Reishi Polysaccharides Induce Immunoglobulin Production through the TLR4/TLR2-mediated Induction of Transcription Factor Blimp-1*

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The polysaccharides of *Ganoderma lucidum* (Reishi) possess immunomodulation activities; however, their mode of molecular action in regulating each cellular subset in the immune system is still not clear. Here, we investigate the function of the main polysaccharide fraction of Reishi (Reishi-F3) in B lymphocyte activation/differentiation. We find that Reishi-F3 causes mouse splenic B cell activation and differentiation to IgM-secreting plasma cells, and the process depends on Reishi-F3-mediated induction of Blimp-1, a master regulator capable of triggering the changes of a cascade of gene expression during plasmacytic differentiation. In human peripheral B lymphocytes, although Reishi-F3 fails to induce their activation, it is able to enhance antibody secretion, which is associated with Blimp-1 mRNA induction. The function of Reishi-F3 depends on the Toll-like receptors TLR4/TLR2 as neutralizing antibodies against TLR4/TLR2 block Reishi-F3-mediated induction of Blimp-1 mRNA and Ig secretion. We have shown that interaction of Reishi-F3 with TLR4/TLR2 followed by signaling through p38 MAPK is involved in the induction of Blimp-1 mRNA, whereas signaling through ERK, p38 MAPK, JNK, and IKK complex is involved in Reishi-F3-mediated Ig secretion. Furthermore, the differential mechanism of Reishi-F3 in mouse and human B cell activation is probably due to the presence of Blimp-1 regulatory site in human CD86 promoter. These results establish the signaling and molecular mechanisms of Reishi-F3 on promoting antibody secretion.

*Ganoderma lucidum* (Reishi),2 a popular home remedy, has been known for its beneficial activities in human health for centuries. Accumulated studies attempting to understand the role of Reishi in regulating various body functions revealed that the crude or purified components of Reishi extracts possess anti-tumor and immunomodulating activities (1–3). The effects of Reishi on the immune system have been linked to the induction of cytokine expression and differentiation of macrophages (4, 5), maturation of cultured murine bone marrow-derived dendritic cells, and the immune response initiated by dendritic cells (6, 7), proliferation of murine splenic B cells (8, 9), and natural killer cells activation in treated human cancer patients (10, 11). Despite numerous studies, the precise functional mechanism of Reishi, especially with respect to its cellular receptors and the molecular marker(s) associated with its activities has not been firmly demonstrated. Additionally, it is unclear whether Reishi would directly affect the effector stage of B lymphocytes.

Blimp-1 (B lymphocyte-induced maturation protein-1) has been shown as the master regulator for plasmacytic differentiation (12, 13). Ectopic expression of Blimp-1 is sufficient to induce plasma cell formation and immunoglobulin production from mature B cells (14, 15). Blimp-1 is able to trigger a cascade of gene regulation during plasmacytic differentiation, and several of its direct target genes have been identified, including c-myc, Pax-5, CIITA, Id3, and Spi-B (14, 16–18). Studies from mice deficient in prdm1, the gene encoding Blimp-1, demonstrated that Blimp-1 is required for the formation of antibody-secreting cells and preplasma memory B cells (19). In addition to its crucial role in B lymphocyte terminal differentiation, Blimp-1 has also been shown to play an important role in differentiating U937 cells, a human promonocytic cell line, in response to the stimulation by phorbol 12-myristate 13-acetate (20) and in regulating T cell homeostasis (21, 22). Many signaling pathways involved in immune responses, including IL-2-, IL-5-, IL-6-, and IL-21-induced pathways, and the Toll-like receptor 4 (TLR4)-mediated signaling pathway have been shown to induce the expression of Blimp-1 mRNA (13, 23, 24). The ability of Blimp-1 to promote the differentiation of various cell subsets in response to stimuli led us to test whether the effects of Reishi extracts in B lymphocyte correlate with Blimp-1 expression.

A previous report has demonstrated that incubation of human macrophages with Reishi fraction 3 (Reishi-F3) induced the expression of many phosphotyrosyl proteins and pro-IL-1 production (4). In this study, we used Reishi-F3 (25) to test its functional role in purified murine primary B cells and human peripheral B cells. We found that Reishi-F3 could induce antibody production in purified murine splenic...
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B cells and in human peripheral B cells, both associated with the induction of Blimp-1. The Reishi-F3-mediated process is at least in part through the TLR2 and TLR4 receptors, the key players in recognizing pathogenic microorganisms in the innate immune system, and the signaling pathways downstream to TLR4 and TLR2, like p38 MAPK, appeared to be involved in Blimp-1 induction. Finally, we found that Reishi-F3 could induce mouse splenic B cell proliferation and activation, whereas it failed to do so in human peripheral B cells. Such differential effects of Reishi on CD86 expression between mice and humans might be due to the interaction of Blimp-1 with the human CD86 promoter.

