Comparative Analysis of Transcriptomes of Macrophage Revealing the Mechanism of the Immunoregulatory Activities of a Novel Polysaccharide Isolated from *Boletus speciosus* Frost

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**ABSTRACT**

**Background:** The mechanism of the immunoregulatory activities of polysaccharide is still not clear. **Materials and Methods:** Here, we performed the B-cell, T-cell, and macrophage cell proliferation, the cell cycle analysis of macrophage cells, sequenced the transcriptomes of control group macrophages, and *Boletus speciosus* Frost polysaccharide (BSF-1) group macrophages using illumina sequencing technology to identify differentially expressed genes (DEGs) to determine the molecular mechanisms of immunomodulatory activity of BSF-1 in macrophages. **Results:** These results suggested that BSF-1 could promote the proliferation of B-cell, T-cell, and macrophages, promote the proliferation of macrophage cells by abolishing cell cycle arrests in the G0/G1 phases, and promote cell cycle progression in S-phase and G2/M phase, which might induce cell division. A total of 12,498,414 and 11,840,624 bp paired-end reads were obtained for the control group and BSF-1 group, respectively, and they corresponded to a total size of 12.5 G bp and 11.8 G bp, respectively, after the low-quality reads and adapter sequences were removed. Approximately 81.83% of the total number of genes (8,257) were expressed reads per kilobase per million mapped reads [RPKM ≥1] and more than 1366 genes were highly expressed (RPKM >60) in the BSF-1 group. A gene ontology-enrichment analysis generated 13,042 assignments to cellular components, 13,094 assignments to biological processes, and 13,135 assignments to molecular functions. A Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis showed that the mitogen-activated protein kinase (MAPK) signaling pathways are significantly enriched for DEGs between the two cell groups. **Conclusion:** An analysis of transcriptome resources enabled us to examine gene expression profiles, verify differential gene expression, and select candidate signaling pathways as the mechanisms of the immunomodulatory activity of BSF-1. Based on the experimental data, we believe that the significant antitumor activities of BSF-1 in vivo mainly involve the MAPK signaling pathways. **Key words:** Differentially expressed gene, macrophage, mechanism, polysaccharide, RNA-Seq

**SUMMARY**

- *Boletus speciosus* Frost-1 (BSF-1) could promote the proliferation of B-cell, T-cell, and macrophages, promote the proliferation of macrophage cells by abolishing cell cycle arrests in the G0/G1 phases, and promote cell cycle progression in S-phase and G2/M phase, which might induce cell division.
- Approximately 81.83% of the total number of genes (8257) were expressed reads per kilobase per million mapped reads [RPKM ≥1] and more than 1366 genes were highly expressed (RPKM >60) in the BSF-1 group. A gene ontology-enrichment analysis generated 13,042 assignments to cellular components, 13,094 assignments to biological processes, and 13,135 assignments to molecular functions. A Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis showed that the mitogen-activated protein kinase (MAPK) signaling pathways are significantly enriched for DEGs between the two cell groups.

**INTRODUCTION**

Recently, polysaccharides obtained from microorganisms, fungi, and plants have been regarded as the most effective immune-regulating substances, and they have been shown to be clinically effective. Polysaccharides have anti-inflammatory, antihypoglycemic, antibacterial, and antitumor activities, and the basic mechanisms underlying the therapeutic effects of fungal polysaccharides, including their antitumor and immunostimulatory activities, likely occur through the modulation of reads per kilobase per million mapped reads [RPKM ≥1] and more than 1366 genes were highly expressed (RPKM >60) in the BSF-1 group. A gene ontology-enrichment analysis generated 13,042 assignments to cellular components, 13,094 assignments to biological processes, and 13,135 assignments to molecular functions. A Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis showed that the mitogen-activated protein kinase (MAPK) signaling pathways are significantly enriched for DEGs between the two cell groups.

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**Abbreviations used:** BSF-1: *Boletus speciosus* Frost polysaccharide.

