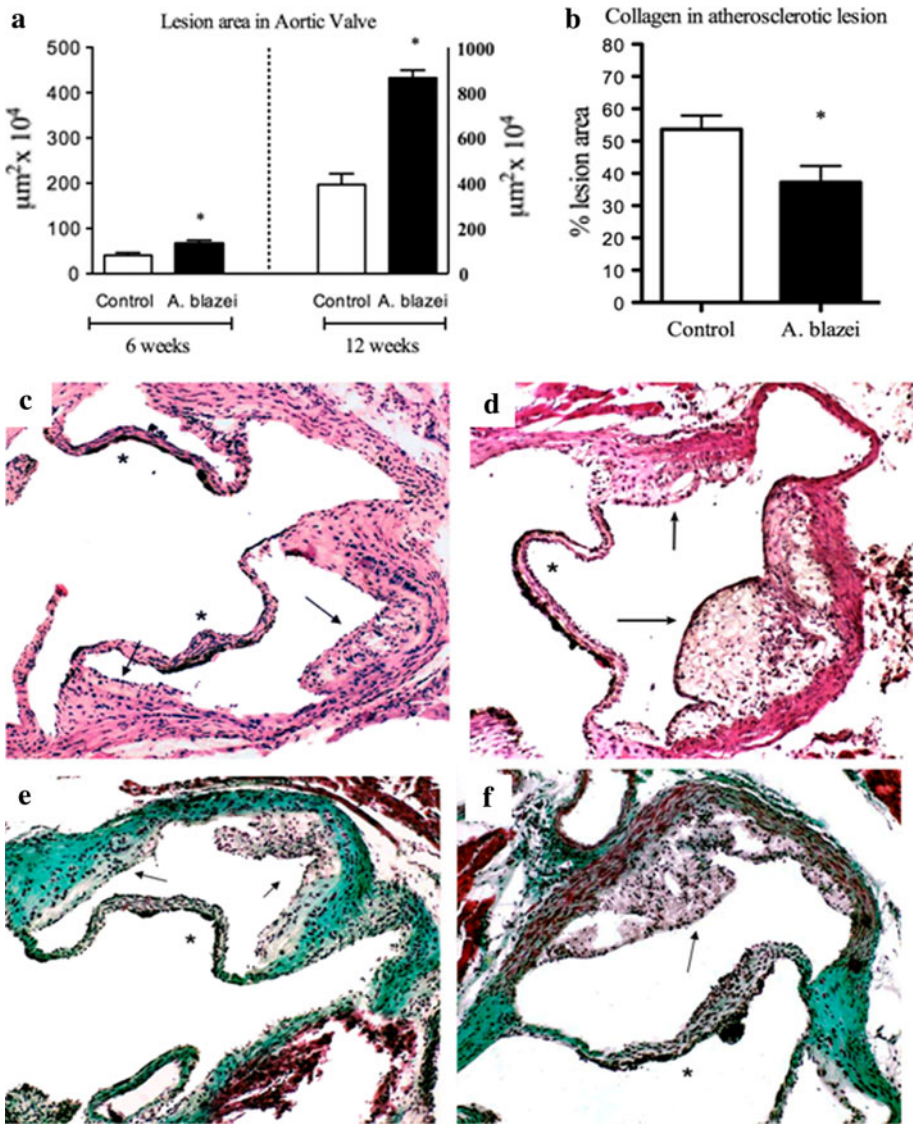

Fig. 3 Atherosclerotic lesion in the aortic valve. Lesion area in the aortic valve for 6 and 12 weeks of dietary treatment (**a**) and collagen content as percentage of total lesion area for 6 weeks of dietary treatment (**b**). Aspect of typical lesions in the aorta valve of control (**c**) and *A. blazei* group (**d**) stained with HE (6th experimental week). The lesions are more developed in *A. blazei* group, with the presence of well-defined fatty streak, constituted by xantomized macrophages. There is also the presence of a fibrous layer characterizing a more advanced stage of lesion. Aspect of collagen content of total lesion area in the aorta valve of control (**e**) and *A. blazei* group (**f**) stained with Gomori Trichrome (6th experimental week). *Arrows* indicate histological aspect (increased 100X). *Stars* indicate anatomic reference (aortic valve). *Bars* represent *average* and *vertical lines* SE. Student's *t* test $^{*}p < 0.05$, $n = 10$

intake, we suggest that the stimulation of NKT cells, neutrophils, and macrophages triggers faster atherogenesis.