**EXPERIMENTAL PROCEDURES**

Reishi-F3 Preparation—The preparation of the polysaccharide-containing F3 fraction of G. lucidum (Reishi-F3) was performed essentially as previously described (4, 25). Briefly, a crude powder of Reishi extract (6 g) was dissolved in a small volume of double-distilled water, stirred at 4 °C for 12 h, and centrifuged (1000 r.p.m.) for 1 h to remove the insoluble materials. The resulting solution was concentrated at 40–50 °C to a small volume and lyophilized to generate 5 g of dark brown powder. This water-soluble residue was stored at −20 °C for further purification. Briefly, a 2.1-g sample was dissolved in a small volume of 0.1 N Tris buffer (pH 7.0) containing 0.1% sodium azide and purified by gel filtration chromatography at 4 °C using a Sephacryl S-500 column (95 × 2.6 cm) with 0.1 N Tris buffer (pH 7.0) as the eluent. The flow rate was at 0.6 ml/min, and 6.0 ml/tube was collected. After the chromatography, each fraction was subjected to carbohydrate detection with phenol–H₂SO₄. Five fractions were collected (fractions 1–5), concentrated at 40–50 °C to give a small volume, and dialyzed to remove excessive salts and sodium azide and then lyophilized to give 520 mg (25%) of Reishi-F3.

Cell Culture and Reagents—Mouse splenic B cells were purified using B220 microbeads (Miltenyi Biotec) from 6–8-week-old C57/B6 mice, prdm1f/f/CD19Cre⁺ or prdm1f/f/CD19Cre⁻ as previously described (19, 26). Purified splenic B cells (purity >95%) were cultured at RPMI 1640 medium (Invitrogen) containing 10% heat-inactivated fetal bovine serum (Invitrogen), penicillin/streptomycin (100 units/ml), 2 mM l-glutamine, and 50 μM 2-mercaptoethanol at the density of 2 × 10⁶ cells/ml. Cells were stimulated with lipopolysaccharide (LPS) at 2000 rpm at 37 °C. The plates were subsequently read on a plate reader (Molecular Devices) at 450 nm. For the detection of multiple cytokines from Reishi-F3 treated human peripheral B cells, we essentially followed the protocol provided by the manufacturer (Upstate Cell Signaling Solutions). The reading was performed on a Luminex® 100™ instrument.

Flow Cytometry—B cells were harvested and washed in phosphate-buffered saline once and then further resuspended in phosphate-buffered saline plus 2% fetal bovine serum at a density of 10⁶ cells/ml. A total of 10⁵ cells were used for each staining. Antibodies used in this study were as follows: phycoerythrin-conjugated anti-mouse syndecan-1 (BD PharMingen), phycoerythrin-conjugated mouse anti-human CD19 (BD PharMingen), and phycoerythrin-conjugated anti-human CD86 (eBioscience). Cells were then analyzed by FACScalibur (BD Biosciences) and CellQuest software.

Transfection and Luciferase Assay—The human CD86 promoter fragment was isolated from SKW genomic DNA by PCR according to the published sequences (accession number G. lucidum (Reishi-F3) was prepared essentially as previously described (4, 25). Briefly, a crude powder of Reishi extract (6 g) was dissolved in a small volume of double-distilled water, stirred at 4 °C for 12 h, and centrifuged (1000 r.p.m.) for 1 h to remove the insoluble materials. The resulting solution was concentrated at 40–50 °C to a small volume and lyophilized to generate 5 g of dark brown powder. This water-soluble residue was stored at −20 °C for further purification. Briefly, a 2.1-g sample was dissolved in a small volume of 0.1 N Tris buffer (pH 7.0) containing 0.1% sodium azide and purified by gel filtration chromatography at 4 °C using a Sephacryl S-500 column (95 × 2.6 cm) with 0.1 N Tris buffer (pH 7.0) as the eluent. The flow rate was at 0.6 ml/min, and 6.0 ml/tube was collected. After the chromatography, each fraction was subjected to carbohydrate detection with phenol–H₂SO₄. Five fractions were collected (fractions 1–5), concentrated at 40–50 °C to give a small volume, and dialyzed to remove excessive salts and sodium azide and then lyophilized to give 520 mg (25%) of Reishi-F3.