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and stimulation of the complement system through macrophages.[1]
The antitumor and immunomodulatory properties are generally related to their ability to activate immune system.[2] The immune system is a system of many biological structures and processes within an organism that protects against disease. Most importantly, the macrophages occupy a unique position in the immune system because they can initiate natural immune responses and then act as effector cells that help manage immune responses,[3,4] such as inflammation, angiogenesis, and fighting an infection. Macrophages can eliminate the advanced stage of tumors because of their powerful functions, including phagocytosis and the release of numerous proinflammatory cytokines (interleukin), tumor necrosis factor, cytotoxic and inflammatory molecules (nitric oxide), and (reactive oxygen species) that contribute to direct and/or indirect antitumor activities.[3,4]

Our group recently isolated a novel polysaccharide from Boletus species Frost (BSF-1), which has a backbone of (1 →4)-α-L-mannopyranose residues, which branches at O-6 and the branches were mainly composed of one with (1 →1)-α-D-galactopyranose residue.[5] BSF-1 also exhibits significant antitumor activities in vivo and it can significantly promote the macrophage cells in vitro. However, the immunomodulatory activity and mechanism of BSF-1 remain unclear. Here, the goal of this study was to determine the molecular mechanisms underlying the immunomodulatory activity of BSF-1 in macrophages.

MATERIALS AND METHODS

Materials

The reagent 2-(2-methoxy-4-nitrophenyl)- 3-(4-nitrophenyl)-5-
(2,4-disulfonic acid benzene)-2H-tetrazolium monosodium salt (Cell
Counting Kit [CCK]-8) was purchased from Dojindo Molecular
Technologies, Inc., (Tokyo, Japan); D-Hanks solution, RPMI 1640
medium, fetal calf serum, and dimethyl sulfoxide were purchased from
Gibco (NY, USA). Penicillin G and streptomycin were purchased from
Sigma (China). All other chemicals and solvents were of analytical grade,
and BSF-1 was prepared in our laboratory as previously described.[7]

Cell lines and reagents

The RAW264.7 cell line, B(Raji) cell line, and T (Jurkat) cell line were
cultured in RPMI 1640 medium containing 10% fetal bovine serum, 1%
penicillin (100 IU/mL), and streptomycin (100 mg/L) in a humidified
atmosphere with 5% CO₂ at 37°C before use.

Pharmacological evaluation for B-cells, T-cells, and
RAW264.7 cells’ stimulation

The cytotoxic effects of BSF-1 on B-cells, T-cells, and RAW264.7 cells were
determined by CCK-8-based colorimetric method. In brief, B-cells,
T-cells, and RAW264.7 cells suspended in RPMI-1640 medium at a
density of 1 × 10⁵ cells/ml were pipetted into a 96-well plate (100 μL/well)
and inoculated at 37°C in a humidified 5% CO₂. After incubation for 24,
100 μL of test sample with different concentrations (0.3125–20 μg/mL
in fresh growth medium) was added into each well separately in an
incubator at 37°C in a humidified 5% CO₂ for 48 h. RPMI-1640 and
2 μg/mL lipopolysaccharides (LPS) were used as negative and positive
controls, respectively. Then, 20 μL of CCK-8 reagent was added to each
well, and the plate was further incubated for another 1–4 h. Absorbance
of the colored solution at 490 nm was measured on a 96-well microplate
reader (Bio-Rad, Tokyo, Japan). All experiments were performed in triplicate, and the inhibitory rate was calculated as follows:

\[
\text{Cell proliferation activity (\%) = } \frac{[A2 - A0]/[A1 - A0]}{100}
\]

Where, A2 is the average optical density of BSF-1-treated cells, A0 is the
average optical density of the control wells (culture medium without
cells), and A1 is the average optical density of the negative control (culture medium containing cells).

Macrophage phagocytosis assay

The RAW264.7 cells were inoculated in the presence of varying
concentrations of BSF-1 as mentioned above. RPMI-1640 and LPS
were used as negative and positive controls, respectively. After 24 h,
supernatants were removed, 100 μL of 0.075% neutral red solution was
added to each well, and the cells were cultured for a further 1 h. And then,
the plate was washed three times with phosphate-buffered saline (PBS)
and patted gently on tissues to let them drain. Finally, 100 μL of cell lysis
buffer (0.1 mol/L acetic acid and ethanol in the ratio of 1:1) was added to
each well at 4°C for 2 h. The absorbance at 540 nm was determined using
microplate ELISA reader. All determinations were conducted in triplicate.