The pro-inflammatory cytokines IFN-γ and TNF-α are also involved in atherogenesis. IFN-γ is known to contribute to plaque vulnerability, because it propitiates metalloproteinase-9 production, which in turn degrades the collagen content of the plaque's fibrous cap [39]. In our study, the high levels of IFN-γ in the spleen were associated with a reduction in the collagen content in aortic lesion area of *A. blazei*-treated animals and overexpression of matrix metallopeptidase 9 (MMP9) in the aortic valve, reinforcing the deleterious effects of *A. blazei* on atherosclerosis.

The involvement of TNF-α in atherosclerosis is supported by its presence in human plaques [40]. In the present study, it could be assumed that TNF-α circulating levels of *A. blazei* group are also increased, as a consequence of increased splenic production, contributing to the pro-inflammatory environment created by *A. blazei*. Furthermore, circulating TNF-α levels is associated with an increased risk of recurrent myocardial infarction [41].

Although we did not measure INF-γ levels in the blood, *A. blazei* induction of enhanced splenic cytokine production and circulating levels of monocytes (including activated monocytes) are consistent with previous studies showing *A. blazei*-induced TNF-α and IL-8 secretion in macrophages, IL-8, IL-1β, TNF-α, and IL-2 expression in monocytes and IFN-γ secretion by spleen cells [5, 11]. Altogether, these findings suggest that *A. blazei* acts by facilitating leukocyte recruitment and stimulating the immune system, an important component of atherogenesis.

Studies on inflammatory activation in atherosclerotic lesions suggest that neutrophils could contribute to the

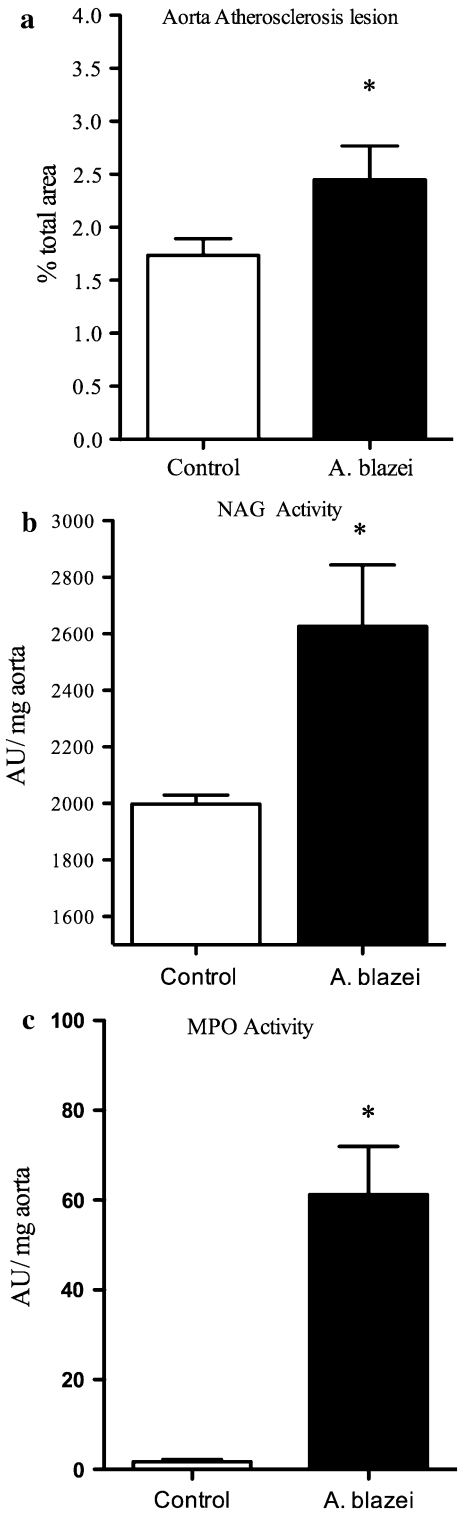

Fig. 4 Atherosclerotic lesion and inflammatory infiltration in aorta. Lesion area stained with Sudan IV in the aorta ($n = 10$) (**a**); *N*-acetyl-beta-D-glucosaminidase (*NAG*) activity ($n = 5$) (**b**) and myeloperoxidase (*MPO*) activity ($n = 5$) (**c**) in ApoE$^{-/-}$ mice fed a control diet or an *A. blazei*-rich diet (5%) for 6 weeks. *Bars* represent the *average*, and *vertical lines* represent the SE. Student's *t* test $^{*}p < 0.05$