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AF099105) (28). 5′-AGCGCTCGATTCTTGGGATATTATATG-3′ and 5′-CGACAAGCTCTAGTGGTCTATGTTATT-3′ under conditions of 94 °C for 30 min, 50 °C for 30 min, and 72 °C for 30 min for 35 cycles. The amplified product was cloned into Xhol and HindIII sites of pGLO-3B vector (Promega). The transfection procedure is essentially performed as previously described (17). Basically, a total of 2 × 10^5 3T3 cells were seeded in 6-well plates 1 day before transfection. 2 μg of luciferase reporter construct driven by CD86 promoter were cotransfected with various amounts of Blimp-1 expression vectors (pBDP1-F) or the control vector carrying the reverse sequence of Blimp-1 (pBDP1-R) by calcium phosphate method. 0.5 μg of Renilla luciferase reporter driven by thymidin kinase promoter was used in each transfection to normalize the transfection efficiency. After 2 days of transfections, cells were lysed and used for firefly luciferase and Renilla luciferase assay using a Promega Dual-Luciferase Reporter® assay kit. The luminescence was measured with TopCount NXT™.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extract from P3X was prepared as previously described (16) and stored at −80 °C until used. DNA oligonucleotides containing Blimp-1 binding sites were synthesized and labeled with biotin using the LightShift chemiluminescent EMSA kit (Pierce) following the manufacturer’s provided protocol. EMSA was performed essentially following the protocol provided by the LightShift chemiluminescent EMSA kit (Pierce). 10 μg of nuclear extract and 30 fmol of labeled probes alone or with various amounts of excess equivalents of unlabeled oligonucleotides were incubated in the 10× binding buffer plus 1 μg of poly(dl-dc) in 20-μl reaction systems at room temperature for 20 min. For supershift EMSA, 1 μl of rabbit anti-Blimp-1 antibody (29) or control antibody from rabbit was incubated with 10 μg of nuclear extract 5 min before the addition of labeled probes and further incubation on ice for 20 min as described above. The entire binding reaction was loaded on a 6% polyacrylamide gel and run at room temperature in 0.25× TBE at 110 V for 1–1.5 h. The binding complex was then transferred to nylon Hybond-N membrane (Amersham Biosciences) in a Trans-Blot SD semidry electrophoretic transfer cell (Bio-Rad) at 15 V for 30 min and cross-linked in UV Stratalinker 1800 (Stratagene). The method for detection of biotin-labeled probe was according to the manufacturer’s suggestion. The oligonucleotides corresponding to various Blimp-1 binding sites or control in this study were as follows: PRF, 5′-CGCGTACAGAAGGGAAAAGGACTAGCGCG-3′; CD86, 5′-AAATAATTAGAAAGAGAAAACAAACCTC-3′; nonspecific, 5′-AGCTTTAGCCTGCAAATGCGGATATCC-3′.

RESULTS

Reishi-F3 Induces PlasmaCELL Differentiation in Mouse Primary Splenic B Cells—Reishi has been shown to stimulate murine splenic B lymphocyte proliferation (8, 9), but whether Reishi has the activity to promote the plasma cell formation has not been formally addressed. Following our previous published protocols (4, 25, 30), as described under “Experimental Procedures,” we used Reishi-F3 to investigate its mode of action in B lymphocytes. We first tested whether Reishi-F3 could have an effect on inducing the antibody secretion in purified murine B cell culture. As previously described (9), we observed that Reishi-F3 treatment results in B cell activation as shown by the increased expression of the activation marker, CD86, on surfaces (data not shown) and the increased proliferation of murine splenic B cells after 3 days of treatment (about 3.5-fold). Significantly, as shown in Fig. 1A, compared with cells without Reishi-F3 treatment, a dramatic induction of IgM began to be detected by ELISA after treatment of splenic B220+ B cells with Reishi-F3 for 3 days. A further increased IgM induction was observed at day 4 (Fig. 1A). A dose-dependent induction of IgM by Reishi-F3 determined at day 3 was shown in Fig. 1B. Treatment of primary murine splenic B cells cultured ex vivo with the polyclonal mitogen LPS gives rise to a burst of proliferation followed by subsequent differentiation to immunoglobulin-secreting plasma cells (31, 32). We found that the induction of IgM secretion by Reishi-F3 is comparable with the stimulation by LPS (Fig. 1A). A robust induction of expression of plasma cell surface marker, syndecan-1 (CD138), was observed in splenic B cell culture stimulated with Reishi-F3 for 3 days (Fig. 1C). The possibility of LPS contamination during the Reishi-F3 preparation has been carefully eliminated in our studies (4). We used sodium azide during the initial preparation procedures of Reishi to prevent the growth of bacteria. NMR analysis also revealed differential signature of components between LPS and Reishi-F3.3 These results suggest that Reishi-F3 may have a role in promoting plasmacytic differentiation.

The ability of Reishi-F3 to induce plasma cell differentiation in ex vivo splenic B cell culture led us to investigate whether the plasma cell-dependent transcription factor is induced during this process. Blimp-1, a master regulator for plasma cell differentiation, was studied for this purpose. RT-QPCR analysis demonstrated that, like the positive control with LPS, Reishi-F3 induced the expression of Blimp-1 mRNA after 3 days of treatment (Fig. 1D). The induction of Blimp-1 protein was also detected by Western blot analysis after 3 days of Reishi-F3 treatment (Fig. 1E). Studies of B lymphocyte lineage-specific deletion of prdm1 have shown that Blimp-1 is essential for generation of IgM-secreting cells in response to LPS in ex vivo splenic B cell culture (19). We took advantage of this system and determined whether Blimp-1 is required for Reishi-F3-mediated IgM secretion. We found that Reishi-F3 as well as LPS treatment failed to induce IgM secretion of splenic B cells from prdm1 knock-out B cells (prdm1f/f CD19Cre−); in contrast, control B cells (prdm1f/f CD19Cre+) differentiate normally (Fig. 1F). These data together indicated that Reishi-F3 could trigger a signaling cascade to activate plasmacytic differentiation, which required the induction and expression of the plasma cell transcription factor Blimp-1.