RAW264.7 cell cycle analysis by flow cytometry

The effect of BSF-1 on the cell cycle distribution was assessed by flow
cytometry after staining the cells with propidium iodide (PI). RAW264.7 cells were seeded in 6-well plates (5 × 10⁴ cells/well) and allowed to grow for 1 day
before being exposed to BSF-1 (1, 5, or 10 μg/mL) for 72 h. After incubation,
the treated cells were harvested, washed twice with PBS, and fixed in cold
70% ethanol for 4 h or overnight at 4°C. After an additional wash in cold
PBS, the cells were resuspended in 0.5 mL of staining buffer containing 10
μL of RNase A and 25 μL of PI, and then incubated for 30 min in dark at 37°C.
The DNA content of the cells was measured using a flow cytometer (BD,
Franklin Lakes, NJ, USA), and the population of cells in each phase was
calculated using the ModFit LT software (Verity Software House, Inc, NY,
USA) program. Each experiment was conducted for three times.

RNA extraction, library preparation, and
sequencing

Trizol reagent (Invitrogen, Canada) was used to extract the
total RNA, and 1% agarose gels were used to investigate the RNA
contamination and degradation. RNA purity was detected on a
Nano Photometer spectrophotometer (Implen, Westlake Village,
CA, USA). After examining the RNA purity and concentration, the
RNA 6000 Nano Assay Kit with NanoDrop2000 (Thermo Scientific
NanoDrop 2000c) was used to assess the RNA integrity. A total of 3 μg of RNA per sample was used for the RNA sample preparations as
input material.[8] Following the manufacturer’s recommendations, the
transcriptome libraries were generated using the Illumina TruSeq™
RNA Sample Preparation Kit (Illumina, San Diego, CA, USA).
Clustering of the index-coded samples was completed using the TruSeq PE Cluster Kit
v3-cBot-HS (Illumina) on a Bot Cluster Generation System. The libraries
were sequenced on an Illumina HiSeq 2000 platform after clustering, and
100 bp paired-end reads were generated.[9]

Transcriptome data analysis

In-house Perl scripts were used to process the raw data in FASTQ format
to remove low-quality reads, which contained poly-N stretches (partially
un-sequenced regions) and adapter sequences. All the downstream
analyses are based on the high-quality clean sequences.

Differential expression and quantification analysis of
the transcripts

Prior to performing the differential gene expression analysis, the
read counts were adjusted using an edgeR program package for each
sequenced library through one scaling normalized factor. The reads per
kilobase per million reads (RPKM) method was used to quantify the
transcript expression, and HTSeq version 0.5.3 (European Molecular
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Biology Laboratory (EMBL), Heidelberg, Germany) was used to count the number of reads mapped to each transcript. The RPKM value was calculated based on the mapped transcript fragments, sequencing depth, and transcript length.\(^9\) The edgeR Bioconductor was used to complete the read counts with one scaling normalized factor before the analysis of differential gene expression, which was completed using the DESeq R package, release 1.12.0. A log2-fold change of ±1 and \(P < 0.005\) were set as the threshold of statistically significant differential expression. A large fold-change value (\(|\log_2\text{-fold-change}| > 5\)) was also used to identify differentially expressed genes (DEGs).

