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RESEARCH ARTICLE

Rubinoboletus ballouii polysaccharides exhibited immunostimulatory activities through toll-like receptor-4 via NF-κB pathway

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The biological activities of water-soluble components of edible mushroom *Rubinoboletus ballouii* (RB) were seldom reported. Polysaccharides of RB (RBP) were prepared and well-characterized using chemical analyses. The immunomodulatory properties of RBP were investigated using human monocyte-derived dendritic cells (moDC) *in vitro*, and cyclophosphamide (CTX)-induced immunosuppressive mouse model. Results showed that RBP was found to contain 80.6% (w/w) of neutral sugars including D-fucose, D-mannose, D-glucose and D-galactose (1.7:1.4:1.0:1.8), and 12.5% (w/w) of proteins, which composed of glutamine, threonine, serine, etc. RBP could promote the maturation of moDC and increase the secretion of IL-12p40, IL-10, and TNF-α. Furthermore, the stimulation of IL-12p40 production was inhibited by pretreatment with toll-like receptor (TLR)-4 blocker or NF-κB pathway blocker, suggesting that the activation of moDC by RBP was mediated through NF-κB pathway via TLR-4 receptor. On the other hand, in CTX-treated mice, RBP restored the loss of $CD34^{bright}CD45^{dim}$ hematopoietic stem cells and increased IL-2 production in sera and splenocytes culture supernatant, as well as up-regulated the percentage of $CD4^{+}$ T helper lymphocyte in mice splenocytes. These findings strongly suggested that RBP are the active ingredients of RB responsible for its immunostimulatory actions and deserved to be further investigated as cancer supplements.

Abbreviations: BCA, bicinchoninic acid; ConA, concanavalin A; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinases; FBS, fetal bovine serum; FITC, fluorescence isothiocyanate; GC, gas chromatography; IL, interleukin; JNK, c-Jun N-terminal kinases; LPS, lipopolysaccharide; LPS-RS, lipopolysaccharide from *Rhodobacter sphaeroides*; MAPK, mitogen-activated protein kinases; PBMC, peripheral blood mononuclear cells; PI3K, phosphatidylinositol 3-kinases; RBP, *Rubinoboletus ballouii* polysaccharides.

KEYWORDS
immunostimulatory effects, immunosuppression, myelosuppression, polysaccharide, *Rubinoboletus ballouii*, toll-like receptor-4

1 | INTRODUCTION

Polysaccharides are considered to be the major active components in mushrooms. Modern pharmacological studies indicated that polysaccharides from mushrooms exhibited a variety of biological activities, including the immunomodulatory (Friedman, 2016), liver protection (Liu et al., 2017), hypotensive and neurometabolic effects (Shevelev et al., 2018), and anti-tumor activities (Meng, Liang, & Luo, 2016). Kozarski et al. (2011) demonstrated that polysaccharide extracts from *Agaricus bisporus*, *A. brasiliensis* fruiting bodies and *Ganoderma lucidum* spores extracts expressed immunostimulatory effects in terms of activating human PBMCs and inducing the release of IFN-γ. Recently, Barad et al. (2018) reported that the polysaccharide fraction of *Paxillus involutus*, the basidiomycete fungus from British Columbia showed anti-proliferative activities against several cancers and triggered apoptosis. Polysaccharides are considered as one group of immunomodulatory biological response modifiers, which possess the potential role in treating various cancers and other immune-related disorders (Ferreira, Passos, Madureira, Vilanova, & Coimbra, 2015; Ina, Kataoka, & Ando, 2013).

Most polysaccharides from mushrooms are composed of beta-structure of monosaccharides, which cannot be directly digested in human body (Friedman, 2016). One important theory of how polysaccharides affect the immune system in human beings is that these polysaccharides can be captured by the antigen-presenting cells, such as dendritic cells (DC) and macrophages (Cordeiro Caillot et al., 2018; Kikete et al., 2018), whose main function is phagocytosis, digesting and presenting antigens to lymphocytes, natural killer cells, and other immune cells. DCs, normally in immature format, have a broad distribution in our human body including skin, blood, mucosal surface, and portals of human body (Steinman & Banchereau, 2007). Once encountering the invading pathogens, immature DCs can mature, capture and process antigens, then migrate to other lymphoid organs, and present the antigens to other immune cells (Morel & Butterfield, 2015). In this process, they also express high levels of cytokines, chemokines, costimulatory molecules such as IL-12, IL-10 to activate T-lymphocyte and other immune cells (Banchereau & Steinman, 1998). Kim and his group found that polysaccharides from the mushroom *Cordyceps militaris* induced the maturation of immature DCs not only on phenotypic changes but also on the functional changes. They also observed that these polysaccharides were able to increase the production of pro-inflammatory cytokines such as IL-12 and TNF-α, to enhance the stimulation of allogenic T cell, and to decrease the endocytosis ability of DCs (Kim et al., 2010).

Rubinoboletus ballouii (RB) is an edible mushroom broadly found in Yunnan Province, China (Li et al., 2013). In our previous study, we demonstrated that the ethanolic extract of this mushroom exerted antiinflammatory actions in human peripheral blood mononuclear cells (PBMC). Two active compounds, namely 1-ribofuranosyl-s-triazin-2 (1H)-one and pistillarin, with immunosuppressive activities were further isolated and characterized using bioassay-guided fractionation from the ethanolic extract (Li et al., 2013). *R. ballouii* is belonging to the family Boletaceae. The hot water extracts of fruiting bodies of *Boletus edulis* were first evaluated by Byerrum *et al.* as early as in 1957. They found that the extract from *B. edulis* exerted significant inhibitory effects against Sarcoma S-180 tumor cells (Byerrum et al., 1957). However, until now, little information was known about the water-soluble components from the fruiting body of *R. ballouii*, for example, polysaccharides of RB (RBP), and their biological activities. Therefore, this study aimed to evaluate the immunomodulatory activities of RBP in human monocytes-derived dendritic cells (moDC) *in vitro* and cyclophosphamide (CTX)-induced immunosuppressive mouse model *in vivo*. Furthermore, the structural characterization of RBP was also carried out by various chemical and instrumental analyses.