3 W.-B. Yang, unpublished data.
B cells from C3H/HeJ mice with mutated TLR4 signaling failed to respond to Reishi in the proliferation assay (8). Additionally, rat anti-mouse TLR4 monoclonal antibody inhibited the proliferation of BALB/c mouse B cells under Reishi treatment (8). Since a recent paper indicated that TLRs have a direct role in B-cell activation and antibody production (33), we tested Reishi-mediated Ig Production through Blimp-1 Induction.
whether TLRs, such as TLR2 and TLR4, which recognize carbohydrate ligands, are involved in the effects of Reishi-F3 on B cells. TLR2 can recognize a broad range of ligands, including peptidoglycan, lipoteichoic acid, or the lipopolysaccharide component present in various pathogens such as *Leptospira interrogans* (34–36). We found that the induction of IgM secretion upon Reishi-F3 treatment is blocked by the treatment of anti-TLR4 and anti-TLR2 antibodies in a dose-dependent manner (Fig. 2A). The effect of inhibition is neither additive nor synergetic upon the treatment of anti-TLR4 and anti-TLR2 together (Fig. 2A), suggesting that a common signaling pathway was utilized upon the conjugation of TLR4/TLR2 with the ligands in Reishi-F3. LPS-dependent induction of IgM secretion could only be blocked by anti-TLR4 antibody (Fig. 2B). These results demonstrated that, unlike LPS, Reishi F3 clearly uses an additional set of receptor(s) for stimulation of plasmacytic differentiation in B cells.

To determine whether Reishi-F3-mediated induction of Blimp-1 is through the TLR4 and/or TLR2, the levels of Blimp-1 mRNA induction by Reishi-F3 plus the treatment of anti-mouse TLR4 or TLR2 antibody were monitored by RT-QPCR. Similar to the effects of anti-mouse TLR4 antibody in LPS-treated B cells, the addition of neutralizing antibodies to TLR4 and TLR2 resulted in about 2-fold repression of Blimp-1 mRNA induction in response to Reishi-F3 treatment (Fig. 2B), indicating that both TLR4 and TLR2 signaling pathways ultimately trigger the expression of the master regulator of plasma cell differentiation, Blimp-1, in B cells.

Signaling through TLR2 or TLR4 can activate the signaling cascade of MyD88/TIRAP-IRA1-TRAF6-TAK1 (36, 37), which is involved in the activation of mitogen-activated protein kinases (MAPKs), including p38 (38), MEK-ERK1/2 (39), and JNK (40). In parallel, IKK complexes are activated and then cause the activation of NF-κB (36, 37). Since a relatively common upstream signaling pathway is involved upon the initiation of TLR2/TLR4 signaling, we sought to determine which downstream pathway in TLR4/TLR2 signaling is involved in Blimp-1 induction and Ig secretion. The effects of p38 MAPK inhibitor (SB203580) (41), MEK-ERK1/2 inhibitor (PD98059), JNK inhibitor (SP600125), and NF-κB inhibitor on Reishi-F3-dependent splenic B cell differentiation and Blimp-1 mRNA induction were examined. We found that SB203580 (1 μM) dramatically inhibits Blimp-1 mRNA induction by Reishi-F3 and LPS stimulation (Fig. 3A), suggesting that the p38 MAPK pathway, may, in part, play a role in inducing Blimp-1 expression in this setting (Fig. 3A). SB203580 significantly blocked the IgM secretion by Reishi-F3 in a dose-dependent manner (Fig. 3A).

In parallel, we examined whether other above mentioned pathways are involved in Blimp-1 induction. Treatment of JNK inhibitor, SP600125, resulted in reduction of the Reishi-F3-mediated IgM secretion, whereas it had no effect on the expression of Blimp-1, when compared with its negative control group (Fig. 3B). Similarly, MEK-ERK1/2 inhibitor, PD98059 at 50 μM, caused a slight reduction of Reishi-F3-mediated Ig production (to 72%) but had no influence on Blimp-1 mRNA expression (data not shown). Finally, we examined the role of NF-κB in Reishi-F3-mediated effect. Three different agents known to block the activity of NF-κB, MG132 (42), helenalin (43), and NF-κB activation inhibitor (44), were used with this purpose. We found that all three agents caused significant inhibition of Ig production mediated by Reishi-F3 (Fig. 3C), whereas they had practically no influence on the levels of Blimp-1. Altogether, these data suggested that Reishi-F3-induced Blimp-1 expression by interaction with TLR4/TLR2 and signaling through p38 MAPK in B cells, whereas a broader signaling regulation, including p38 MAPK, JNK, ERK1/2, and NF-κB, was used by Reishi-F3 to produce Ig.