Gene ontology annotation and gene ontology/Kyoto Encyclopedia of Genes and Genomes enrichment analyses
The protein functions of all the genes were annotated using BLASTX and InterProScan against the NCBI database. The resulting BLAST and InterPro annotations were then converted into gene ontology (GO) annotations. All the GO terms were mapped to the GO slim categories. Fisher’s exact test within Blast2GO (false discovery rate [FDR] <0.05) was used to determine the statistical significance of the functional GO slim enrichment. A hyper geometric test and the Benjamini–Hochberg FDR correction were used to identify significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with KEGG Orthology-based Annotation System version 2.0 (KOBAS 2.0) (Peking University, Beijing, China).\(^8\)

RESULTS AND DISCUSSION

Effect of Boletus speciosus Forst -1 on B-cell activation in vitro
B-cells, also known as B-lymphocytes, are a type of white blood cell of the lymphocyte subtype. They function in the humoral immunity component of the adaptive immune system by secreting antibodies. The stimulation of BSF-1 on B-cells is shown in Figure 1. Cell proliferation activity was lowest when B-cells were exposed to medium alone, whereas incubation of these cells with increasing concentrations of BSF-1 was associated with a dose-dependent increase in cell proliferation activity. Compared with the control group, the low concentration of BSF-1 significantly promoted B-cell proliferation (0.625–20 \(\mu\)g/mL, **\(P < 0.01\)). Furthermore, cell proliferation activity at 1.25 \(\mu\)g/mL concentration of BSF-1 was comparable to or even greater than that elicited by 5 \(\mu\)g/mL LPS.

Effect of Boletus speciosus Forst -1 on T-cell activation in vitro
T-cells are a type of lymphocyte which plays a central role in cell-mediated immunity. They can be distinguished from other lymphocytes, such as B-cells and natural killer cells, by the presence of a T-cell receptor on the cell surface. They are called T-cells because they mature in the thymus from thymocytes. The stimulation of BSF-1 on T-cells is shown in Figure 2. Compared with the control group, the low concentration of BSF-1 significantly promoted T-cell proliferation (1.25 \(\mu\)g/mL, *\(P < 0.05\); 2.5–20 \(\mu\)g/mL, **\(P < 0.01\)). Cell proliferation activity at 2.5 \(\mu\)g/mL concentration of BSF-1 was comparable to or even greater than that elicited by 5 \(\mu\)g/mL LPS.

Effect of Boletus speciosus Forst -1 on macrophage activation in vitro
Macrophages are a type of white blood cell that engulfs and digests cellular debris, foreign substances, microbes, cancer cells, and anything else that does not have the types of proteins specific of healthy body cells on its surface in a process called phagocytosis. The stimulation of BSF-1 on macrophage is shown in Figure 3. Compared with the control group,
the low concentration of BSF-1 significantly promoted RAW264.7 cell proliferation (0.3125–20 µg/mL, **P < 0.01). It is worth noting that cell proliferation activity at 20 µg/mL concentration of BSF-1 was lower than that elicited by 5 µg/mL LPS.

### Macrophage phagocytosis

One of the most distinguished features of macrophage activation would be an increase in pinocytic activity. Pinocytic activity of BSF-1-activated macrophages was examined by the uptake of neutral red (0.075%). From Figure 4, it can be observed that after 24 h incubation with varying concentrations of BSF-1, the BSF-1 enhanced the phagocytic activity of RAW264.7 in the testing dose range with a dose-dependent way compared to the negative control. Furthermore, pinocytic activity at 0.3125–20 µg/mL concentrations of BSF-1 was comparable to or even greater than that elicited by 5 µg/mL LPS, a positive control.

### Effects of *Boletus speciosus* Forst-1 on the cell cycle distribution of RAW264.7 cells

The cell cycle analysis was performed on RAW264.7 cells using flow cytometry to examine the effects of BSF-1 on cell cycle progression. Figure 5 shows the effects of BSF-1 on the cell cycle phase (G0/G1, S, and G2/M) distribution of RAW264.7 cells using flow cytometry with PI staining. The treatment of RAW264.7 cells with BSF-1 at 10 µg/mL for 72 h induced a concentration-dependent increase in the G2/M phase population from 15.7% of the control group to 19.5% of the BSF-1 group (P < 0.05 or P < 0.01), with a concomitant decrease in the percentage of cells in the G0/G1 phase from 71.9% of the control group to 66.3% of the BSF-1 group. At the tested concentrations, BSF-1 also induced a significant change in the S-phase population, from 13.2% of the control group to 15.2% of the BSF-1 group. These results suggested that BSF-1 could promote the proliferation of macrophage cells by abolishing cell cycle arrested in the G0/G1 phase and promote cell cycle progression in S-phase and G2/M phase, which might induce cell division.