2 | MATERIALS AND METHODS

2.1 | Materials and chemicals

The fruiting bodies of *R. ballouii* were collected from Yunnan province, China and authenticated by Professor Zhuliang Yang, at Kunming Institute of Botany, China. The mushroom specimen was deposited in the herbarium of the Institute of Chinese Medicine, the Chinese University of Hong Kong, with voucher specimen number 2009-3232. Bovine serum albumin (BSA), polymyxin B, lipopolysaccharide (LPS), the amino acid standards, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO) and the monosaccharide standards were from Merck (Germany). RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Waltham, MA). SB202190 (a specific blocker of p38 MAPK), PD98059 (an inhibitor of the ERK pathway), LY294002 (an inhibitor of the PI3K pathway), SP600125 (a specific blocker of the JNK pathway), and dexamethasone (positive control for isolated compounds) were purchased from Sigma-Aldrich. Bay11-7082 (a specific blocker of NF-κB) was purchased from Calbiochem® (Merck, Germany). All chemicals and reagents used were of analytical grade.

2.2 | Preparation of mushroom polysaccharides

The air-dried *R. ballouii* (100 g) was cut into pieces and extracted twice with boiling water (1.0 L × 2) under reflux for 1 hr. The solution

was filtered and concentrated under reduced pressure. The solution was precipitated with four volumes of absolute ethanol for 24 hr. After deproteinized by Sevag method, the solution was dialyzed against distilled water for 72 hr. Finally, the retentate was lyophilized with Virtis Freeze Dryer (The VirTis Company, New York, NY) to yield crude polysaccharide (RBP, 1.45 g). RBP was dissolved in a culture medium for *in vitro* experiments or in distilled water for *in vivo* experiments.

2.3 | Structure characterization of RBP

2.3.1 | Sugars content and monosaccharide composition analysis

The carbohydrate concentration of RBP was determined by the phenol-sulphuric acid method, using D-glucose as the standard (Zeng et al., 2017). For monosaccharides analysis, in brief, 5 mg of RBP was hydrolyzed by 2.0 M TFA at 120°C for 2 hr in a sealed tube. The acid was removed in vacuum by repeated evaporation with methanol, then the hydrolysate was converted into alditol acetates (Zeng et al., 2017). The hydrolysated monosaccharides were identified and quantified by GC–MS (Agilent HP6890GC/5973MS equipment, Agilent Technologies, Santa Clara, CA). Briefly, the produced alditol acetates from hydrolysated monosaccharides were separated by BPX70 column (SGE Analytical Science, Australia). Helium was used as carrier gas at 1 ml/min. A total of 1 μl of sample with split (1/10) injection was loaded using an autosampler and injection temperature was set at 240°C. Oven conditions were set at initial temperature of 170°C, holding for 2 min, and then ramping at 3°C/min to 260°C and holding for 3 min. For MS analysis, the MS quad was maintained at 106°C and the MS source at 230°C, using 70 eV electron impact ionization and acquiring the data in scan mode from 100 to 350 m/z at 2.14 scans/s and with a solvent delay of 3 min (Pettolino, Walsh, Fincher, & Bacic, 2012).

2.3.2 | Protein content and hydrolyzed amino acid analysis

The protein concentration of RBP was determined by BCA assay using BSA as the standard (Zeng et al., 2017). The amino acids in RBP were determined with an automatic amino-acid analyzer (Hitachi 835, Tokyo, Japan) by the standard physiological program described in the manual after the acid hydrolysis and ninhydrin procedure. Five milligrams of RBP were hydrolyzed with 1 ml of 6 M HCl at 110°C for 24 hr in a sealed tube. The acid was removed in vacuum by repeated evaporation and re-dissolved in 1 ml of 0.02 M HCl before loading into the amino acid analyzer.

2.3.3 | FT-Infrared analysis

FT-Infrared spectrum of RBP was recorded at the absorbance mode ranged from 4,000 to 400 cm^{-1} with a Thermo Nicolet 5700 infrared spectrometer (Madison, WI), using KBr disks method.

2.4 | *In vitro* immunomodulatory activity on human PBMC

PBMC was isolated from blood buffy coat (Li et al., 2013), which was obtained from Hong Kong Red Cross Blood Transfusion Service. The use of human blood samples in the experiments was approved by the Joint Chinese University of Hong Kong—New Territories East Cluster Clinical Research Ethics Committee. Buffy coat was diluted with PBS, after centrifugation, the resulting cell pellet was resuspended in RPMI medium. The cell suspension was then layered onto an equal volume of Ficoll-Paque™ Plus gradient solution (Amersham Pharmacia Biotech, Uppsala, Sweden) in a 15-ml centrifuge tube and centrifuged at 800 ×*g* for 20 min. PBMCs were collected at the interface between the two layers and then washed twice with RPMI medium. The cells were seated in 96-well plates at the density of 3×10^5 cells/ml and treated with RBP (0.01–10 μg/ml) for 18 hr. Polymyxin B, an endotoxin blocker, was added to each well at 10 μg/ml to eliminate the effects of endotoxin contamination. Cell-free supernatants were collected and tested for the concentrations of TNF-α, IL-1β, IL-2, and IL-4 by commercially available human cytokine ELISA kits (BD OptEIA, San Jose, CA) according to the manufacturer instruction.