The Function of Reishi-F3 in Primary Human Peripheral B Cells—It is important to evaluate whether Reishi-F3 has effects on the activation and/or differentiation of human peripheral B cells isolated from healthy donors. Purified human CD19+ cells from peripheral blood were treated with Reishi-F3, and the expression of B cell activation marker CD86 was analyzed by flow cytometry after various time points. Cells treated with CD40 ligand (CD40L) and IL-4 were used here as the positive control for B cell activation. Treatment with Reishi-F3 caused only a slight increase of cell number at day 6 (about a 10% increase), whereas the treatment with CD40L plus IL-4 resulted in around a 50% increase of cell number, compared with cells without treatment. Although the significant induction of B-cell activation marker, CD86, was observed in the positive control groups, CD40L plus IL-4–stimulated B cells (Fig. 4A, shown as a gray bar), peripheral B cells stimulated with Reishi-F3 showed no effect on the expression of CD86 at either the early (16-h) or late (6-day) time points of treatment (Fig. 4A, black bar). A similar observation was found in detection of the expression of another activation marker, CD69 (data not shown). Therefore, in contrast to the observation with mouse splenic B cells, Reishi-F3 could not activate human peripheral B cells.

The effect of Reishi-F3 on the production of various cytokines or chemokines in peripheral B lymphocytes was monitored by analysis of the array of their expression. Cultured supernatants from CD19+ B cells treated with Reishi-F3, CD40L + IL-4, or sham treatment for 6 days were harvested for...
the test run on Luminex 100 instrument. Reishi-F3 failed to induce the expression of B cell activation markers, but it could sufficiently induce the secretion of certain cytokines or chemokines (Fig. 4B). We found no dramatic induction on a panel of cytokines or chemokines, including IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-7, IL-10, IL-12p70, IL-13, IL-15, IP-10, MCP-1, RANTES (regulated on activation normal T cell expressed and secreted), and tumor necrosis factor-α, by Reishi-F3 treatment (data not shown). However, a robust induction of IL-6, IL-8, and MIP-1α by Reishi-F3 treatment was detected (Fig. 4B). The induction of IL-8 appeared to be limited to Reishi-F3 treatment. Combining the data indicating that Reishi-F3 can enhance IL-1 production in human macrophage (4) and elicit global changes of cytokine and chemokine production in primary human dendritic cell culture4 indicates that the production of IL-6, IL-8, and MIP-1α by Reishi-F3 should be derived from purified B cells rather than from other minor cellular subsets.

We next tested whether Reishi-F3 could induce Blimp-1 expression in purified human CD19+ B cells. Fig. 5A showed that treatment of Reishi-F3 could induce the expression of Blimp-1 mRNA by 4-fold from at least three independent donor B lymphocytes after 6 days of culture monitored by RT-QPCR, whereas the treatment with CD40L + IL-4 down-regulated Blimp-1 mRNA expression (data not shown). The ability of Reishi-F3 to induce the secretion of immunoglobulins was monitored by ELISA from the supernatant of cells cultured with Reishi-F3 for 6 days. Interestingly, although Reishi-F3 failed to induce human peripheral B cell activation (Fig. 4A), it caused a dramatic induction of IgM and IgG (Fig. 5B). Treatment of human peripheral B cells with Reishi-F3 could not trigger a global programming of plasma cell differentiation, since we did not observe the up-regulation of plasma cell surface marker, CD138, after Reishi-F3 treatment (data not shown). Therefore, unlike its effect on both activating and differentiating B cells in mouse splenic B cells, Reishi-F3 could only induce the secretion of Ig in human peripheral B cells. To compare the effect of LPS and Reishi-F3 on human B cells, we performed the same

4 A. Yu and H.-H. Lin, unpublished data.
set of experiments with human purified peripheral B cells stimulated with LPS for 6 days. The differences of effects of LPS and Reishi-F3 on Blimp-1 mRNA induction, antibody production, and cytokine/chemokine production are listed in Table 1. Our results show that LPS caused a comparable amount of induction of Blimp-1 and induction of IL-8 and MIP-1α, whereas it

FIGURE 3. Reishi-F3-mediated Blimp-1 induction through p38MAPK. A, Reishi-F3-mediated Blimp-1 mRNA induction and IgM production is through p38 MAPK. Splenic B cells were pretreated with the p38 MAPK inhibitor SB203580 (10, 5, and 1 μM) 1 h before the addition of LPS (2.5 μg/ml) or Reishi-F3 (20 μg/ml). After 3 days of culture, the RNAs from cell pellets were isolated for RT-QPCR analysis to determine the levels of Blimp-1 mRNA. L32 mRNA was used for normalization. The supernatants from the culture were collected for ELISA determination of IgM. B, Reishi-F3 mediated IgM production, in part, through JNK. JNK inhibitor II (SP600125; 5 μM) and its negative control (N’-methyl-1,9-pyrazoloanthrone, stress-activated protein kinase inhibitor II; 5 μM) were used to treat splenic B cells, which were then harvested for analysis of Blimp-1 mRNA and IgM production as described in A. C, NF-κB-mediated Reishi-F3-induced IgM production. MG132 (1 μM), helenalin (1 μM and 100 nM), and NF-κB activation inhibitor (1 μM and 100 nM) were used to pretreat cells, which were then harvested as described in A for determination of IgM. MG132 (1 μM), helenalin (100 nM), and NF-κB activation inhibitor (100 nM) were used for determination of Blimp-1 mRNA. Error bar, S.D. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
failed to promote the production of Ig, again supporting the notion of differential mode of actions between Reishi-F3 and LPS.