### Transcriptome sequencing and *de novo* assembly

High-throughput and low-cost next-generation sequencing technologies, such as RNA-Seq, have become popular and useful not only for *de novo* genome assembly and genome diversity studies, but also to investigate gene expression profiles and discover pharmacological activity mechanisms. In our previous studies, the polysaccharide BSF-1 exhibited significant antitumor activities *in vivo*. The inhibitory rate in mice treated with 40 mg/kg BSF-1 reached 62.449%, which might be comparable to the effects of mannatide. However, the immunomodulatory activity and mechanism of BSF-1 remain unclear. To explore differences in the RAW264.7 cell transcriptomes between the control group and the BSF-1 group, two cell groups were selected for analysis. Two cDNA libraries were constructed with the respective total RNA from the control and BSF-1 groups. The prepared libraries were sequenced on an Illumina HiSeq 2000 platform. After quality control, a total of 12,498,414 and 11,840,624 bp paired-end reads were obtained for the control and BSF-1 groups, respectively, which corresponded to a total size of 12.5 G bp and 11.8 G bp, respectively, after the low-quality reads and adapter sequences were removed [Table 1 and Figure 6]. We mapped the clean reads to the RAW264.7 cell reference genome. The proportion of total reads in the two RAW264.7 cell transcriptome libraries that mapped to the genome ranged from 41.04% to 40.03%. A sequencing saturation analysis showed that the number of genes detected by the library was saturated [Figure 7]. A 5’-3’ sequence preference statistical analysis showed that the sequencing was mainly focused on the gene body region, and the bias at the two ends was limited [Figure 8]. The distribution of gene coverage is shown in Figure 9 and it provides a good basis for the follow-up analysis.

### Transcriptome profiles of the two RAW264.7 cell groups

The abundance of all the genes was calculated and normalized using uniquely mapped reads by the RPKM method. The distribution of the expression levels of all the genes was similar for the two groups. Genes with RPKMs over 60 were considered to be expressed at a high level, whereas genes with RPKMs in the interval of 0–1 were considered to be expressed at low levels or not at all. The results showed that in the
control group, approximately 81.77% of the total number of genes (8208) were expressed (RPKM ≥1) and more than 1333 genes were highly expressed (RPKM >60), whereas in the BSF-1 group, approximately 81.83% of the total number of genes (8257) were expressed (RPKM ≥1) and more than 1366 genes were highly expressed (RPKM >60). The results also showed that eight genes (Rps6, Tpt1, Rpl23, Eef1α1, Fth1, Rpl5, Rps24, and Rplp2) were extremely highly expressed (RPKM >10,000) in the control group [Table 2], whereas nine genes (Eef1α1, Fth1, Rpl23, Rps24, Tpt1, Rps6, Rpl5, Rplp2, and Rps27) were extremely highly expressed (RPKM >9000) in the BSF-1 group [Table 3]. It is worth noting that the RPKM of the Eef1α1 gene was 27073.88 in the control group and 26186.717 in the BSF-1 group.

Table 1: Summary of the mapping results (mapping to reference genome)

| Sample ID | Total reads (%) | Total base pairs (%) | Total mapped reads (%) | Perfect match (%) | ≤2 bp mismatch (%) | Unique match (%) | Multi-position match (%) | Total unmapped reads (%) |
|-----------|-----------------|---------------------|------------------------|-------------------|-------------------|-------------------|-------------------------|-------------------------|
| Control   | 12,498,414 (100.00) | 612,422,286 (100.00) | 5,129,004 (41.04) | 985,094 (7.88) | 4,143,910 (33.16) | 3,166,326 (25.33) | 1,962,678 (15.70) | 7,369,410 (58.96) |
| BSF-1     | 11,840,624 (100.00) | 580,190,576 (100.00) | 4,739,212 (40.03) | 896,331 (7.57) | 3,842,881 (32.46) | 2,988,159 (25.24) | 1,751,053 (14.79) | 7,101,412 (59.97) |