2.5 | *In vitro* immunomodulatory activity on human monocytes-derived dendritic cells

Standard isotonic Percoll solution (SIP) was prepared by mixing nine parts of Percoll with one part of 10× acidic PBS (pH 4.6). Three dilutions of SIP (60, 45, and 34, v/v) were prepared in IMDM medium containing 10% (v/v) FBS. Ficoll isolated PBMCs were suspended (2×10^7 cells/ml) in 60% SIP. Cell suspension (2.5 ml) was layered at the bottom of the 15-ml tube, then overlaid with 5 ml of 45% SIP, followed by 2.5 ml of 34% SIP. After centrifugation at 2,400 ×*g* for 45 min, monocytes in the light fraction were harvested from the upper inter-phase (between 34% SIP and 45% SIP) and washed thrice with RPMI medium. Monocytes were suspended in a 75 cm^2 flask and incubated at 37°C with 5% CO_2 for 45 min. Cells were gently washed to remove the non-adherent cells with RPMI medium twice. Then the monocytes were cultured with fresh RPMI medium containing 50 ng/ml GM-CSF and 40 ng/ml IL-4 to induce immature monocytes derived DCs. On day 3 and day 5 of culture, half of the medium was replaced with the same amount of fresh medium with the same concentrations of GM-CSF and IL-4 (Kalinski, Wieckowski, Muthuswamy, & de Jong, 2010). After one-week incubation, the cells were washed with RPMI medium for three times and seated in 96-well plates at the density of 3×10^5 cells/ml. RBP ranged from 0.08 to 50 μg/ml were added into the wells. The cell-free supernatants were collected after incubating the plates at 37°C for 24 hr. The concentrations of IL-12p40, IL-10, and TNF-α in the supernatant were determined by ELISA kits. Lipopolysaccharide (LPS), a well-known mitogen from Gram-negative bacteria, was used as a positive control for the immunostimulatory effects of RBP.

2.6 | Involvement of TLR4 in RBP-stimulated moDC

In order to evaluate the involvement of TLR4 in the activation of moDC by RBP, lipopolysaccharide from the photosynthetic bacterium *Rhodobacter sphaeroides* (LPS-RS), a potent antagonist of lipopolysaccharide (LPS), was added into the cells at 50–200 ng/ml before treating with RBP. The cell-free supernatants were collected after incubating the plates at 37°C for 24 hr. The concentrations of IL-12p40 in the supernatant were determined by ELISA.

2.7 | Chemical blocking assay

Various blockers were used to study the possible pathways involved. The cells were pretreated with Bay11-7082 (a specific blocker of NF-κB, 5 μM), SB202190 (a specific blocker of p38 MAPK, 20 μM), PD98059 (an inhibitor of the ERK pathway, 40 μM), LY294002 (an inhibitor of the PI3K pathway, 10 μM), SP600125 (a specific blocker of the JNK pathway, 10 μM), or DMSO for 1 hr and subsequently treated with RBP (50 μg/ml) for 24 hr. The cell-free supernatants were collected and the levels of IL-12p40 were determined by ELISA.

2.8 | *In vivo* immunomodulatory activities of RBP

2.8.1 | Animals

Male BALB/c mice, 6–8 weeks of age (18–22 g in weight), were fed and kept in the Laboratory Animal Services Centre, the Chinese University of Hong Kong. All experiments were approved by the University Animal Experimentation Ethics Committee (Ref No. 13/005/MIS).

2.8.2 | Establishment of immunosuppressive mouse model and drug administration

Mice were randomly divided into five groups (n = 8). One group was used as untreated normal control (naïve group). From day 1 to day 3, the other four groups were intraperitoneally administrated with CTX (80 mg/kg/day). From day 4 to day 18, four groups of CTX-treated mice were orally administrated with distilled water (control group), 40 or 200 mg/kg body weight of RBP, and 500 mg/kg body weight of *Ganoderma lucidum* (GL) water extract as positive group (equal to 40 mg/kg GL polysaccharides) respectively. Naïve group was also orally administrated with distilled water. All solutions were orally administrated daily (200 μl).

One day after the last drug administration (day 19), the mice were euthanasia by cervical dislocation. Peripheral blood samples were taken and stored at −20°C for the measurement of IL-2 production. The spleen samples were used freshly for splenocytes proliferation, IL-2 release, and T-lymphocyte subsets determination. Femurs were taken for collection of bone marrow cells.

2.8.3 | Measurement of hematopoietic cells

Hematopoietic cells were collected from femurs by bone marrow aspiration and washed with PBS supplemented with 2% v/v FBS. After treatment with erythrocyte lysis buffer, cells were resuspended in 100 μl of PBS supplemented with 2% v/v FBS. APC rat anti-mouse CD34, PE-Cy™ 7 rat anti-mouse CD45 or IgG isotype controls (BD Pharmingen™, Franklin Lakes, NJ) were stained for 30 min at 4°C in dark. $CD34^{bright}CD45^{dim}$ cells were washed and analyzed by flow cytometry (Becton Dickinson FACSCanto II, Piscataway, NJ).

2.8.4 | Determination of CD4 and CD8 T-lymphocytes in the spleen

Single-cell suspension of splenocytes was produced by gently homogenizing and passing through a 200-mesh sieve as previously described (Luo et al., 2014). After treatment with erythrocyte lysis buffer, the cells were resuspended at a final density of 2×10^6 cells/ml in RPMI-1640 medium supplemented with 10% v/v FBS. Mouse T-lymphocyte subset antibody cocktail: PE-Cy™ 7 rat anti-mouse CD3e, PE rat anti-mouse CD4, and FITC rat anti-mouse CD8a or IgG isotype controls (BD Pharmingen™, Franklin Lakes, NJ) were stained for 30 min at 4°C in the dark. Cells were washed thrice with PBS and analyzed by flow cytometry (Becton Dickinson FACScanto II, Piscataway, NJ).

2.8.5 | Measurement of IL-2 release

The effects of RBP on IL-2 production in sera of mice and supernatants of splenocytes cultures from CTX-treated mice were determined with a mouse IL-2 ELISA kit according to the manufacturer's instructions.