We next sought to investigate whether Reishi-F3 also utilized TLR4 and TLR2 pathways to induce the Ig secretion in human peripheral B cells, since it appeared that the effects of Reishi-F3 in mouse splenic B and human peripheral B cells differed. Fig. 6A showed that Reishi-F3-dependent induction of IgM and IgG was mediated through the TLR4/TLR2 signaling pathways, since a dose-dependent inhibition of production of Ig was observed upon treatment of anti-TRL4 and anti-TRL2 neutralizing Abs. Like the results from mouse splenic B cells, the combined treatment with anti-TRL4 and anti-TRL2 did not result in further inhibition of Reishi-F3-mediated Ig production in human B cells, suggesting a common signaling pathway elicited by both receptors.

We further found that Reishi-F3-mediated induction of IL-6 and MIP-1α signals through TLR2, since neutralizing antibody to TLR2 significantly blocked their induction, whereas the induction of IL-6, IL-8, and MIP-1α did not appear to involve TLR4 (Fig. 6B). Taken together, these results suggested that Blimp-1 could be a molecular marker associated with Reishi-F3-mediated induction of Ig, cytokine, and chemokine secretion in human peripheral B cells, and the effect of Reishi-F3 in promoting Ig production is through TLR2/TLR4, whereas in the production of cytokine/chemokine it may be through TLR2.

**Blimp-1 Represses Human CD86 Promoter**—The fact that Reishi-F3 was able to activate mouse CD86 induction in splenic B cell culture, whereas it failed to do so in human peripheral B cells, despite the presence of Blimp-1 induction in both experiments, led us to investigate the differential molecular mechanisms involved in this process. Previous results from microarray analysis of Blimp-1-dependent gene expression indicated that Blimp-1 could repress CD86 expression in a panel of human B cell lines (18). We therefore tested whether Blimp-1 could repress CD86. Human CD86 promoter fragment was isolated by amplification of the genomic DNA from human SKW cells and cloned to pGL3B-Luc. We found that Blimp-1 was able to repress the luciferase activity driven by the human CD86.
promoter (Fig. 7A), since cells expressing forward cDNA sequences of Blimp-1 showed reduced reporter activity compared with cells expressing reverse sequences. CIITA promoter III was used as a positive control for Blimp-1-dependent repression (14).

One putative Blimp-1 binding site, GAAAGAGAAA, was found at −94 to −103 bp in the human CD86 promoter (Fig. 7B). EMSA using nuclear extracts from P3X, a mouse plasma-cytoma line expressing large amounts of endogenous Blimp-1 protein, and a labeled probe containing the putative Blimp-1 site showed a major binding complex (Fig. 7C, lane 1, indicated by a star). This major binding complex could be blocked by incubation with excess amounts (10 times more) of unlabeled PRF oligonucleotides, a previously identified Blimp-1 binding site located on the mouse c-myc promoter (16) (Fig. 7C, lane 2). This binding complex appeared to be Blimp-1-specific, since antiserum against Blimp-1 showed a supershifted band (indicated by a dot), whereas a control antibody did not (Fig. 7C, lanes 3–5). To compare the binding affinity of Blimp-1 in human CD86 and mouse c-myc genes, we used P3X nuclear extracts incubated with a labeled PRF probe along with various amounts of excess unlabeled oligonucleotides corresponding to either PRF or CD86 sites. Nonspecific, unlabeled oligonucleotides were used here as the negative control. We found that the CD86 site appeared to possess lower affinity (about 8–10 times lower) than the PRF site for Blimp-1 binding in vitro (Fig. 7D). Taken together, we identified a Blimp-1 binding site on the human CD86 promoter that may mediate Blimp-1-dependent transcriptional repression of human CD86. Interestingly, this binding site was not conserved in the mouse Cd86 promoter (GenBank™ accession number AY741809), and we could not find other putative Blimp-1 binding sites in the mouse Cd86 promoter sequences (45). This observation suggested that differential regulation of the B cell activation marker, CD86, by Blimp-1 exists between species and might account for the mechanism of Reishi-F3 in activating mouse but not human CD86 expression.

**DISCUSSION**

It has been shown that Reishi-F3 could enhance mouse splenic B cell proliferation (9, 46), and our results extend the knowledge of Reishi-F3 effect on B lymphocytes (i.e. Reishi could enhance immunoglobulin production via activation of Blimp-1). The importance of Blimp-1 in enhancing Ig secretion by Reishi-F3 was determined in prdm1 knockout splenic B cell culture that failed to induce Ig production by Reishi-F3 (Fig. 1F). We found that the mechanism of Reishi-F3 function in enhancing antibody production in mouse splenic and human peripheral B cells appears to be different, because Reishi-F3 enhanced mouse splenic B cell proliferation, activation, and

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**FIGURE 5. Reishi-F3-induced Blimp-1 expression and Ig production in human peripheral B cells.** A, purified human peripheral B cells were treated with Reishi-F3 (20 μg/ml) for 6 days, and the levels of Blimp-1 mRNA expression were determined by RT-QPCR using the primer and probe sets for Blimp-1 and normalized to the expression level of PPIA. The results shown are relative -fold induction of Blimp-1 mRNA compared with B cells with sham treatment from three independent experiments. B, B cell culture supernatants were harvested as described in the legend to Fig. 4B, and the levels of IgM and IgG were determined by ELISA. The results shown are relative -fold induction normalized to B cells without Reishi-F3 addition from three independent donors.