BSF-1: Boletus speciosus Frost-1
Differentially expressed genes between the control and *Boletus speciosus* Forst-1 groups

The reads were adjusted using the edgeR program with one scaling normalized factor, and the DEGs between the two cell groups were identified using the DEGSeq R package. Values of FDR ≤0.001 and |log2 Ratio| ≥1 were set as the thresholds for significant differential expression. Our research group performed hierarchical clustering for all the DEGs based on the log10 RPKMs of the two cell groups to observe the gene expression patterns [Figure 10]. A total of 13 unigenes were identified.
as DEGs, and approximately 5 genes were upregulated, whereas 8 genes were downregulated [Figure 10], which might have contributed to the proliferation and phagocytic activities of RAW264.7 cells following BSF-1 treatment in vitro. The number of DEGs in the control versus BSF-1 were 12 for transcripts detected with $|\text{log}_2\text{-fold-change}| > 1$ and 3 for transcripts detected with $|\text{log}_2\text{-fold-change}| > 2$. Among the DEGs within the $|\text{log}_2\text{-fold-change}| > 1$ threshold, 4 genes were upregulated, including Trim23, Znhit6, Lamc1, and Zbtb7a, among others, whereas 8 genes were downregulated, including LOC100360880, Dusp1, Fos, Zfp36, Ier3, Ier2, Atp6v0d2, and Cars [Tables 4 and 5].

**Gene ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analyses of the differentially expressed genes**

GO analyses were used to confirm the functional classifications of the annotated unigenes and classify the transcripts with known proteins. A total of 39,271 genes were annotated with GO terms, which were converted into generic GO slim terms. The GO enrichment analysis was performed using Fisher's exact test in Blast2GO to analyze the gene functions of the DEGs. The analysis generated 13,042 assignments to cellular components, 13,094 assignments to biological processes, and 13,135 assignments to molecular functions [Figure 11]. In the category of cellular components, 96.60% and 96.60% of the unigenes were located in cell parts (GO: 0044464) and cells (GO: 0005623), respectively. Under the molecular functions, the majority of the GO terms were grouped into intracellular part (GO: 0044424), intracellular (GO: 0005622), intracellular membrane-bounded organelle (GO: 0043231), membrane-bounded organelle (GO: 0043227), intracellular organelle (GO: 0043229), and organelle (GO: 0043226) [Table 6]. These results suggested that the immune mechanisms may present additional differences between the control and BSF-1 groups.

The pathway analysis was conducted using the KEGG pathway database to further understand the biological function of the gene products. The KEGG pathway enrichment analysis was performed using KOBAS. A KEGG analysis records the molecular interaction networks in cells with variants that are specific to particular organism. Our analysis identified 22.22% DEGs in the mitogen-activated protein kinase (MAPK) signaling pathway [Figure 12], including MKP (K04459: 114856) and c-fos (K04379: 314322). The MAPK signaling cascade is a common signal transduction module that connects different receptors/sensors to nuclear and cellular responses. The classical MAPK signaling cascade consists of three types of phosphorylated kinases: MAPK, MAPK kinase (MAPKK/MEK), and MAPKK kinase (MAPKKK/MEKK).[9] In the

Table 4: Differentially expressed genes: Upregulated ($|\text{log}_2\text{-fold-change}| > 1$)

| Gene ID | Gene_length | log2 ratio (BSF-1/control) | Symbol | Description | KEGG Orthology |
|---------|-------------|---------------------------|--------|-------------|----------------|
| 81002   | 3308        | 1.483016642              | Trim23 | Tripartite motif-containing 23 | K07963 |
| 292160  | 1586        | 1.310254989              | Znhit6 | Zinc finger, HIT-type containing 6 | K06492 |
| 117036  | 7636        | 1.131341204              | Lamc1  | Laminin, gamma 1 | K05635 |
| 117107  | 2817        | 1.024963199              | Zbtb7a | Zinc finger and BTB domain containing 7a | K10494 |

BSF-1: *Boletus speciosus* Frost-1; KEGG: Kyoto Encyclopedia of Genes and Genomes
MAPK signaling pathway, epidermal growth factor (EGF) acts by binding to EGF receptors on the cell surface, which leads to cell proliferation, differentiation, and survival. This process stimulates ligand dimerization and starts a signal transduction cascade reaction that results in a series of biochemical changes in cells as well as increased intracellular calcium levels, glycolysis, and protein synthesis, with these changes eventually causing cell proliferation and DNA synthesis.\(^{[13-16]}\) This process would adequately explain the mechanism of BSF-1’s proliferation activity on macrophages.