2.9 | Statistical analysis

Data were expressed as the mean ± the standard error of the mean. Statistical analyses and significance were determined by one-way analysis of variance (ANOVA) using GraphPad PRISM software version 8.0 (GraphPad Software, USA). In all comparisons, $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Structure characterization of RBP

The overall yield of RBP was 1.45% w/w based on the dried fruiting body. The results of the chemical analysis indicated that RBP contained 80.6% neutral sugars and 12.5% protein in terms of hydrolysates (Table 1A). The monosaccharide compositions of *R. ballouii* polysaccharides are shown in Table 1B. The polysaccharide was

TABLE 1 Sugar and amino acid composition of RBP

A: Composition			
Components	**w/w %**	**Components**	**w/w %**
Sugar	80.6	Total	93.1
Protein	12.5		
B: Neutral sugars			
Sugars	**mol %**	**Sugars**	**mol %**
Glucose	16.95	Fucose	28.81
Galactose	30.51	Total	100
Mannose	23.73		
C: Amino acids			
Amino acids	**μM/mg**	**Amino acids**	**μM/mg**
Asparagine	4.75	Leucine	3.08
Threonine	5.30	Tyrosine	1.70
Serine	5.14	Phenylalanine	2.51
Glutamine	7.77	Lysine	2.80
Glycine	3.53	Histidine	0.85
Alanine	3.77	Arginine	2.19
Valine	3.16	Proline	3.86
Methionine	1.17	Total	52.87
Isoleucine	1.29		

mainly composed of D-fucose, D-mannose, D-glucose, and D-galactose, in the ratio of 1.7:1.4:1.0:1.8. As shown in Table 1C, the amino acid composition analysis showed that RBP contained glutamine, threonine, serine, asparagine, proline, alanine, glycine, valine, and leucine in descending order. As shown in Figure 1, the FT-IR spectrum of RBP is used for the determination of their structural features. The broad band of absorption around 3,424 cm^{-1} was due to O—H stretching. The bands at 1,642 cm^{-1}, 1,423 cm^{-1}, 1,040 cm^{-1} and 926 cm^{-1} are characteristic for the carboxylic groups. A weak absorption at around 2,926 cm^{-1} was attributed to asymmetrical stretching vibration of CH_2-group and bending vibration.

3.2 | *In vitro* immunostimulatory activity of RBP on human PBMC

The *in vitro* immunostimulatory activities of RBP were preliminarily evaluated on human PBMC. To eliminate the effects of endotoxin contamination, a final concentration of 10 μg/ml of polymyxin B was added into each well. As shown in Figure 2, RBP exhibited high immunostimulatory activity in terms of increasing the release of pro-inflammatory cytokines with the increase of sample concentration. At concentrations ranging from 0.1 to 10 μg/ml, the concentrations of TNF-α and IL-1β in the cell-free supernatant were increased rapidly in a dose-dependent manner. However, the concentration of T-lymphocytes related cytokines such as IL-2 and IL-4 was not significantly changed when treating with 10 μg/ml of RBP compared with the control group.

3.3 | *In vitro* immunostimulatory activity of RBP on human monocytes-derived dendritic cells

Further *in vitro* immunostimulatory activities of RBP were investigated on human monocytes-derived dendritic cells. As shown in Figure 3, RBP showed high immunostimulatory activity on human monocytes-derived dendritic cells in a concentration-dependent manner. At concentrations ranging from 2 to 50 μg/ml, the concentrations of IL-12p40, IL-10, and TNF-α in the cell-free supernatant were increased rapidly with the increase of sample concentration. LPS, a known inducer of dendritic cells, was used as the positive control.

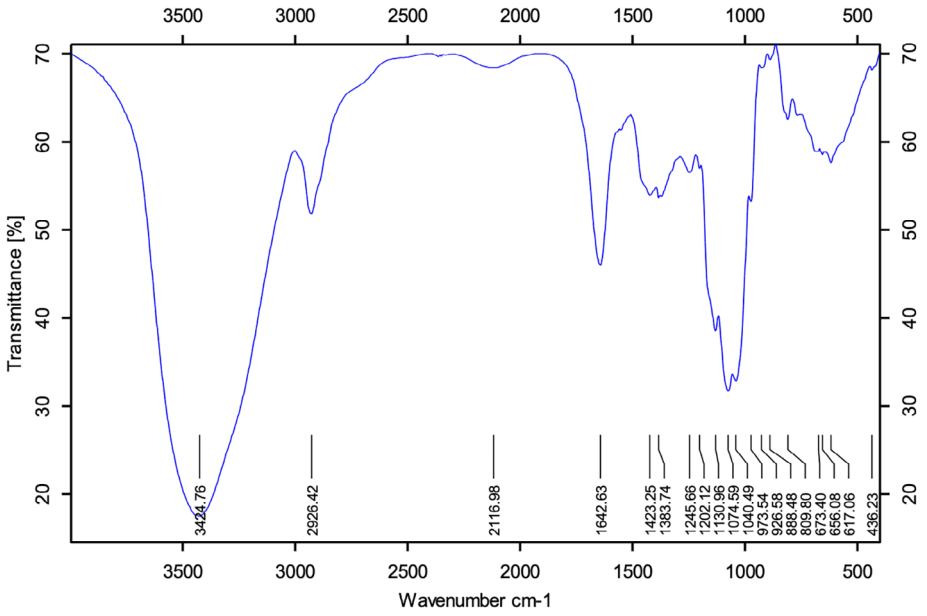

FIGURE 1 FT-IR spectrum of polysaccharides from *Rubinoboletus ballouii* (RBP) [Colour figure can be viewed at wileyonlinelibrary.com]