**TABLE 1**

Comparison of the effects of LPS and Reishi-F3 on human peripheral B cells

|                | LPS | Reishi-F3 |
|----------------|-----|-----------|
| Blimp-1 mRNA induction | +   | +         |
| Ig induction   | −   | −         |
| Cytokine/chemokine production | IL-6 | IL-8 | MIP-1α | IL-6 | IL-8 | MIP-1α |
|                | +   | +         | +     | +     | +     | +     |

Purified human peripheral B cells were treated with LPS (2.5 μg/ml), Reishi-F3 (20 μg/ml), or sham treatment for 6 days. The levels of Blimp-1 mRNA induction, Ig production, and cytokine/chemokine production were measured as described under “Experimental Procedures.” The differential effects between LPS and Reishi-F3 were compared. +, detection of induction; −, uninduced.
differentiation, whereas it only had an effect on promoting human peripheral B cells to secret IgGs. However, the effect of Reishi-F3 on promoting Ig secretion in both systems seemed to depend on TLR4 and TLR2 signaling. Since Blimp-1 can trigger a cascade of regulatory gene expression during plasmacytic differentiation (12), it will be interesting to determine and compare the difference of changes of gene expression profile in primary human B cells upon treatment with Reishi-F3 and other immunomodulators like cytokines.

Similar to and in agreement with the observations of others showing that TLR4-neutralizing antibody suppressed the proliferation of Reishi-F3-induced splenic B cell proliferation (8), we observed that B cell proliferation, Blimp-1 induction, and Ig production by Reishi-F3 were, in part, dependent on TLR4 sig-

FIGURE 6. Reishi-F3 functions in human peripheral B cells through TLR4 and TLR2 signaling. A, purified human B cells were pretreated with anti-TLR4 (5 and 1 µg/ml), anti-TLR2 (5 and 1 µg/ml), anti-TLR4 + anti-TLR2 (5, 2.5, and 1 µg/ml), or equal amounts of control antibody for 1 h and then added with Reishi-F3 (20 µg/ml) for 6 days. The cultured supernatants were collected for ELISA determination of the levels of IgM and IgG. B, the B cell culture supernatants harvested as described in A were subjected to analysis of the production of cytokines or chemokines. Data shown represent relative-fold production upon TLR2/4 antibody treatment after normalization to the control antibody treatment group. Error bar, S.D. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Reishi-mediated Ig Production through Blimp-1 Induction

A

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aattatatctatatttttttttatttttttattttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt

B

C

D

FIGURE 7. Blimp-1 represses human CD86 expression. A, the sequence of human CD86 promoter. The transcriptional start site is indicated by an arrow as +1. The Blimp-1 binding site, −94 to −103 bp, is underlined. B, Blimp-1 represses CD86 promoter-dependent luciferase activity. Human CD86 promoter reporter plasmid was co-transfected with the indicated amounts of Blimp-1 expression plasmid (F) or control plasmid (R) and Renilla luciferase reporter plasmid to 3T3 cells. 48 h after the transfection, cell lysates were harvested for determining the luciferase activity. CIITA promoter III-dependent luciferase plasmid (CIITA-Luc) was used as the positive control. The results are representative of one of three independent experiments. S.D. is shown. C, Blimp-1 binds to the CD86 promoter in vitro. Nuclear extracts from P3X plasmacytoma cells were incubated with a labeled CD86 probe and subjected to EMSA. A protein complex indicated as a star was observed (lanes 1 and 3). Cold competitors, including oligonucleotides containing the mouse c-myc promoter with the Blimp-1 binding site (PRF), showed reduced binding (lane 2). The complex was altered by the addition of Blimp-1-specific antibody but not by the control antibody (lanes 4 and 5). D, measurement of the affinity for Blimp-1 in the CD86 promoter by competitor titrations. EMSA was performed using the nuclear extracts from P3X and the PRF-labeled probe. Molar excess equivalents of the unlabeled oligonucleotide corresponding to the PRF site, the Blimp-1 binding site in the CD86 promoter, and nonspecific oligonucleotide (NS) are indicated.

