International Immunopharmacology 73 (2019) 502–514

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Immunopathological effects of *Agaricus blazei* Murill polysaccharides against *Schistosoma mansoni* infection by Th1 and NK1 cells differentiation

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ARTICLE INFO

Keywords:
Agaricus blazei Murill polysaccharides
Immunomodulation
NK cell
Schistosoma mansoni
T1/T2 doubly transgenic mice

ABSTRACT

In this study, we examined the ability of *A. blazei* Murill polysaccharides (AB-PS) to activate the immune system *in vivo* and the protective activity exhibited against parasitic *S. mansoni* in the murine model. AB-PS treatment significantly reduced the worm and egg burden in infected BALB/c and C57BL/6 mice with dose- and time-dependent manners. Additionally, a dose- and time-dependent expression of IL-2, INF-γ, and TNF-α cytokines was also observed in both strains of mice treatments. Using T1/T2 doubly transgenic mice, we demonstrated that AB-PS-treated mice splenocytes initiated early differentiation of Th1 and NK1 cells, which was consistent with the reduction course of *Schistosoma* infection. Although AB-PS treatment enhanced the Th1 response, it did not suppress Th2 cell activity in treated mice. Histopathological data of the livers showed AB-PS treatment significantly attenuated the liver fibrosis induced by *S. mansoni* eggs. AB-PS augmented type-1 responses by inducing Th1 and NK1 cell differentiation to effectively decrease the infection rate of *S. mansoni*. Furthermore, AB-PS treatment may not only inhibit the schistosome infection, but also improving the pathological effects of granulomas formation. This study provides evidence for a novel therapeutic potential, by which *A. blazei* Murill may be used to treat or prevent schistosome infection.

1. Introduction

Traditional medicinal mushrooms have been reported to induce various immune responses [1], for example, *Ganoderma lucidum* stimulates both macrophages and T-lymphocytes to release various type-1 cytokines [2,3], *Antrodia cinnamomea* exhibits anti-inflammatory and anti-oxidative effects [4–6], and *Agaricus blazei* Murill (AbM) is beneficial in the treatment of cancer (*e.g.* leukemia), infections (*e.g.* HBV), allergy/asthma, and inflammatory disorders (*e.g.* inflammatory bowel diseases) [7,8]. AbM originates from the Brazilian rainforest and is known to be rich in immunomodulatory substances such as branched β-1, 3-/1, 6-glucans and proteoglycans [9,10]. Liu et al. found that AbM enhanced helper T cell expansion and NK cell activation, and helped to decrease the percentage of body fat, visceral fat, blood cholesterol levels, and blood glucose levels in human volunteers of clinical research [11]. This evidence strongly supports the use of AbM as a health-promoting supplement for immunomodulation.

AbM stimulates factors of the innate immune system, such as monocytes, NK cells, and dendritic cells, to mediate the amelioration of a skewed Th1/Th2 balance and symptoms of inflammatory bowel diseases [7,8]. When examining the release of signaling substances from monocyte-derived dendritic cells (MDDC), the presence of AbM increased the production of pro-inflammatory, chemotactic and Th1-type cytokines *in vitro* [12]. Recent research has also revealed that AbM activates the NLRP3 inflammasome in human macrophages, resulting in the secretion of pro-inflammatory cytokine IL-1β, which is crucial for

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https://doi.org/10.1016/j.intimp.2019.05.045
Received 16 August 2018; Received in revised form 24 January 2019; Accepted 14 May 2019
Available online 04 June 2019

stimulating innate immune responses and recruiting phagocytic cells to defend against tumors and infection [13]. Oral treatment with AbM extracts induced the activation of macrophages by interacting with intestinal epithelial cells, and then promoted the differentiation of naive T cells into Th1 cells, thereby increasing anti-allergic activity and suppressing OVA-sensitized allergies of mice [14]. The further investigation of the mechanism by which AbM regulates the immune response against pathogens would be both interesting and commercially valuable.

The innate immune system serves as the host's prime barrier and plays a crucial role in activating the acquired immune system. Mechanisms of innate immunity include enhancement of antigen presentation, phagocytosis by antigen-presenting cells (APCs) and cytotoxicity induced by natural killer (NK) cells [15]. NK cells are specialized lymphocytes of the innate immune system. They are activated by the adaptive immune system of the host and play a role in a variety of inflammatory, autoimmune and allergic diseases [16]. Furthermore, NK cells are endowed with the ability to distinguish and lyse tumor cells [17]. Some reports have found AbM to have antitumor properties *in vitro* and *in vivo* [18,19] and others have reported quality of life improvement in cancer patients following AbM treatment [20,21]. AbM mediates antitumor activity by activating and augmenting the cytotoxicity of NK cells [22–24] and inducing apoptosis [25]. However, the effect of AbM on the differentiation of NK cells *in vivo* has not been examined.

Different cytokine environments serve to regulate endogenous Th1/Th2 imbalances in the body [26]. Direct detection of T helper cell differentiation *in vivo* is made possible by utilizing a T1/T2 doubly transgenic mouse model, which has previously been used to monitor the activation of Th1/Th2 cells by infection with *Listeria monocytogenes* or *Schistosoma mansoni* [27]. We have previously reported that the extracts from *A. camphorata* polysaccharides inhibit infection of *S. mansoni* in this transgenic mouse model by inducing the development of a Th1 response by the immune system [28]. Thus, this *in vivo* model may be used as a predictive assay to asses this immunomodulatory agent. The immunoregulatory role of AbM in the treatment of parasitic infection has been studied in protozoan leishmania [29–31] and malaria [32], but not in helminth parasites. In this study, we first examined whether AbM polysaccharide extract has the capacity to activate the immune system *in vivo* and to protect against *S. mansoni* in a murine model.

2. Methods

2.1. Preparation of Agaricus blazei Murill polysaccharides

The polysaccharides of *A. blazei* Murill were isolated based on a previously published method, with little modification [33]. Briefly, fresh air-dried *A. blazei* Murill mycelia were donated from the Biotechnology Center, Grape King Inc., Chungli, Taiwan. Mycelia were filtered through grade 1 Whatman filter paper with boiling water three times before being air-dried. For the preparation of the aqueous extracts, all air-dried mycelia samples were ground and then shaken with isotonic phosphate saline buffer (PBS) (154 mM NaCl and 10 mM phosphate buffer at pH 7.4) in a ratio of 1:25 (w/v) at 25 °C for 10 h. The mixture was centrifuged at 3000 × *g* for 10 min and then passed through a 0.45-μm pore size filter. The water-soluble polysaccharide-rich fraction was isolated using ethanol precipitation from the concentrated extract. The crude polysaccharides were then passed through a PolySep-GFC-P4000 (Phenomenex, Torrance, CA) gel filtration column. The stock solution was lyophilized and stored for treatment. The quantitative analysis of polysaccharide ingredients has previously been described [34]. The AbM preparations were then tested for gram negative bacterial endotoxin contamination using the limulus amebocyte assay QCL-1000 kit (Cambrex, Walkersville, MD) according to the protocol stated in the US FDA guidelines: “Guideline on Validation of the LAL test as an end-product endotoxin test for human and animal parenteral drugs, biological products, and medical devices” [35].

2.2. Experimental animal treatment

Male BALB/c or C57BL/6 mice (6–8 weeks old) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). The IFN-γ: hThy1, IL-4:mThy1.1 doubly transgenic mice (BALB/c genetic background) were generated and maintained as described previously [27]. Animals were provided with water and mouse chow (Labdiet 5001, PMI Nutrition International LLC, MO) *ad libitum*. Animals were housed in a rodent facility at 22 ± 1 °C with a 12-h light-dark cycle for acclimatization. Each strain of mice was respectively divided into seven groups, including one no AB-PS-received control group and six groups that received two different doses of AB-PS treatment per day for 1, 2 and 4 weeks. Treatment regime was performed entirely before infection was initiated. For treatment, 1.0 and 2.5 mg of lyophilized AB-PS dissolved in 0.2 ml of distilled water was orally administered daily, respectively. Then the mice were received infection on one day after treatment. In addition, the IFN-g: hThy1, IL-4: mThy1.1 doubly transgenic mice were also divided into six groups, including the untreated control group and those that received AB-PS treatment for 2, 3, 4, 5 and 6 weeks. For treatment, 2.5 mg of lyophilized AB-PS dissolved in 0.2 ml of distilled water was orally administered daily.

2.3. Ethics statement

Animal experiments were carried out under humane conditions with approval with license number: LAC-101-0304 from the Institutional Animal Care and Use Committee (IACUC) of Taipei Medical University, and conducted in accordance with NIH Guide for the Care and Use of Laboratory Animals (DHHS publication No. NIH 85–23, revised 1996).

2.4. Isolation of spleen cells

One day after administration, the mice were sacrificed and the spleens were removed, placed in a Petri dish and flushed out any splenocytes. The splenocytes were dispersed using a PBS-filled syringe equipped with a 23-g needle. Residual red blood cells were hypotonically lysed in PBS containing 150 mM ammonium chloride, 1 mM potassium bicarbonate, and 0.1 mM ethylenediaminetetraacetic acid (EDTA) (all from Sigma-Aldrich). The intact splenocytes were recovered by centrifugation (at 1800 rpm, for 3 min, 4 °C) and resuspended in PBS for later examination. Approximately 95% of the isolated cells were viable, as determined using Trypan Blue.