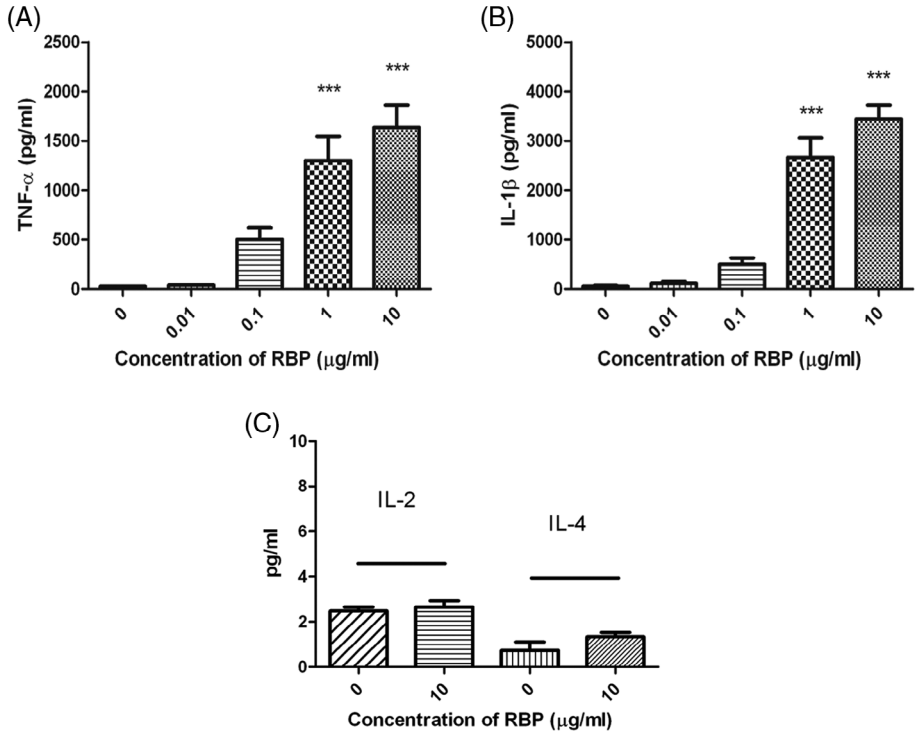

FIGURE 2 Effect of RBP on human blood mononuclear cells (PBMC). The concentrations of TNF-α (A), IL-1β (B), and IL-2 and IL-4 (C) in the supernatant of treatment with different concentrations of RBP. Polymyxin B (PMB) was added into each sample at 10 μg/ml to eliminate the effects of endotoxin contaminations. All results were presented as mean ± SEM (n = 6). Differences among the treated and untreated control groups were determined by one-way ANOVA. ***p < .001 as compared to the control group

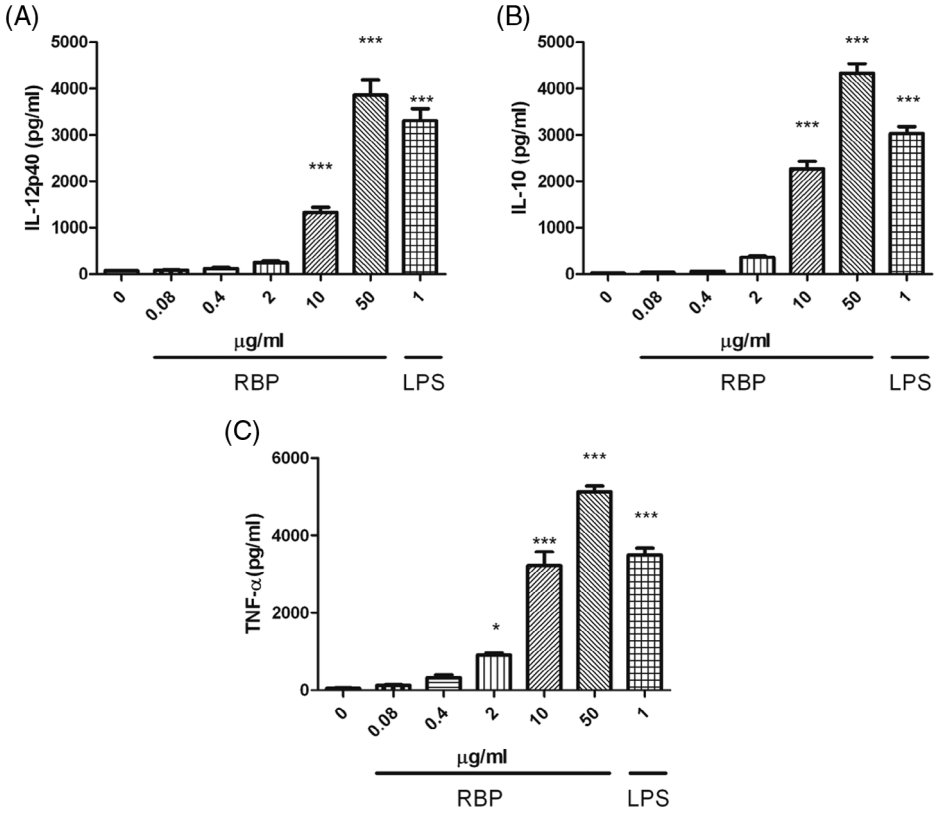

FIGURE 3 Effect of RBP on DC maturation. Immature DC were treated with RBP (0.08–50 μg/ml) or LPS (1 μg/ml) for 24 hr. Cell-free supernatants were collected and tested for the concentrations of IL12p40 (A), IL-10 (B), and TNF-α (C). All results were presented as mean ± SEM (n = 6). Differences among the treated and untreated control groups were determined by one-way ANOVA. *p < .05, ***p < .001 as compared to the control group

3.4 | RBP stimulated moDC maturation through TLR-4 via NF-κB pathway

As shown in Figure 4, the RBP-stimulated IL-12p40 production was inhibited by pretreatment with LPS-RS (a TLR-4 blocker, Figure 4A), or Bay11-7082 (an NF-κB pathway blocker, Figure 4B), which suggested the activation of moDC by RBP might have mediated through NF-κB pathway via TLR-4 receptor. While among other tested inhibitors LY294002 (an inhibitor of the PI3K pathway, 10 μM), SP600125 (a specific blocker of the JNK pathway, 10 μM), SB202190 (a specific blocker of p38 MAPK, 20 μM), or PD98059 (an inhibitor of the ERK pathway, 40 μM), none of them significantly alter the RBP-stimulated IL-12p40 production (Figure 4B).