pathogenesis of the atherosclerotic disease [42]. Neutrophils are the first leukocytes to infiltrate inflamed tissues, which also occurs during spontaneous atherogenesis, although macrophages account for the majority of leukocytes in plaques [42, 43]. The numbers of neutrophils and macrophages in the aorta were increased in *A. blazei*-treated mice, as indirectly measured by the activity of MPO and NAG, respectively. PMN-derived pro-oxidative enzymes such as MPO not only mediate acute inflammatory responses, but also catalyze reactions that consume local vascular nitric oxide, resulting in impaired endothelial function [44]. Consequently, the increased levels of MPO in the aorta are consistent with the increased in circulating neutrophils and the increased severity in atherosclerotic lesions. Moreover, higher NAG activity associated with the increased expression of SRA2, CD36, and TLR4 indicates that the elevated macrophage infiltration in aorta contributes to the inflammation and foam cell formation observed.

In some cases, the effects of functional foods are transient and return to basal levels after long periods of supplementation. We found that the lesions were proportionally more severe in the *A. blazei*-supplemented group after 12 weeks of supplementation, when compared to lesions seen in mice receiving 6 weeks of treatment, suggesting that this effect is maintained even after chronic supplementation. Moreover, the *A. blazei*-induced transcriptional upregulation of some important molecules linked to macrophage activation (CD36 and TLR4), neutrophil chemoattractant activity (CXCL1), leukocyte adhesion (VCAM-1), and plaque vulnerability (MMP9) after 12 weeks of supplementation confirmed the long-term effect of *A. blazei*.

IFN-γ is produced by immune cells, such as helper and cytotoxic T cells and NK cells, and exerts a multitude of immunoregulatory functions. It influences the selective recruitment and extravasation of circulating leukocytes by modulating the expression of endothelial cell-derived chemokines and adhesion molecules, such as VCAM-1 [45]. Moreover, exposure of monocytes and macrophages to IFN-γ increases TLR4 transcription and protein levels when compared with medium alone [46]. Based on this and other studies, the activity of *A. blazei* is linked to the induction of IL-12 production and is dependent on CD14 and TLR4, which activate macrophages via the CD14/TLR4/MD2 receptor complex [46, 47].

In conclusion, to the best of our knowledge, this is the first study showing that the immunostimulatory effects of *A. blazei* supplementation have pro-inflammatory repercussions, consequently deleterious effects on atherosclerosis evolution. This supplementation is able to activate immune cells in the spleen, which results in sustained