2.5. Isolation of RNA, cDNA synthesis, and real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR)

Real-time polymerase chain reaction (PCR) was used for mRNA quantification assessment. Total RNA was extracted using TRIzol™ reagent (Life Technologies, Carlsbad, CA, USA) and reverse transcribed using the Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA) to generate cDNA. Each cDNA pool was stored at −20 °C until real-time PCR analysis was completed. Primer pair specificity was validated by performing RT-PCR using common reference RNA (Stratagene, La Jolla, CA, USA) as a DNA template. The primers used were shown in Table 1, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18 s were used as endogenous reference genes. Real-time PCR was performed using the lightcycler® nano real-time PCR system (Roche Diagnostics, Mannheim, Germany) using LightCycler® 480 SYBR Green I Master (Roche Diagnostics). Briefly, 10-μl reactions were utilized, containing 2 μl of Master Mix, 2 μl of 0.75 μM forward primer and reverse primer, and 6 μl of the cDNA sample. Each sample was run in triplicate. The real time PCR program included 3 min at 95 °C; 45 cycles of 10-s durations at 95 °C,

Table 1
Primer pairs for candidate genes used in real time PCR.

Gene name	Primer sequences	Melting Tm (°C)
Interleukin (IL)-2	forward 5′-ATGGACCTACAGGAGCTCCTG-3′ reverse 5′-TCAAATCCAGAACATGCCGCAG-3′	60
Interferon-gamma (INF-γ)	forward: 5′-CTTCCTCATGGCTGTTTCTG-3′ reverse: 5′-TGTCACCATCCTTTTGCCAG-3′	60
IL-4	forward: 5′-CAGAGAGTGAGCTCGTCTG-3′ reverse: 5′-GGTGCAGCTTATCGATGAATC-3′	60
IL-10	forward: 5′-ATGCAGGACTTTAAGGGTTAC-3′ reverse: 5′-CCTGAGGGTCTTCAGCTTC-3′	60
Tumor Necrosis Factor -alpha (TNF-α)	forward: 5′-CCTCACACTCAGATCATCTTC-3′ reverse: 5′-CGGCTGGCACCACTAGTTG-3′	60
GAPDH	forward: 5′-TCCTGGTATGACAATGAATACGG-3′ reverse: 5′-GATGGAAATTGTGAGGGAGATG-3′	60
18 s	forward: 5′-AAGACGGACCAGAGCGAAAGCA-3′ reverse: 5′-ATCGCCAGTCGGCATCGTTTATG-3′	60

and 30 s at 60 °C. At the end of the program, a melt curve analysis was done. Data analysis was performed using the LightCycler Nano software version 1.0 (Roche).

2.6. Flow cytometric analysis

Three-color immunolabeling was performed using fluorochrome isothiocyanate (FITC)-, phycoerythrin (PE)-, Cy-chrome (Cy)-conjugated monoclonal antibodies. Appropriate isotype controls were purchased from Serotec (Pharmingen, San Diego, CA) and were used for characterization of T, B, and NK cells as follows: anti-CD4-FITC, anti-CD8α-Cy, anti- CD45R/B220-FITC, anti-CD49b/Pan-NK-FITC, anti-human CD90-PE, streptavidin-Cy and anti-mouse thy1.1-biotin. Isolated cells were blocked with rat anti-mouse CD16/CD32 antibodies (2.4G2, Pharmingen, San Diego, Calif.) in PBS for 30 min on ice to reduce nonspecific antibody binding by Fc receptors. They were then incubated with saturating concentrations of color-conjugated monoclonal antibodies and primary monoclonal antibodies, followed by immunoglobulin G (IgG)-FITC at 4 °C for 30 min. After washing twice with PBS, 1×10^6 cells were applied to a FACS flow cytometer (Becton Dickenson Biosciences, San Jose, CA). Data were collected and analyzed using CellQuest Software (Becton Dickenson Biosciences).

Table 3
Effect of AB-PS on egg burdens in *Schistosoma mansoni* infected mice.

Oral administration	Egg burden (Reduction rate, %)	
	C57BL/6j	BALB/c
Control	15,897.32 ± 1572.78	13,607.92 ± 3208.22
1 week 1 mg	12,455.24 ± 2409.15* (21.65%)	10,125.99 ± 1768.82* (25.59%)
2.5 mg	12,387.09 ± 3719.77 (22.08%)	8277.73 ± 2067.32** (39.16%)
2 weeks 1 mg	9872.19 ± 3418.77** (37.90%)	8067.79 ± 3253.78* (40.71%)
2.5 mg	8984.74 ± 4109.92** (43.48%)	7495.14 ± 2642.14* (44.92%)
4 weeks 1 mg	8774.75 ± 3951.92** (44.80%)	7039.36 ± 1799.12** (48.27%)
2.5 mg	7465.31 ± 2557.94*** (53.04%)	6860.04 ± 3048.11** (49.59%)

Following treatment with 1 or 2.5 mg AB-PS for a duration of 1, 2 or 4 weeks, C57BL/6 and BALB/C mice were infected with 120 *S. mansoni* cercariae (n = 10). Eight weeks after the time of infection, all mice were sacrificed concurrently and the egg burdens in the liver were determined. The ten mice that received no AB-PS treatment were used as a control group. Data from 3 separate experiments are expressed as mean ± SD. ** $p < 0.01$, *** $p < 0.001$ when compared with the control value.

2.7. Infection of Schistosoma mansoni and assessment of infection rate

BALB/c or C57BL/6 mice were respectively divided into control ($n = 10$) and AB-PS treatment (1, 2, and 4 weeks) groups (n = 10). The T1/T2 doubly transgenic mice were also divided into untreated control ($n = 7$) and AB-PS treatment (2, 3, 4, 5 and 6 weeks) groups (n = 7). One day after treatment, one hundred and twenty *S. mansoni* cercariae, freshly isolated from their intermediate host *Biomphalaria glabrata,* were used to infect each mouse through direct skin penetration into the tail vein. The life cycle of parasite was maintained in our laboratory by cycling through the planorbid snail and BALB/c mice. Eight weeks later, the adult worms in portal and mesenteric veins were isolated and counted. Sex determination of the worms was performed simultaneously. Part of the mouse liver was cut, weighed, and digested with 5 ml of 5% KOH at 37 °C overnight, and then the egg number per gram was determined under microscope and calculated. The reduction rate

Table 2
Effect of AB-PS on *Schistosoma mansoni* infection.

Oral administration	Adult worm burden (Reduction worm rate, %)		Female worm burden (Reduction worm rate, %)	
	C57BL/6j	BALB/c	C57BL/6j	BALB/c
Control	79.00 ± 5.20	67.67 ± 13.06	39.22 ± 8.97	34.75 ± 2.99
1 week 1 mg	75.50 ± 5.07 (4.43%)	60.40 ± 6.27 (10.74%)	31.10 ± 8.76 (20.70%)	23.71 ± 2.50*** (31.76%)
2.5 mg	66.40 ± 13.41 (15.95%)	52.33 ± 14.32 (22.66%)	27.71 ± 13.03 (29.34%)	22.20 ± 7.46* (36.12%)
2 weeks 1 mg	65.33 ± 8.50 (17.30%)	45.83 ± 2.79** (32.27%)	23.80 ± 2.59** (39.32%)	21.25 ± 6.24** (38.85%)
2.5 mg	52.33 ± 4.80***, # (33.76%)	34.33 ± 4.18***, ## (49.26%)	17.00 ± 2.94***, # (56.65%)	13.30 ± 4.19***, # (61.73%)
4 weeks 1 mg	46.67 ± 2.08*** (40.93%)	37.00 ± 6.04*** (45.32%)	18.40 ± 4.14*** (53.09%)	19.56 ± 3.84*** (43.73%)
2.5 mg	37.60 ± 4.77***, # (52.41%)	28.33 ± 4.89***, # (58.13%)	12.50 ± 2.62***, ## (68.13%)	12.33 ± 3.27***, ### (64.51%)

Following treatment with 1 or 2.5 mg AB-PS for a duration of 1, 2 or 4 weeks, C57BL/6 and BALB/C mice were infected with 120 *S. mansoni* cercariae ($n = 10$). Eight weeks after the time of infection, all mice were sacrificed concurrently and the adult worm burden and female worm burden were determined. The ten mice that received no AB-PS treatment were used as a control group. Data from 3 separate experiments are expressed as mean ± SD. ** $p < 0.01$, *** $p < 0.001$ when compared with the control value. # $p < 0.01$, ## $p < 0.01$, ### $p < 0.001$ when values obtained from mice treated with 1 or 2.5 mg AB-PS are compared.