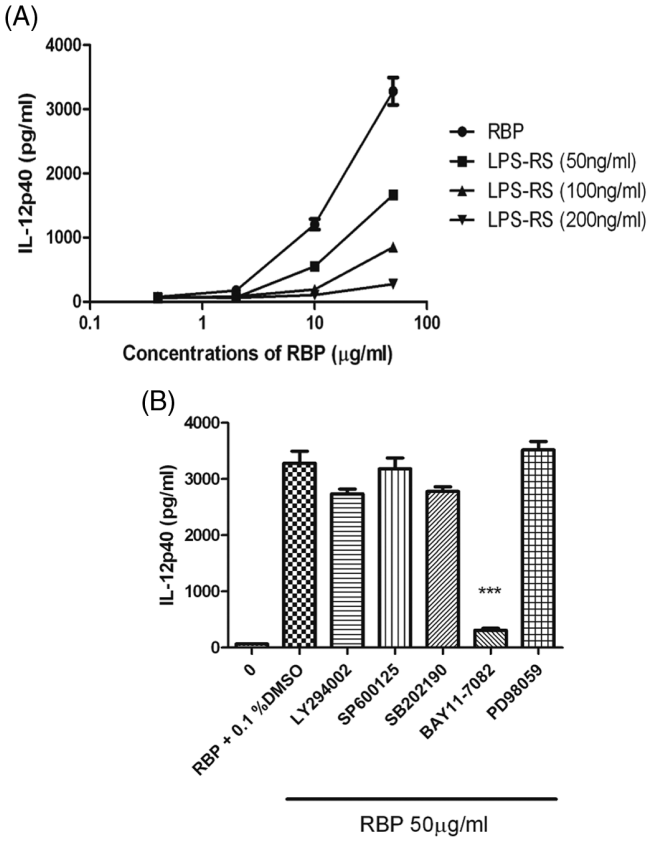

FIGURE 4 The production of IL-12p40 after challenged by LPS-RS and chemical blockers. (A) moDC was pretreated with LPS-RS at 50–200 ng/ml before treating with RBP. (B) The cells were pretreated with Bay11-7082 (a specific blocker of NF-κB, 5 μM), SB202190 (a specific blocker of p38 MAPK, 20 μM), PD98059 (an inhibitor of the ERK pathway, 40 μM), LY294002 (an inhibitor of the PI3K pathway, 10 μM), SP600125 (a specific blocker of the JNK pathway, 10 μM) or DMSO for 1 hr and subsequently treated with RBP (50 μg/ml) for 24 hr. The cell-free supernatants were collected and the levels of IL-12p40 were determined by ELISA. All results were presented as mean ± SEM ($n = 6$). Differences among the treated and untreated control groups were determined by one-way ANOVA. ***$p < .001$ as compared to the control group

3.5 | RBP restores the hematopoietic function of bone marrow inhibited by CTX

The protective effects of RBP on the hematopoietic function of bone marrow inhibited by CTX were evaluated in $CD34^{bright}CD45^{dim}$ cells from bone marrow in Cy-treated mice counted by flow cytometry. As shown in Figure 5A, the percentage of $CD34^{bright}CD45^{dim}$ cells were significantly decreased after giving CTX while the cell number was elevated remarkably by RBP 40 and 200 mg/kg/day. The number of $CD34^{bright}CD45^{dim}$ cells was enhanced 1.9- and 2.4-fold, respectively, indicating that RBP could restore the hematopoietic suppression induced by CTX.

3.6 | Effects of RBP on T-lymphocyte subsets

$CD4^+$, $CD8^+$, and total $CD3^+$ T-lymphocytes from spleens of Cy-treated mice were counted by flow cytometry to investigate the effects of RBP on the distribution of T-lymphocytes subsets. As shown in Figure 5B-E, CTX treatment induced a suppression of $CD3^+CD4^+$ T-lymphocyte count and the CD4/CD8 ratio, compared to the naïve group. RBP treatment (40 and 200 mg/kg/day), however, could partially reverse the CTX suppressive effect by increasing the percentage of $CD3^+CD4^+$ T-lymphocyte and the CD4/CD8 ratio.

3.7 | Effects of RBP on IL-2 production of CTX-treated mice

The amount of IL-2 in the mice sera and supernatants of splenocyte were quantified by ELISA for further elucidation of the immunomodulatory effects of RBP on CTX-treated mice. As shown in Figure 6, a dose-dependent increase of IL-2 secretion in the sera on CTX-treated mice was observed in RBP treatment. Similarly, the production of IL-2 in the supernatant of cultured splenocytes was also increased in RBP-treated groups compared to the control group. After the addition of LPS (1 μg/ml) or ConA (5 μg/ml), the concentrations of IL-2 in supernatants of RBP-treated splenocytes were enhanced remarkably by LPS- or ConA-stimulation. These results also indicated that RBP could partially reverse the suppressive effects of CTX on the IL-2 productions of splenocytes.

4 | DISCUSSION

In Yunnan province, China, *R. ballouii* has been used as food for years, however, little information was known about its chemical and biological properties. RBP was chemically characterized to be protein-binding polysaccharides with 80.6% of polysaccharide and 12.5% of protein. The polysaccharide part was mainly composed of D-fucose, D-mannose, D-glucose, and D-galactose, in the ratio of 1.7:1.4:1.0:1.8; and the protein part mainly contained glutamine, threonine, serine, asparagine, proline, alanine, glycine, valine, leucine, etc.