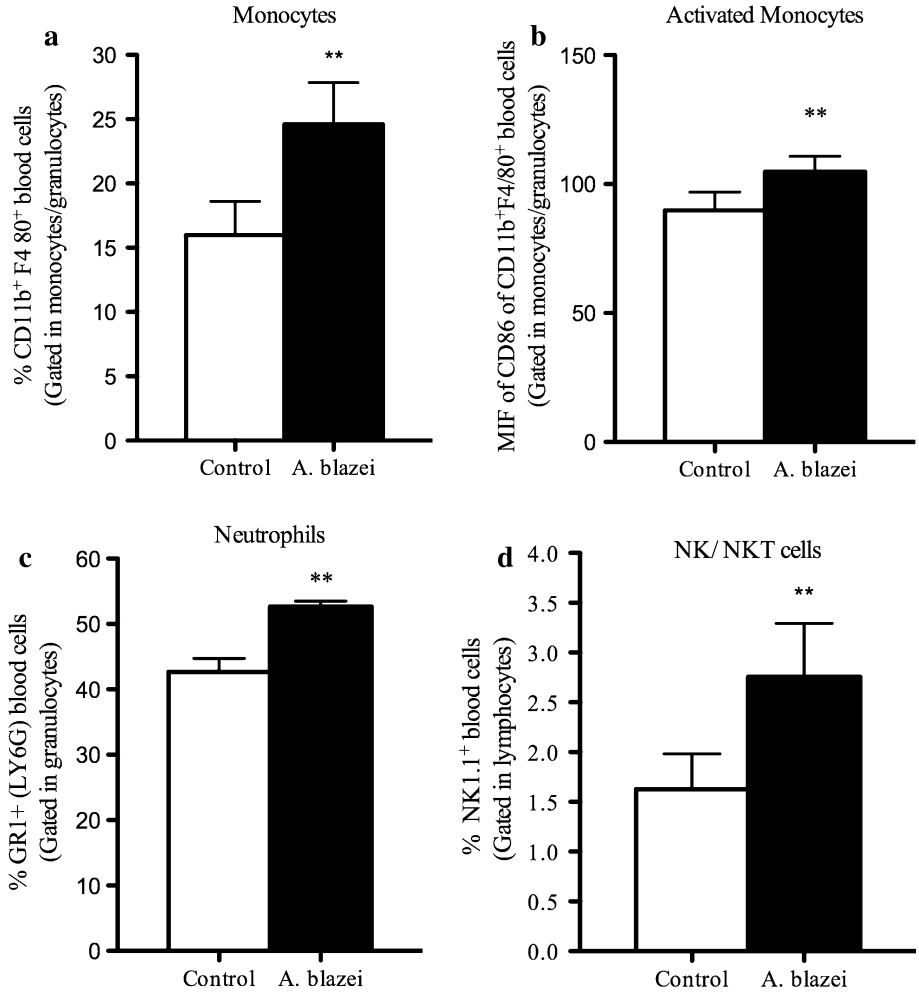

Fig. 5 Circulating inflammatory cells: Monocytes (**a**), Activated Monocytes (**b**), Neutrophils (**c**), and NK/NKT cells (**d**) in ApoE$^{-/-}$ mice fed a Control or *A. blazei*-supplemented diet (5%) for 12 weeks, in the blood. Data assessed using flow cytometry ($n = 7$). *Bars* represent the *average,* and *vertical lines* represent the SE. Student's *t* test $^{**}p < 0.01$

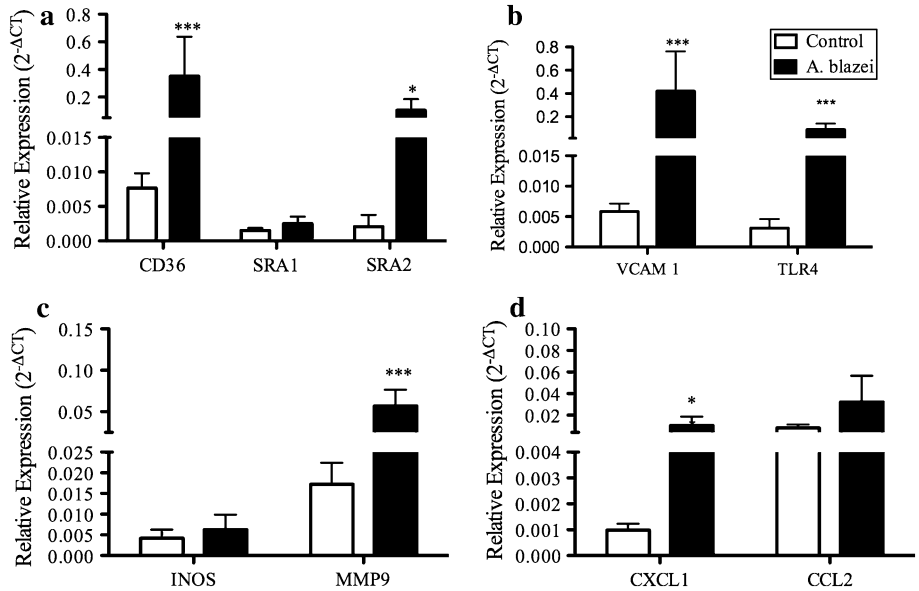

Fig. 6 Proatherogenic molecules expression in the aortic valve: Expression of CD36, SRA1, and SRA2 (**a**); VCAM-1 and TLR4 (**b**); iNOS and MMP9 (**c**); and CXCL1 and CCL2 (**d**) in aorta valve of ApoE$^{-/-}$ mice fed a Control or *A. blazei*-supplemented diet (5%) for 12 weeks. Data assessed using real-time PCR ($n = 6$). *Bars* represent the *average,* and *vertical lines* represent the SE. Student's *t* test $^{*}p < 0.05$, $^{***}p < 0.001$

activation and homing of monocytes and neutrophils to sites of atherosclerosis. In these sites, increased expression of important molecules involved in foam cell formation, leukocyte adhesion, and fibrous cap degradation occurs, culminating in a more advanced and vulnerable atherosclerosic plaque.

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Conflict of interest The authors declare that they have no conflict of interest.

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