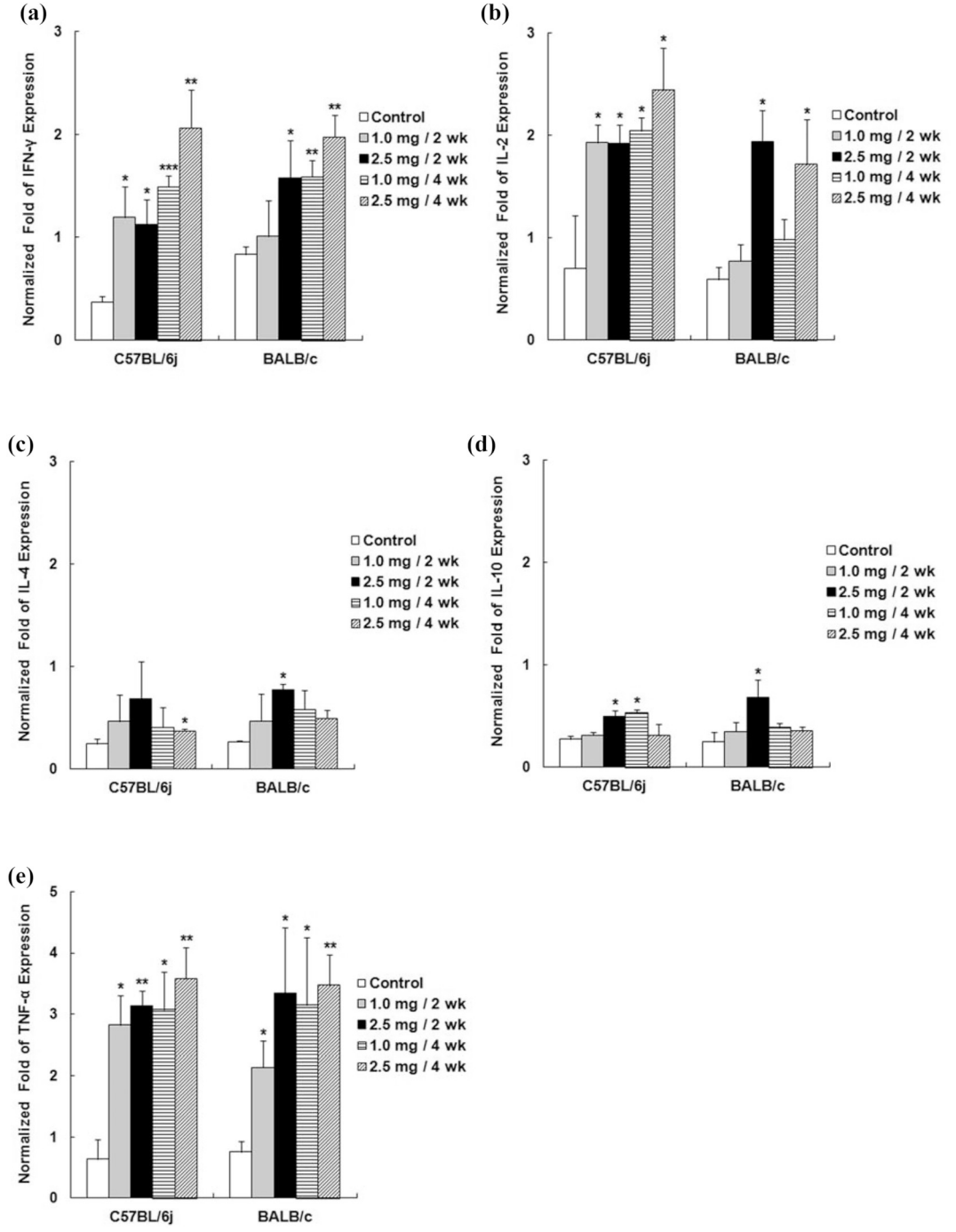

Fig. 1. Expression of various cytokine mRNAs obtained from the spleen cells of C57BL/6j and BALB/c mice treated with AB-PS. cDNA from the splenocytes of C57BL/6j and BALB/c mice in the 1.0 or 2.5 mg treatments of AB-PS for 2 and 4 weeks were analyzed for the expression of (a) IFN-γ, (b) IL-2, (c) IL-4, (d) IL-10 and (e) TNF-α cytokines using real-time PCR. The mice that received no AB-PS treatment were used as a control group. All data were normalized to the internal controls, 18 s and GADPH levels, to ensure that equivalent template amounts were used. The panels represent the mean from three independent experiments, which are expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared to the control.

was calculated using the following formula: Worm burden reduction rate (%) = (1 − mean number of worms in immunized mice / mean number of worms in mice immunized with the control plasmid) × 100. Egg reduction rate (%) = (1 − mean number of eggs per gram in immunized mice / mean number of eggs per gram in mice immunized with the control plasmid) × 100.

2.8. Histopathological examination of liver tissue for granulomas

The livers of infected and control mice were collected, fixed, and then stained with hematoxylin and eosin, as described previously [36]. Briefly, liver specimens were fixed in 10% formalin, embedded in paraffin, and sectioned at 3-μm thickness. The sizes of non-confluent granulomas formed around a single egg were measured in the stained sections using the ImageJ software (NIH). For all histological quantification, 7 mice livers from each group were analyzed and at least 3