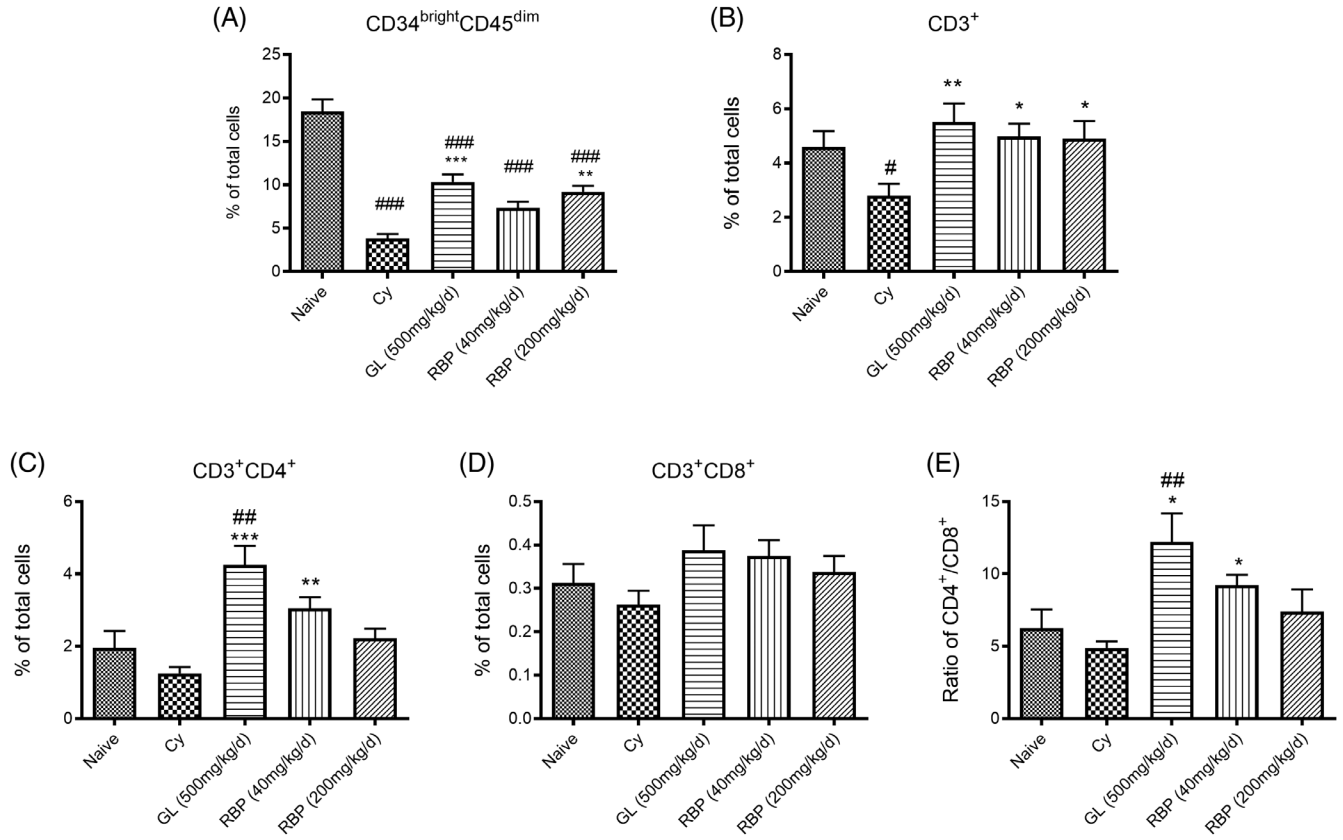

FIGURE 5 Effect of RBP on the hematopoietic function and T-lymphocyte subsets in Cy-induced immunosuppressive mouse model. Hematopoietic cells were collected from femurs by bone marrow and stained with APC rat anti-mouse CD34, PE-Cy™ 7 rat anti-mouse CD45. Single-cell suspension of splenocytes was produced by gently homogenizing and passing through a 200-mesh sieve. Mouse T-lymphocyte subset antibody cocktail: PE-Cy™ 7 rat anti-mouse CD3e, PE rat anti-mouse CD4 and FITC rat anti-mouse CD8a (BD Pharmingen™, Franklin Lakes, NJ) were used to stain the splenocytes. $CD34^{bright}CD45^{dim}$ cells (A), $CD3^+CD4^+$ (B), $CD3^+CD8^+$ (C), and $CD4^+/CD8^+$ cells were analyzed by flow cytometry (Becton Dickinson FACScanto II, Piscataway, NJ). All results were presented as mean ± SEM (n = 8). Differences among groups were determined by one-way ANOVA. $^{\#,}$ $^{*}p < .05$, $^{\#\#,}$ $^{**}p < .01$, and $^{\#\#\#,}$ $^{***}p < .001$ as compared to naïve or CTX group. No significant difference among groups could be observed in graph D

In the present study, we evaluated the *in vitro* immunomodulatory effects of RBP on human PBMC and human moDC, and on CTX-induced immunosuppressive mouse model *in vivo*. Human PBMC represent a heterogeneous population of immune cells including B cells, T cells, monocytes, NK cells, and various granulocytes. In a close interplay, these cells orchestrate innate and acquired immune responses that might either be enhanced or reduced by the addition of mushroom compounds to these human PBMC cultures (Yue, Fung, Leung, & Lau, 2008). RBP showed potential immunostimulatory properties *in vitro* in terms of increasing the proinflammatory cytokines TNF-α and IL-1β in human PBMC for 18 hr. However, T-lymphocytes related cytokines such as IL-2 and IL-4 were not affected indicating that RBP might directly and selectively affect monocytes.

Dendritic cells (DCs) are professional antigen-presenting cells (APCs), which play a critical role in the activation and regulation of the adaptive immune system (Steinman & Banchereau, 2007). One important function of dendritic cells is to present antigen and to activate both $CD4^+$ and $CD8^+$ T-lymphocytes. It is believed that exogenous macromolecules including polysaccharides were firstly captured by DC, then further affect other immune cells. Our results showed that RBP could stimulate the immature DC into the mature stage in terms of the releases of IL-12p40 and IL-10 and TNF-α in human moDC.

The toll-like receptors (TLRs) are a group of pathogen recognition receptors expressed in antigen-presenting cells, which play a vital role in recognize pathogens and initiate the innate immune response (Yamamoto et al., 2003). TLR4 is identified on cell membranes, which can identify lipopolysaccharides of Gram-negative bacteria. TLR4 also plays an important role in many natural polysaccharide-induced events. The involvement of this receptor also triggers an innate immune response and the production of various cytokines (Zhang, Qi, Guo, Zhou, & Zhang, 2016). Polysaccharides bind to TLR4 can activate diverse signaling pathways including MAPKs and NF-κB pathways, and mediate series of intracellular actions to induce the morphological changes and cytokines productions in APC (Wei et al., 2016; Zhang et al., 2016). In the present study, we used LPS-RS, a TLR-4 blocker, to inhibit the activation of TLR4 in moDC, and various chemicals to block MAPK and NF-κB pathway. The production of IL-12p40 in RBP-treated moDC was interfered with LPS-RS or Bay11-7082 (rather than other chemical blockers), strongly suggesting that the activation of moDC by RBP were mediated through NF-κB pathway via TLR-4 receptor.