naling in mouse splenic B cell culture (Fig. 2). We also observed that TLR4 was involved in Blimp-1 induction and Ig secretion by Reishi-F3 in human peripheral B cells (Fig. 6). In addition to TLR4, we identified, for the first time, that Reishi functions on either mouse or human B cells also via TLR2 (Figs. 2B and 6), which differs from the function of Reishi in dendritic cells and macrophages through the TLR4 receptor only (7, 30). This observation suggested that multiple biologically active components are present in Reishi-F3 for B lymphocytes. The carbohydrate compositions of Reishi-F3 are 7.1% L-fucose, 3.1% D-xylose, 15.1% D-Man, 13.5% D-Gal, 1.2% D-GalNac, and 58.1% D-Glc, as described previously (30). It will be interesting to further determine the definitive bioactive components as well as the ligands for TLR4/TLR2 in Reishi-F3 for B cell activation/differentiation. Additionally, it has been demonstrated that the simultaneous addition of ligands for TLR4 and TLR2 revealed antagonism between those stimuli during mouse B cell maturation (47), suggesting a MyD88-independent pathway for TLR4 signal transduction. Since the simultaneous addition of neutralizing Abs for TLR4 and TLR2 did not further inhibit Reishi-F3-mediated Ig production (Figs. 2A and 6A), it appears that the unidentified ligands for TLR4 and TLR2 from Reishi polysaccharide extract transduce a common MyD88-dependent signaling pathway.

Blimp-1 is known as a master regulator during plasmacytic differentiation (12, 13). Using Reishi-F3-mediated function in B lymphocytes as a model, we identified the molecules that could regulate Blimp-1 expression during plasmacytic differentiation. Our results suggest that the p38 MAPK pathway was involved in the induction of Blimp-1 expression, since the inhibitor of p38 MAPK, SB203580, blocked Reishi-F3- and LPS-dependent induction of Blimp-1 mRNA (Fig. 3A). This result suggested that the downstream effectors of p38 MAPK, such as MEF-2C, ATF-2, and Elk-1 (48), could be involved in regulating Blimp-1 transcription, which provides a clue for understanding the signaling pathways leading to the induction of this important transcription factor. Although we observed that other MAPK-relevant pathways are not involved in the regulation of Blimp-1 expression and that various NF-κB inhibitors failed to influence Blimp-1 expression, despite their effects on suppression of Ig (Fig. 3C), it remains likely that these pathways might have redundant functions and impact the significance of each individual pathway/molecule in this context of Blimp-1 induction. The signaling pathway elicited by Reishi-F3 in the production of Ig and induction of Blimp-1 mRNA in B lymphocytes is summarized in Fig. 8.

The effect of Reishi-F3 on the up-regulation of IL-1 secretion and IL-1-converting enzyme expression in murine and human macrophages has been investigated (4). It has been shown that Reishi-F3 mediated production of IL-1 is through the receptor TLR4 followed by a cascade of protein kinase C and p38 MAPK signaling in macrophages (4). The signaling pathways utilized by Reishi-F3 in inducing IL-1 production in macrophages seemed to differ from that of Blimp-1 induction in B lymphocytes. We have observed that in addition to TLR4, TLR2 is also involved in Blimp-1 induction in B cells. This observation suggests that the immunomodulating agent, such as Reishi-F3, uses different receptors and pathways in regulating various
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FIGURE 8. The signaling pathway utilized by Reishi-F3 in the production of Ig and induction of Blimp-1 mRNA in B lymphocytes. Reishi-F3 utilizes TLR4/TLR2 to initiate a common upstream signaling pathway, which in turn activates IKK complex and MAPKs, including ERK, JNK, and p38. Only p38 is involved in the induction of transcript of Blimp-1, which is a master regulator for plasma cell formation, whereas NF-κB and MAPKs are all required for the production of Ig.

downstream genes in each cell type. Blimp-1 has been previously shown to be important for triggering macrophage differentiation (20). We did not observe a significant induction of Blimp-1 mRNA after 48 h of Reishi-F3 treatment in U937, in contrast to its dramatic induction (about 40-fold) by phorbol 12-myristate 13-acetate (data not shown), suggesting that the role of Reishi-F3 in macrophages may be Blimp-1-independent. The biologically active components from Reishi-F3 for each cellular subset, such as macrophages or B lymphocytes, remain to be identified.

We have found that Reishi-F3 selectively signals through TLR2 in production of cytokines or chemokines in human B cells, suggesting its additional roles in regulating the proliferation of plasma cells and activating leukocytes recruitment in the immune system. The signaling pathway leading to the induction of IL-6, IL-8, and MIP-1α by Reishi-F3 in human B cells, however, awaits further characterization. Taken together, we report here that the immunomodulating agent, Reishi-F3, can enhance the production of Ig, and this induction is dependent on the activation of the plasma cell master regulator, Blimp-1, in mouse splenic and human peripheral B cells. Blimp-1 induction is mediated, in part, through the TLR4 and TLR2 receptor and the p38 MAPK pathway. In summary, given the ability of Reishi-F3 to enhance immunoglobulin production, it is possible that Reishi-F3 may be developed as a therapeutic candidate for the treatment of immunoglobulin-deficient diseases. In addition, the differential regulation of CD86 by Reishi-F3 between mouse and human B cells observed in this study suggests that precautions must be taken when extrapolating the results of animal studies to human.

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