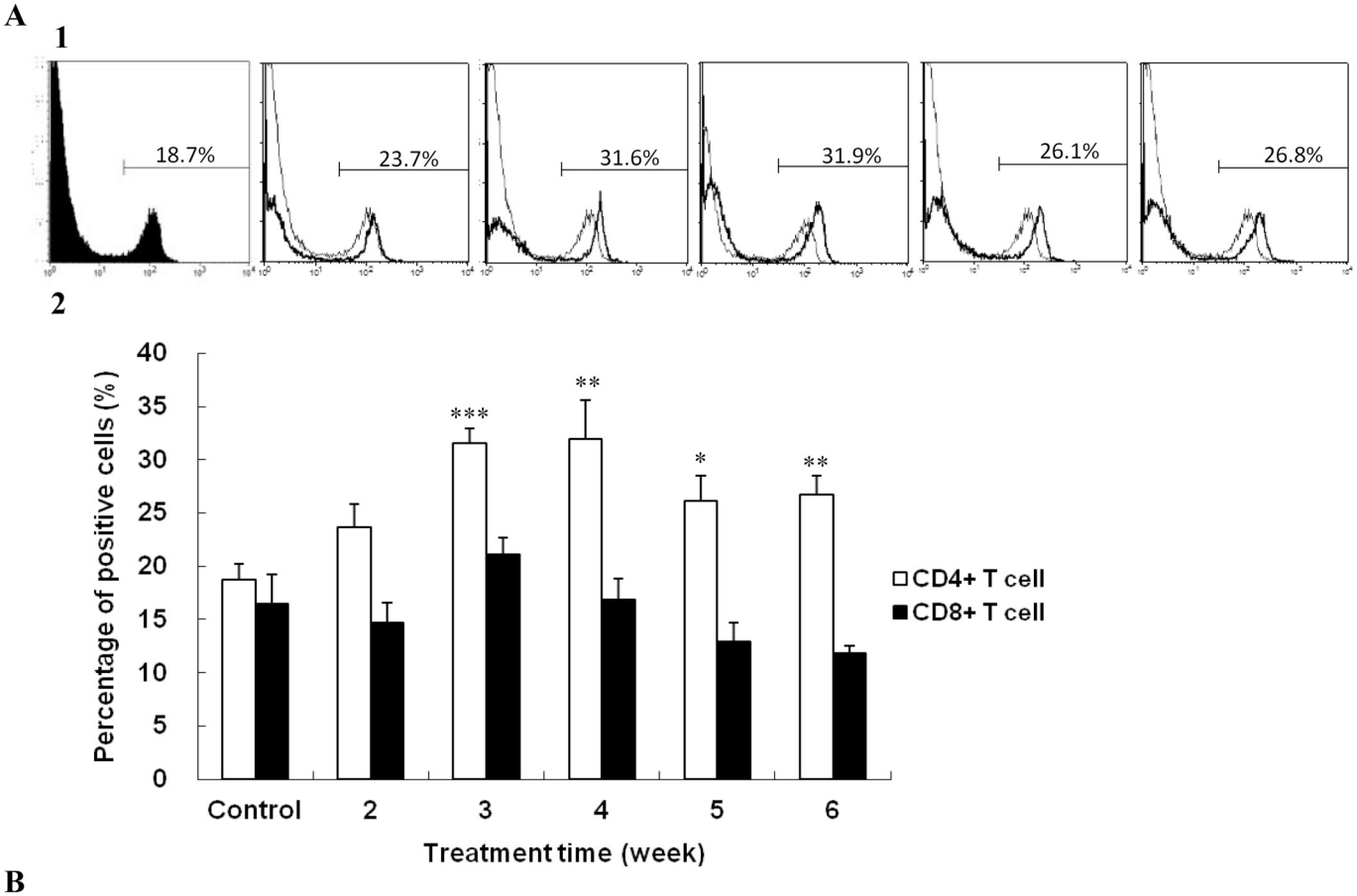

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Fig. 2. The percentages of surface $CD4^+$ and $CD8^+$ expression on splenocytes and surface type1 and type2 marker molecule expression on $CD4^+$ Th cells. Splenocytes from mice treated with no AB-PS (control) or orally with 2.5 mg AB-PS for 2, 3, 4, 5, and 6 weeks were collected and further bound with (A) primary CD4/CD8 monoclonal antibodies, or (B) primary type1 (hThy1) and type2 (mThy1.1) marker monoclonal antibodies. For FACS, lymphocytes were gated and the percentages of cells expressing $CD4^+$ or $CD8^+$ marker were estimated. The histograms and bars depict the percentage of $CD4^+$ (A1) at different weeks following the AB-PS treatment. The numbers on each histogram represent the mean percentage. Actual percentages of cells were determined for each group. The data shown is representative of a typical result. Cells of control group were presented as gray line and those of experimental group were shown as black line, respectively. A2 showed splenocytes were identified and analyzed for CD4+ and CD8+ expression by FACS. $CD4^+$ cells were identified and analyzed for type1 (hThy1) and type2 (mThy1.1) expression by flow cytometry. For FACS, $CD4^+$ cells were gated and the percentage of cells expressing type1 (hThy1) and type2 (mThy1.1) was estimated. Data of type1 (hThy1) and type2 (mThy1.1) expressing $CD4^+$ at different weeks following the AB-PS treatment are shown as dot blots from all the analyzed samples (B1). The quadrants were set according to the IgG isotype controls. Numbers in the right or upper quadrants show the positive frequency of type1 or type2 expressing cells, respectively. $CD4^+$ cells were gated and the percentage of cells expressing type1 (hThy1) or type2 (mThy1.1) markers were calculated (B2). Actual percentages of the cells were determined for each group. The data shown is representative of a typical result. Data from 3 to 5 independent experiments are expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared to the control.

slides were used to determine the width of the granuloma borders of each specimen by referring the previous description [37]. Two liver sections, selected to be sufficiently distant from each other to ensure that a granuloma was not measured twice, were used for all granuloma measurements.

2.9. Statistics

Data were expressed as mean ± standard deviation (SD). Analysis of variance was used to examine the differences in surface marker expression between various treatment and control groups. The changes in schistosome infection rates and cells frequency were analyzed using the Mann-Whitney *U* test, and were expressed as *P*-values. A p value of ≤ 0.05 was considered statistically significant.

3. Results

3.1. Quality analysis of AB-PS

The eluted fractions were assayed for hexose by the phenol-sulfuric acid method and the percentage of carbohydrate content in lyophilized extract of *A. blazei* Murill was 97.52%. The quantitative percentage of polysaccharides in the extract was 5.11%. In addition, the concentration of gram negative bacterial endotoxin contamination in the sample is in direct proportion with absorbance and is calculated from a standard curve by the limulus amebocyte assay QCL-1000 kit. The concentration found was significantly below the following limits are approved by the US FDA Devices. So the influence of endotoxin contamination could be ignored.

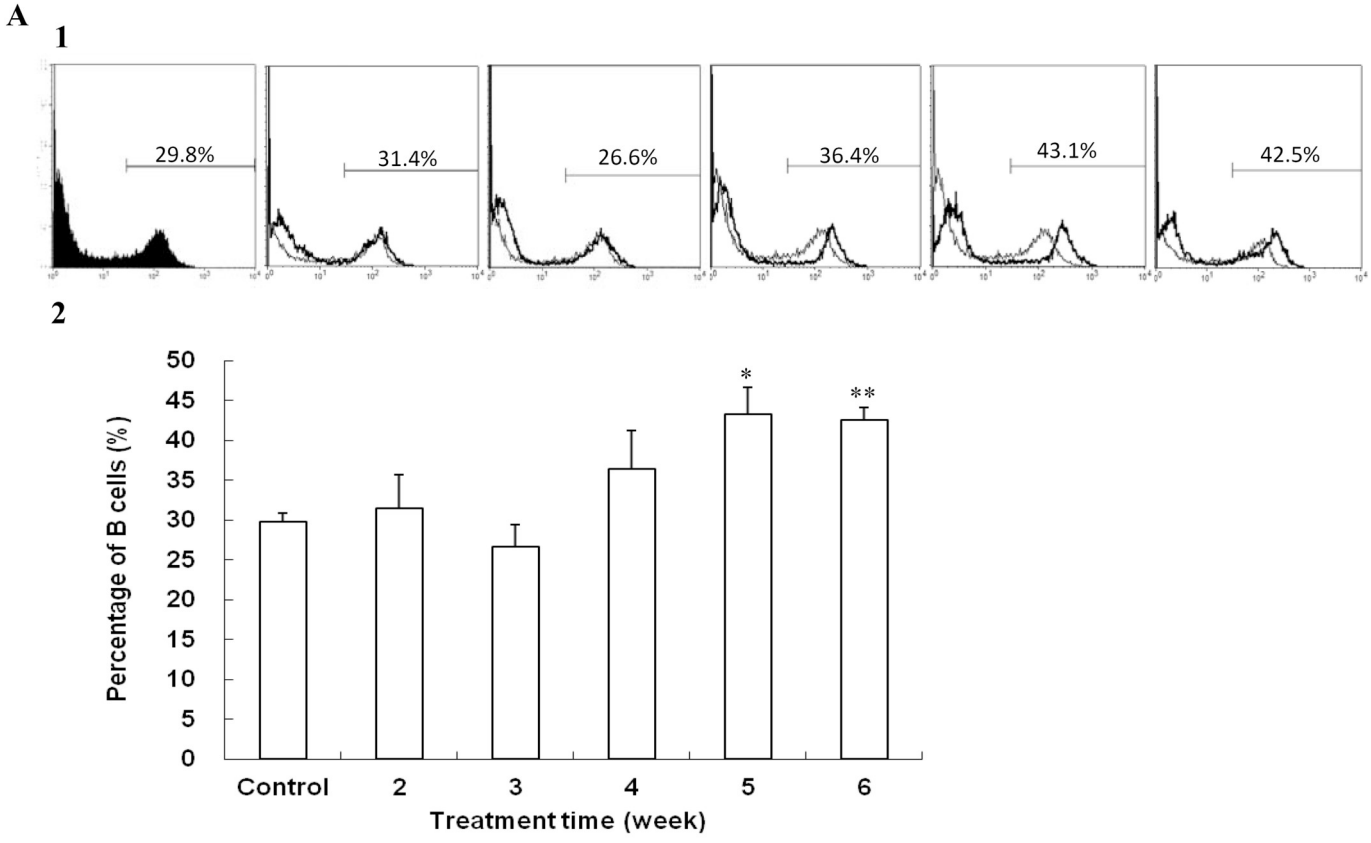
3.2. Protective effects of AB-PS against Schistosoma mansoni infection

As shown in Table 2, the protective effects of AB-PS against *S. mansoni* infection, increased in not only a dose-dependent but also a time-dependent manner. The worm burden of C57BL/6 mice in the control group was 79.00 ± 5.20 and significantly decreased to 52.33 ± 4.80 with 2 weeks of 2.5 mg AB-PS treatment ($p < 0.001$). Worm reduction was more significant after 4 weeks of treatment, with 1 and 2.5 mg AB-PS treated groups down to 46.67 ± 2.08 and 37.60 ± 4.77, respectively ($p < 0.001$). Meantime, the worm burdens of BALB/c mice in the 2 week AB-PS-treated groups (45.83 ± 2.12 of 1 mg; 34.33 ± 4.18 of 2.5 mg) were both significantly less than the control group (67.67 ± 13.06) ($p < 0.01$; $p < 0.001$). The 4 week treated groups exhibited the greatest reduction to 37.00 ± 6.04 and 28.33 ± 4.89 with the 1 mg and 2.5 mg treatments, respectively ($p < 0.001$). On the other hand, the effects of AB-PS treatment on female worms were more rapid and obvious (Table 2). The reduction in the number of female worms in C57BL/6j mice was significant, with rates reaching 39.32% for the 1 mg condition ($p < 0.01$) and 56.65% for the 2.5 mg condition ($p < 0.001$) after 2 weeks of AB-PS treatment, and even up to 53.09% (1 mg) and 68.13% (2.5 mg) for the 4 week AB-PS treated groups ($p < 0.001$). Moreover, these effects in BALB/c mice were significant even after 1 week of AB-PS treatment (31.76% of the 1 mg condition, $p < 0.001$; 36.12% of the 2.5 mg condition, $p < 0.05$). The significant reduction in the number of female worms can also be represented in comparison with the control groups for both the 2 week-treated groups (38.85% of the 1 mg condition, $p < 0.01$; 61.73% of the 2.5 mg condition, $p < 0.001$) and the 4 week-treated groups (43.73% of the 1 mg condition, $p < 0.001$; 64.51% of the 2.5 mg condition, $p < 0.001$). It is also worth noting that there was an evident dose-dependent female worm decrease in both the 2-week and 4-week treated groups ($p < 0.05$).