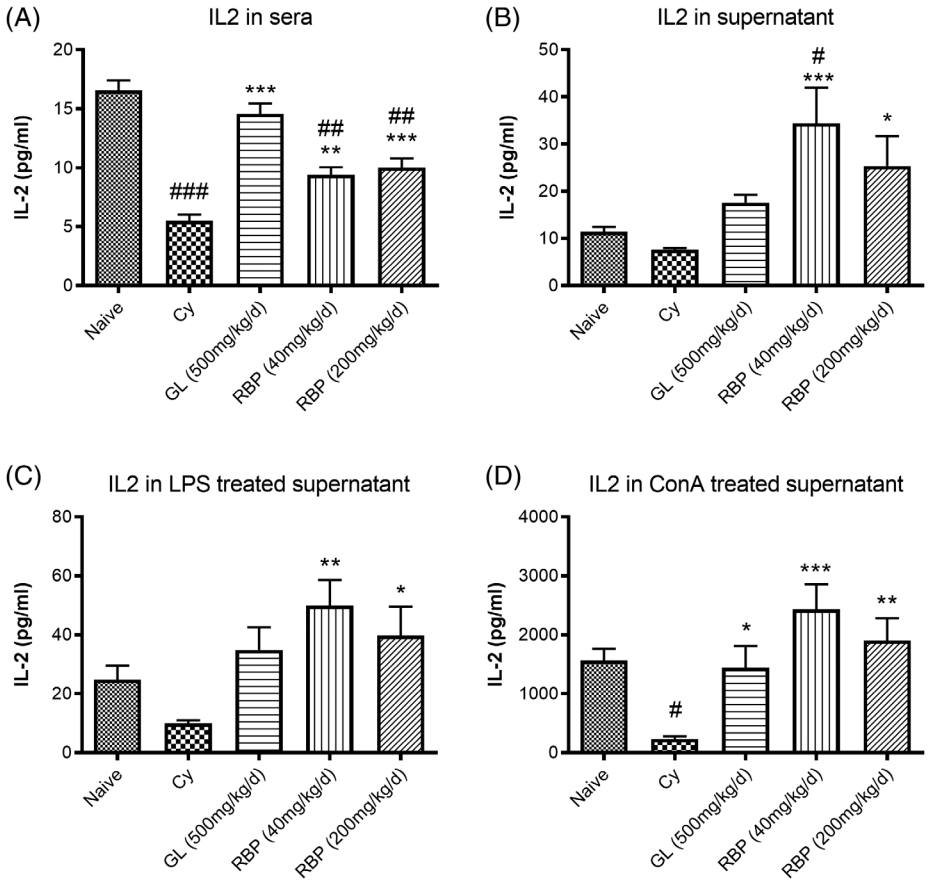

FIGURE 6 Effect of RBP on IL-2 release in sera or supernatant of cultured splenocyte from Cy-treated mice. The production of IL-2 was remarkably increased in the sera (A), the supernatant of cultured splenocytes (B), the supernatant of LPS-stimulated splenocytes (C) and the supernatant of ConA-stimulated splenocytes (D) of Cy-treated mice. All results were presented as mean ± SEM (n = 8). Differences among each group were determined by one-way ANOVA. $^{\#,}$ $^{*}p < .05$, $^{\#\#,}$ $^{**}p < .01$, and $^{\#\#\#,}$ $^{***}p < .001$ as compared to naïve or CTX group

Myelosuppression and immunosuppression are the main side effects of many chemotherapeutic drugs including CTX in cancer treatment (Manente et al., 2017). Polysaccharides are found to have great potential in treating with myelosuppression and immunosuppression (Yang, Hsieh, & Lin, 2015; Ye et al., 2011). Hematopoietic stem cells (HSCs) enable themselves to renew, and to produce mature blood cells including erythrocytes, leukocytes, platelets, and lymphocytes. CD34 is one of HSC cell surface markers, and all colony-forming activity of bone marrow cells is found in the $CD34^{+}$ fraction (Knapp, Strobl, Scheinecker, Bello-Fernandez, & Majdic, 1995). RBP treatment remarkably increased the percentage of $CD34^{bright}CD45^{dim}$ cells from the bone marrow of CTX-treated mice, compared to the model group. The recovery of decreasing of $CD34^{bright}CD45^{dim}$ cells in RBP-treated groups indicated that RBP could protect the myelosuppression caused by chemotherapeutic drugs including CTX. Our results also demonstrate that RBP showed a direct mitogenic activity on splenocytes from immunosuppressive mice in a dose-dependent manner. Besides, RBP also exerted remarkable co-mitogenic effects on LPS- and ConA-stimulated splenocytes. The increased $CD4^{+}$ T-lymphocytes demonstrated that helper T-lymphocytes were activated by RBP. IL-2 is secreted by helper T-lymphocytes, which can promote immune cell proliferation and differentiation. RBP significantly unregulated IL-2 production in CTX-treated mice, indicating RBP could recover the immunosuppressive function *in vivo*. Many requirements considered to be relevant in recent guidelines for best practice in natural products pharmacological research (Heinrich et al., 2020; Izzo et al., 2020) have been taken into account in the present study.

Researchers have found that *Ganoderma lucidum* polysaccharides (GLPS) could enhance the activity of immunological effector cells in CTX-treated mice. GLPS could accelerate the recovery of bone marrow cells and white blood cells and enhance T and B cell proliferation responses (Zhu, Chen, & Lin, 2007). Therefore, GL extract, which contained 8% of polysaccharides (w/w), was used as a positive control in the present study. Our results indicated that the *in vivo* immunostimulatory effects of RBP were comparable to GL extract.

In conclusion, RBP was shown to exert immunostimulatory effects *in vitro*, and restore or enhance the hematopoietic function and modulate cellular and humoral immunity in CTX-treated mice. The underlying mechanisms of the immunostimulatory effects of RBP warrant further investigation. These results indicated that RBP might be useful for boosting the immune system. Therefore, RBP could be developed as an immunomodulator for treating cancer or other immune-related diseases.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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