The number of eggs in the liver tissue of each group is presented in Table 3. Compared to infected C57BL/6j and BALB/c control mice, the numbers of eggs per gram of liver in 1 week AB-PS treatment were respectively reduced by 28.91% for the 1 mg condition to 39.37% for the 2.5 mg condition, and 25.59% for the 1 mg condition to 39.16% for the 2.5 mg condition ($p < 0.05$). Moreover, the 2 weeks AB-PS treatment led to a significant reduction of the hepatic egg burden in both C57BL/6j and BALB/c mice (37.90% and 40.71% for the 1 mg condition, 43.48% and 44.92% for the 2.5 mg condition, respectively, $p < 0.05$). The 4 weeks AB-PS treated mice showed more significant decreases of egg burdens in both strains of infected mice (44.80% and 53.04% for 1 mg in C57BL6j mice, 48.27% and 49.59% for 2.5 mg in BALB/c mice, $p < 0.01$). However, similar differences in the worm burdens were not observed in the hepatic egg burdens between the groups treated with 1 mg AB-PS and the groups treated with 2.5 mg AB-PS. Thus, treated mice with AB-PS can significantly reduce the worm and hepatic egg burdens.

3.3. Effects of AB-PS on cytokine mRNA expression in C57BL/6j and BALB/c mice splenocytes

In order to elucidate the effects of AB-PS on inducing cytokine expression, mRNA were extracted from splenocytes after sacrificing mice treated with 1.0 or 2.5 mg AB-PS for 0, 2, and 4 weeks. Subsequently, the cytokine mRNA expression levels of IFN-γ, IL-2, IL-4, IL-10, and TNF-α were detected and evaluated. The levels of mRNA expression were normalized with housekeeping genes 18 s and GAPDH, which have internal RNA control properties. As shown in Fig. 1, the quantities of IFN-γ, IL-2, and TNF-α were significantly increased in the 2 or 4-week treatment groups as compared to the control group levels in both strains of mice ($p < 0.05$, Fig. 1a, b and e). In addition, the expression levels of these genes all reached maximum levels after 2.5 mg daily AB-PS treatment for 4 weeks, except for IL-2 in BALB/ c mice. These results demonstrate that AB-PS induces similar IFN-γ and TNF-α expression profiles in C57BL/6j and BALB/c mice. However, the mRNA expression of IL-2 in C57BL/6j mice was much higher than that in BALB/c mice, especially at the 1 mg dosage level. On the other hand, the results revealed no significant difference in expressions of IL-4 and IL-10 in BALB/c mice treated with AB-PS (Fig. 1c and d). The observed results for the 2 cytokines were also similar in C57BL/6j mice, while the amount of mRNA was mildly higher in BALB/c mice treated with 2.5 mg for 2 weeks ($p < 0.05$). These data indicate that AB-PS



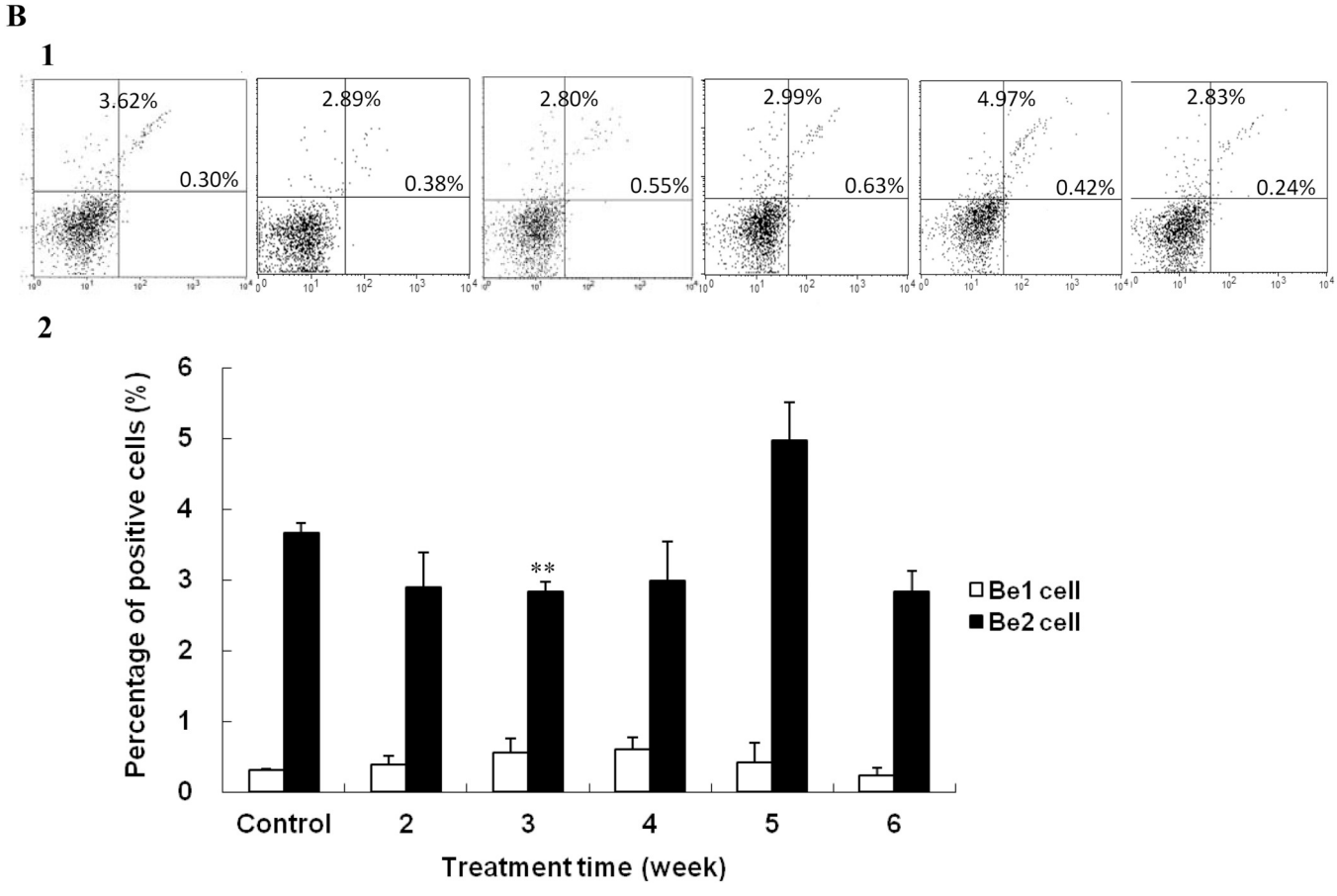


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Fig. 3. The percentages of surface B220$^+$ expression on splenocytes and surface type1 and type2 marker molecule expression on B220$^+$ cells. Splenocytes from mice treated with no AB-PS (control) or orally with 2.5 mg AB-PS for 2, 3, 4, 5, and 6 weeks were collected and further bound with (A) primary B220$^+$ monoclonal antibodies, or (B) primary type1 (hThy1) and type2 (mThy1.1) marker monoclonal antibodies. For FACS, lymphocytes were gated and the percentages of cells expressing B220$^+$ marker were estimated. The histograms and bars depict the percentage of B220$^+$ (A1) at different weeks following the AB-PS treatment. The numbers on each histogram represent the mean percentage. Actual percentages of cells were determined for each group. The data shown is representative of a typical result. Cells of control group were presented as gray line and those of experimental group were shown as black line, respectively. A2 showed splenocytes were identified and analyzed for B220$^+$ expression by FACS. B220$^+$ cells were identified and analyzed for type1 (hThy1) and type2 (mThy1.1) expression by flow cytometry. For FACS, B220$^+$ cells were gated and the percentage of cells expressing type1 (hThy1) and type2 (mThy1.1) was estimated. Data at different weeks following the AB-PS treatment are shown as dot blots from all the analyzed samples (B1). The quadrants were set according to the IgG isotype controls. Numbers in the right or upper quadrants show the positive frequency of type1 or type2 expressing cells, respectively. B220$^+$ cells were gated and the percentage of cells expressing type1 (hThy1) or type2 (mThy1.1) markers were calculated (B2). Actual percentages of the cells were determined for each group. The data shown is representative of a typical result. Data from 3 to 5 independent experiments are expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared to the control.

Table 4
Expression of surface CD4 and CD8 on spleen cells.

Oral administration time	CD4 (%)	CD8 (%)	CD4/CD8 ratio
Control	18.72 ± 3.99	16.50 ± 7.45	1.13
2 week	21.83 ± 5.76	14.90 ± 5.32	1.47
3 week	27.94 ± 1.70*	17.97 ± 1.75	1.55
4 week	28.87 ± 4.29*	16.14 ± 2.03	1.79
5 week	27.80 ± 2.23*	10.72 ± 1.61	2.59
6 week	26.11 ± 3.02*	11.85 ± 1.91	2.20

Spleen cells, obtained from the various treatment conditions (control, AB-PS treatment for 2–6 weeks), were harvested and further incubated with saturating concentrations of primary CD4 monoclonal antibodies, followed by FITC-conjugated IgG and Cy-conjugated anti-mouse CD8 at 4 °C for 30 min. After washing twice with PBS, cells were applied to a FACS caliber flow cytometer. Data from 5 separate experiments are expressed as mean ± SD. * $p < 0.05$ when compared with the control value.

stimulates predominant Th1 responses (IFN-γ and IL-2) and TNF-α expression in the splenocytes of treated mice. According to these results, mice in the following experiments were treated with 2.5 mg AB-PS for further examination.

3.4. AB-PS immunomodulation of CD4$^+$/ CD8$^+$ cells and Th1/Th2 differentiation

The percentages of CD4$^+$ cells from mice splenocytes, indicated in Table 3 and Fig. 2, illustrate an upward trend upon treatment with AB-PS, with values increasing from 18.72 ± 1.46% in the control group to 31.57 ± 1.39% and 31.91 ± 3.74% in the 3 and 4 week of AB-PS treatment conditions, and then slightly pulling back to 26.17 ± 2.36% and 26.77 ± 1.76% in the 5 and 6 week treatment conditions, respectively. There was a significant difference between the number of CD4$^+$ cells in the control condition and the 3–6 week AB-PS treatment conditions, whereas there was no significant change in CD8$^+$ cell levels at similar time points ($p < 0.05$, Fig. 2A). In addition, an established T1/T2 doubly transgenic mouse model was used to investigate the differentiation of Th1/Th2 cell populations, as stimulated by AB-PS (Fig. 2B). The percentage of Th1 cells in the 2–6 week AB-PS treatment groups were as follows: 1.76 ± 0.24%, 1.78 ± 0.58%, 1.99 ± 0.16%, 3.17 ± 0.89% and 2.00 ± 0.26%. These values were all significantly greater than the control group value, 0.07 ± 0.02% ($p < 0.05$). The percent of Th2 cells was 0.93% ± 0.02% in the control group, with significantly higher values observed in the 3–5 AB-PS treatment groups: 1.75 ± 0.23%, 1.76 ± 0.30% and 2.03 ± 0.22% ($p < 0.05$).

3.5. AB-PS immunomodulation of type 1/type 2 effector B cells

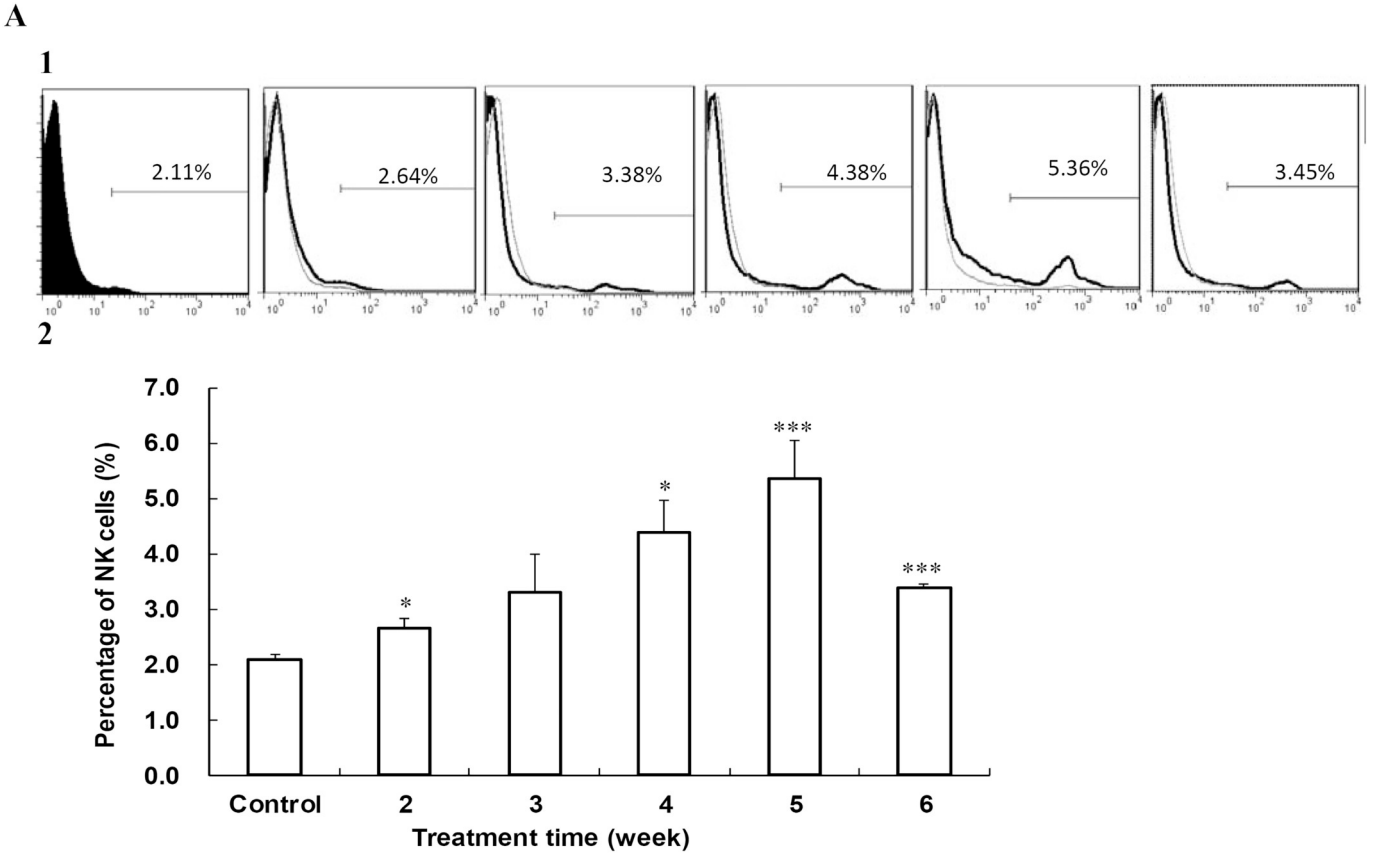
AB-PS treatment for 2 to 4 weeks had no significant effect on the percentage of B cells when compared to the control group value of 29.80 ± 1.10%. However, until the 5 and 6 week treatment B cell percentages 43.25 ± 3.38% and 42.55 ± 1.63%, respectively, were significantly increased ($p < 0.05$) (Fig. 3A). Similar to T lymphocytes, B cells may also differentiate into two subsets of effector B cells (Be1 and Be2), depending on the cytokine environment. Our data showed the Be1 cells in all of the AB-PS-treated groups were not statistically varied as comparing with the control group, although it seems to slightly increase in 3–4 weeks. Similar results were also appeared in the changes of Be2 cells, while only in the 3-week AB-PS-treated group was reduced to 2.84% ± 0.14% (Fig. 3B). These results suggest that neither Be1 nor Be2 cells may be involved in the immune responses mediated by AB-PS treatment (Table 4).

3.6. AB-PS immunomodulation of type 1/type 2 NK cell differentiation

As shown in Fig. 4A, AB-PS treatment induced an increase in NK cell population in a time-dependent manner. The percentage of NK cells was significantly raised from 2.10 ± 0.07% in the control group to 2.65% ± 0.18%, 4.38% ± 0.59%, 5.37% ± 0.70%, 3.40% ± 0.06% in the 2, 4, 5 and 6 weeks treatments, respectively ($p < 0.05$). Differentiation of NK cell subpopulations was also taking place during the course of AB-PS treatment (Fig. 4B). The prevalence of Type-1 NK cell (NK1) in the control group was 0.22% ± 0.03% and significantly increased to 0.70% ± 0.11%, 0.77% ± 0.22%, 1.46% ± 0.25%, 0.93% ± 0.04%, 0.84% ± 0.06% in the 2 to 6 week AB-PS treated groups, respectively ($p < 0.05$). Meanwhile, the percentage of NK2 cells following AB-PS treatment for 2, 3, and 6 weeks (1.48% ± 0.39%, 1.14% ± 0.08%, 1.26% ± 0.06%) were significantly lower than that of the control group (3.67% ± 0.61%) ($p < 0.05$). These results indicate AB-PS treatment in mice effectively stimulates splenic NK cell proliferation, with a trend in differentiation from NK2 to NK1.

3.7. Histopathological effect of the AB-PS treatment

The immune pathological changes of granuloma formation are more apparent in infected BALB/c mice than that in the C57BL/6 mice [38]. To investigate the immunopathological effect of AB-PS treatment to granuloma, the liver tissue slides of BALB/c mice treated with 1.0 or 2.5 mg AB-PS for 1, 2, and 4 weeks were determined. As shown in Fig. 5A, the deposition of schistosome eggs and severe granuloma formation were observed in liver tissue of mice with normal infection control. However, the granuloma reactions under morphological observation seem to decrease not only in a dose- but also time-dependent manner after the AB-PS treatment as comparing to the positive control. Further, we measured the size of granulomas observed in the hepatic tissue samples as shown in Fig. 5B. Our results showed mice treated with 2.5 mg AB-PS for 2, and 4 weeks both exhibited significantly smaller granulomas than mice in the normal infected control group ($p < 0.05$; 0.01). Moreover, the size of granulomas from liver of mice in 4 weeks 2.5 mg AB-PS treated group was significantly reduced than that in mice with 2.5 mg AB-PS treatment for only 1 week or 2 weeks groups ($p < 0.05$). This data suggests that AB-PS treatment may suppress the hepatic granuloma formation by *S. mansoni* infection time-dependently.



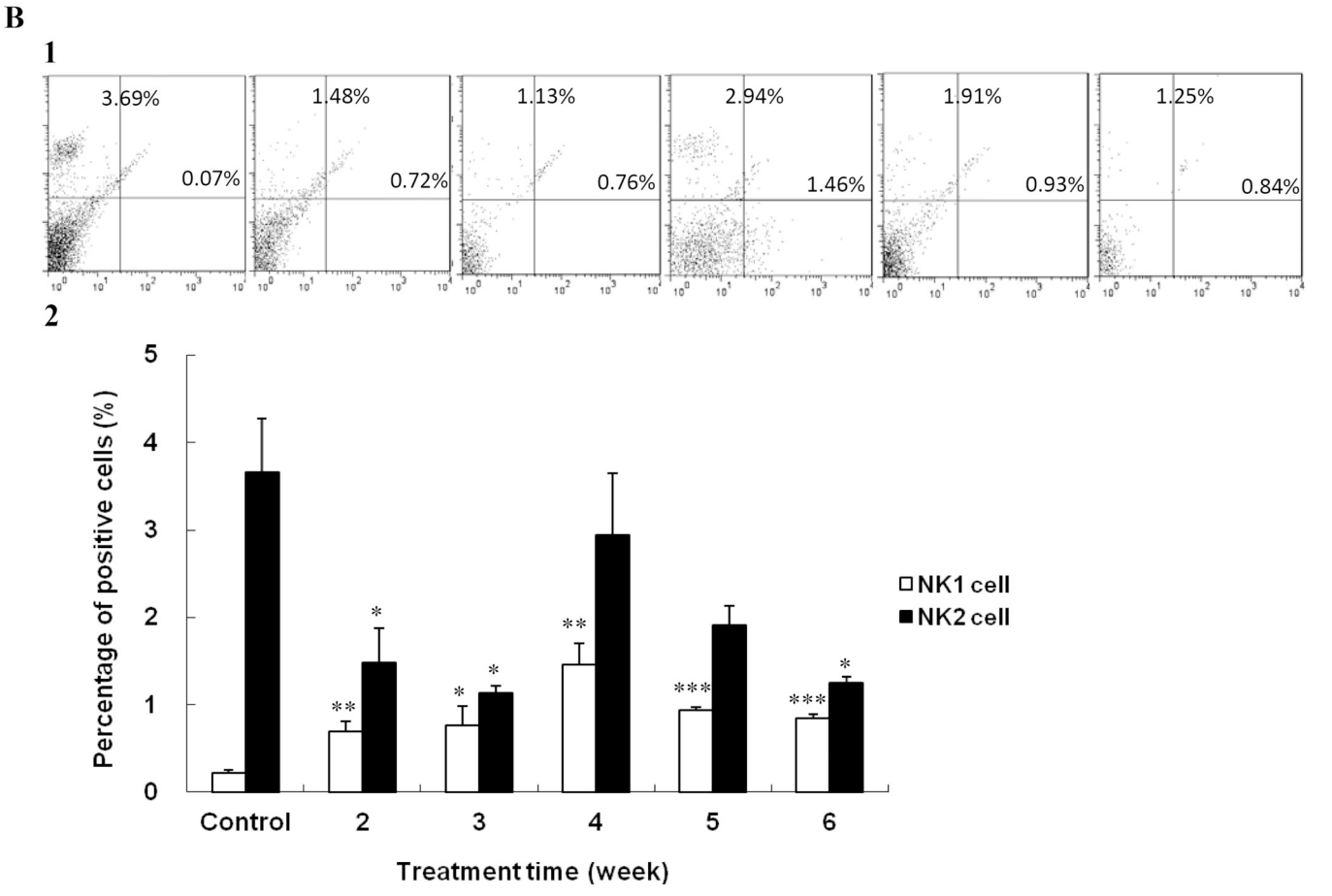


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Fig. 4. The percentages of surface NK1.1$^+$ expression on splenocytes and surface type1 and type2 marker molecule expression on NK1.1$^+$ cells. Splenocytes from mice treated with no AB-PS (control) or orally with 2.5 mg AB-PS for 2, 3, 4, 5, and 6 weeks were collected and further bound with (A) primary NK1.1$^+$ monoclonal antibodies, or (B) primary type1 (hThy1) and type2 (mThy1.1) marker monoclonal antibodies. For FACS, lymphocytes were gated and the percentages of cells expressing NK1.1$^+$ marker were estimated. The histograms and bars depict the percentage of NK1.1$^+$ (A1) at different weeks following the AB-PS treatment. The numbers on each histogram represent the mean percentage. Actual percentages of cells were determined for each group. The data shown is representative of a typical result. Cells of control group were presented as gray line and those of experimental group were shown as black line, respectively. A2 showed splenocytes were identified and analyzed for NK1.1$^+$ expression by FACS. NK1.1$^+$ cells were identified and analyzed for type1 (hThy1) and type2 (mThy1.1) expression by flow cytometry. For FACS, NK1.1$^+$ cells were gated and the percentage of cells expressing type1 (hThy1) and type2 (mThy1.1) was estimated. Data at different weeks following the AB-PS treatment are shown as dot blots from all the analyzed samples (B1). The quadrants were set according to the IgG isotype controls. Numbers in the right or upper quadrants show the positive frequency of type1 or type2 expressing cells, respectively. NK1.1$^+$ cells were gated and the percentage of cells expressing type1 (hThy1) or type2 (mThy1.1) markers were calculated (B2). Actual percentages of the cells were determined for each group. The data shown is representative of a typical result. Data from 3 to 5 independent experiments are expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared to the control.

4. Discussion

The medicinal mushroom *Agaricus blazei* Murill has been used for the inhibition of parasitic diseases, and reports on its usage as a treatment for leishmaniasis and cerebral malaria have indicated positive results [29–32]. This present study revealed that AbM polysaccharides modulate immune responses to activate type-1 Th and NK cells to secrete specific cytokines, subsequently reducing the infection rate and the pathological lesions of *S. mansoni* in treated mice. This is the first study that examined the possibility of *A. blazei* Murill as a treatment for the helminthic schistosome infection.

We previously had illustrated that *A. camphorate* polysaccharide extract induces type-1 immune responses that protects against *S. mansoni* infection [28]. In the present study, we demonstrated that AB-PS treatment leads to a significant decrease in the worm and egg burdens of infected mice, particularly the female worms' reduction (Tables 2 and 3). Maximal reduction worm rates were up to 58% of total worm burden and 68% of female worms, as well as the reduction egg rates that were near to 50%. The data also showed that only one-week AB-PS treatment in BALB/ c mice was able to effectively reduce the number of female schistosome worms. The anti-fecundity effect against female adult worms is a key point of both the anti-pathologic characteristics and transmission-blocking application. The host's immune response to female schistosome worms is also associated with their pathogenesis and laying of eggs [39,40]. Our results suggest that AB-PS has the potential to be developed as active antischistosomal drugs with more benefits than A. camphorate polysaccharide.

It is interesting that protective activities against *S. mansoni* were more significant in predominant Th2 phenotype BALB/c mice than in the Th1 predisposed C57BL/6 mice. With regard to reducing the number of female worms, these two strains of mice showed a significant difference only after 1 week of AB-PS treatment. This result is consistent with that of our previous *A. camphorate* experiment, that BALB/c mice were more susceptible to the effects of medicinal mushrooms and exhibited greater protective effects than C57BL/6j mice [28]. Additionally, the cytokine expression trends were similar to those found in the *A. camphorate* study, revealing a time- and dose-dependent increase in the Th1 cytokines (IL-2 and INF-γ). However, AB-PS only enhanced the Th1 responses for killing schistosome, and did not suppress the expression of Th2 IL-4 and IL-10 cytokines in BALB/c mice. In the acute infection stage, tolerance to worm egg antigens led to the enhancement of the Th1 response and a reduction of the Th2 response, as well as an increase in host mortality rates [41]. Therefore, inducing the appropriate Th2 response, combined with effective Th1 immunity, may decrease unnecessary immunopathological damage to host tissue. TNF-α also plays a complex role in the pathophysiological changes that accompany schistosome infection, such as causing hepatocyte apoptosis, early fecundity, granuloma formation, and hepatic fibrosis [42,43]. We have observed rapid and significant increases in TNF-α expression in both strains of mice treated with AB-PS. AbM polysaccharides have also been found to stimulate myeloid derived suppressor cells by blocking Toll-like receptor 2 signals, and resulted in a switch of cell type from M2 to M1. This produces cytokines IL-6, IL-12, TNF-α and iNOS, which further affect NK and T cells [12,44].

A. blazei Murill has potential immunomodulatory effects, including antitumor, anti-inflammatory, and anti-parasitic properties, mediated by the enhancement of type-1 cytokine secretion by T cells [7,12,31]. In the present study, we utilized a T1/T2 doubly transgenic mice model that was previously used in the *A. camphorate* experiment to examine the potential effects of AB-PS on type1/type2 differentiation of lymphocytes [27,28]. Our results illustrated that AB-PS treated mice splenocytes not only significantly increased CD4$^+$ T cells populations following 3 to 6 weeks of treatment, but also initiated earlier differentiation of Th1 cells from two to six weeks of treatment administration. However, unlike the strong Th1-induction effects of *A. camphorate*, AB-PS also resulted in the increase of Th2 cell numbers in the mice treated for 3–5 weeks. Recent reports indicated that *A. blazei* Murill polysaccharides as adjuvants, combined with OVA, elicited greater antigen specific Th1 cell activation and produced specific IgG2b antibodies against OVA in the vaccinated mice [45]. Moreover, it is worth noting that AB-PS did not produce any robust effects with regards to B cell differentiation, while cell proliferation also being delayed until the 5–6 week treatment time point. In contrast, a marked induction of type-1 effector B cells was observed in the *A. camphorate* treatments. Several studies have indicated that *A. blazei* extract has anti-allergy activities, through the promotion of Th1-dominant immune responses to prevent allergy development [14,46]. Our data suggest that *A. blazei* participated in alleviating allergen-related humoral responses such as IgE production may not directly involve B cells.

The natural killer cell is another important lymphocyte that causes cytotoxicity and is involved in polarizing Th1 responses that result from microbial organisms [47]. Here we demonstrated that AB-PS stimulated NK cell proliferation in a time-dependent manner and caused the differentiation from NK2 to NK1 cells during the 2 to 6 week durations of treatments. More importantly, the time sustained for NK1 cell activation was consistent with the induction of Th1 cells, as well as the reduction course of schistosome infection. The antitumor properties of *A. blazei* have been described in a number of studies and clinical tests [44,48,49]. The relationship between the tumor inhibition effects and *A. blazei* Murill polysaccharides may also support our NK1 cell enhancement results. Although the numbers of splenic NK cells were induced during the *S. japonicum* infection, but the NKG2D and CD94 expressions on NK cells were decreased, and also produced more IL-4 and IL-10, accompanying with less IFN-γ [50]. The activated NK cells in the liver after *S. japonicum* infection might negatively regulate egg-induced liver fibrosis *via* producing IFN-γ and killing activated stellate cells. The NK cells recognized and then killed activated HSCs by retinoic acid early inducible 1 (RAE1)/NKG2D dependent, to inhibit fibrosis [51]. Our data showed AB-PS treatment also attenuated the liver fibrosis induced by *S. mansoni* eggs, especially in the mice of 2 and 4 weeks 2.5 mg AB-PS treated groups. It has demonstrated *A. blazei* Murill treatment could augment NK cell activation through IL-12-mediated IFN-γ production [23]. Another study also reported treating hexane extract from *A. blazei* could restore the decrease of NK activity

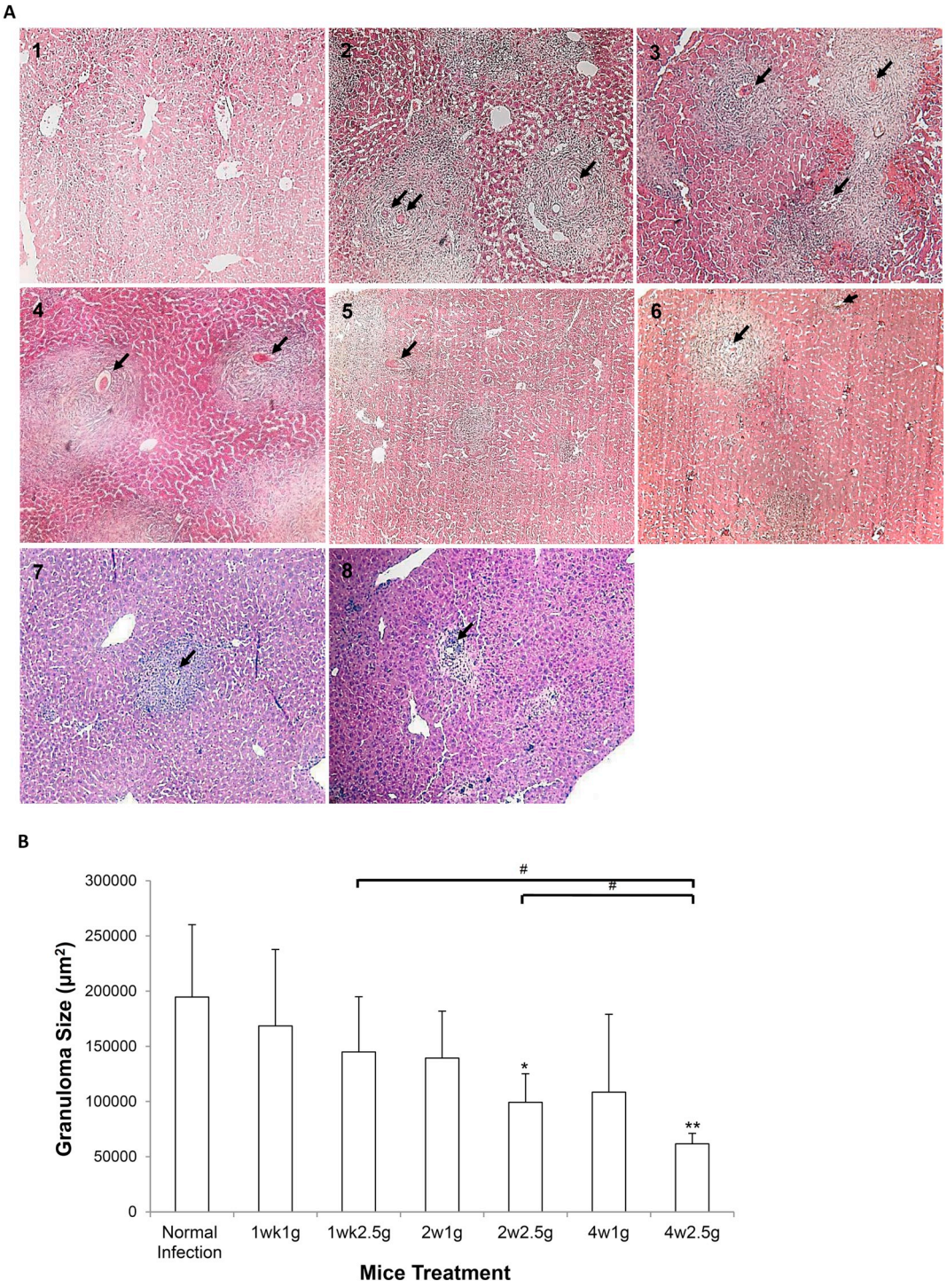

Fig. 5. Histopathology of liver tissue sections of mice in AB-PS treated groups. (A) Histopathological differences were observed between the livers of mice in different AB-PS treated groups. Images 1 and 2 respectively show the livers tissue of an uninfected mouse and a normal infected BALB/c mouse at 8 weeks. Images of 3, 5 and 7 show that the liver tissues of mice with 1.0 mg AB-PS treatment for 1, 2 and 4 weeks before *S. mansoni* infection, respectively. Images of 4, 6 and 8 show that the liver tissues of mice with 2.5 mg AB-PS treatment for 1, 2 and 4 weeks before infection, respectively. AB-PS treatment reduced the pathogenesis of egg-induced granulomas and showed fewer granuloma formations in 2 and 4 weeks treated groups. Paraffin-embedded liver tissues from *S. mansoni*-infected mice were stained using H&E. Tissue images were captured with a microscope (Leica) under 100× magnifications. Sites of egg deposition are shown with arrows. (B) Hepatic average granuloma size in AB-PS treated mice challenged with *Schistosoma mansoni.* Size distribution of hepatic granulomas from mice after AB-PS treatment followed by 8 weeks challenge was measured. The size of each granuloma surrounding an egg was quantitated using ImageJ software (NIH). Results represent the average of 30 granulomas (mean ± SD, in mm^2) from 7 animals per group. * $p < 0.05$, **$p < 0.01$, compared with an untreated control, respectively. # $p < 0.05$, for comparisons between different groups.

in the animals with tumor implantation [52]. Therefore, it suggested that IFN-γ production from NK1 and Th1 cells activation by AB-PS treatment may not only inhibit the schistosome infection, but also improving the pathological effects of granulomas formation. Moreover, AB-PS treatment did not suppress the expression of Th2 cytokines such as IL-4 and IL-10 may also help to attenuate the pathological changes of

hepatic granuloma. Since our previous study indicated the appropriate Th2 responses exist is necessary for decreasing damage to host tissue from immune attacks during the induction of Th1 reactions against Schistosoma [53].

Overall, the data in the present study suggest that *A. blazei* Murill polysaccharides treatment effectively decreases the *S. mansoni* infection rate in mice, particularly through the suppression of adult female worms and hepatic egg deposition. AB-PS also inhibited the development of schistosomiasis by augmenting the type-1 responses and inducing Th1 and NK1 cell proliferation and differentiation, as well as by upregulating the expression of INF-γ and TNF-α cytokines. AB-PS not only drives the lymphocytes into a type 1 response, but also maintains Th2 cell effects for alleviating immune pathological damages in liver. Our study provides evidence for a novel therapeutic potential, by which *A. blazei* Murill may be used to treat or prevent schistosome infection. Further studies are necessary to determine the efficacy of AB-PS treatments in comparison to well-known therapeutic drugs.

Conflicts of interest statement

The authors do not have a commercial or other association that might pose a conflict of interest.

Acknowledgments

This study was partly supported by a grant MOST-106-2320-B-038-023 from Ministry of Science and Technology, Taipei, Taiwan.

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