It is worth noting that the phagosome and oxidative phosphorylation (11.11% DEGs with pathway annotation, respectively) between the two cell groups is also enriched for DEGs. These results indicated that BSF-1 could promote the proliferation of macrophage cells by abolishing cell cycle arrests and might induce cell division.

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**Table 5:** Differentially expressed genes: Downregulated (\(|\text{log}_{2}\text{-fold-change}| > 1\))

| Gene ID   | Gene length | \(\log_{2}\) ratio (BSF-1/Control) | Symbol     | Description                  | KEGG Orthology |
|-----------|-------------|-----------------------------------|------------|------------------------------|----------------|
| 100360880 | 3251        | -7.504347241                      | LOC100360880 | FBJ osteosarcoma oncogene B  | K09029         |
| 114856    | 1940        | -3.736868923                      | Dusp1      | Dual specificity phosphatase 1 | K04459         |
| 314322    | 1589        | -2.982925779                      | Fos        | FBJ osteosarcoma oncogene    | K04379         |
| 79426     | 1811        | -1.713497147                      | Zfp36      | Zinc finger protein 36       | K15308         |
| 294235    | 1114        | -1.547424471                      | Ier3       | Immediate early response 3   | -              |
| 494344    | 1564        | -1.298554254                      | Ier2       | Immediate early response 2   | -              |
| 297932    | 1330        | -1.07331925                       | Atp6v0d2   | ATPase, H+transporting, lysosomal V0 subunit D2 | K02146         |
| 293638    | 2970        | -1.02032673                       | Cars       | CysteinyltRNA synthetase      | K01883         |

FBJ: Finkel-Biskis-Jinkins; BSF-1: Boletus speciosus Frost-1; KEGG: Kyoto Encyclopedia of Genes and Genomes

**Table 6:** Gene ontology enrichment analysis of the differentially expressed genes

| Gene ontology term                      | Cluster frequency | Genome frequency of use | Corrected \(P\) value |
|-----------------------------------------|-------------------|-------------------------|-----------------------|
| Cell - GO: 0005623                      | 11 out of 12 genes, 91.7% | 12,602 out of 13,042 genes, 96.6% | 1                     |
| Cell part - GO: 0044464                 | 11 out of 12 genes, 91.7% | 12,602 out of 13,042 genes, 96.6% | 1                     |
| Intracellular part - GO: 0044424        | 10 out of 12 genes, 83.3% | 9289 out of 13,042 genes, 71.2% | 1                     |
| Intracellular - GO: 0005622             | 10 out of 12 genes, 83.3% | 9321 out of 13,042 genes, 71.5% | 1                     |
| Intracellular membrane-bounded organelle - GO: 0043231 | 7 out of 12 genes, 58.3% | 7081 out of 13,042 genes, 54.3% | 1                     |
| Membrane-bounded organelle - GO: 0043227 | 7 out of 12 genes, 58.3% | 7169 out of 13,042 genes, 55.0% | 1                     |
| Intracellular organelle - GO: 0043229   | 7 out of 12 genes, 58.3% | 7819 out of 13,042 genes, 60.0% | 1                     |
| Organelle - GO: 0043226                 | 7 out of 12 genes, 58.3% | 7918 out of 13,042 genes, 60.7% | 1                     |

GO: Gene ontology

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**Figure 12:** Mitogen-activated protein kinase signaling pathway

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CONCLUSION

Our results suggested that BSF-1 could promote the proliferation of B-cells, T-cells, and macrophages, promote the proliferation of macrophage cells by abolishing cell cycle arrests in the GO/G1 phase, and promote cell cycle progression in S-phase and G2/M phase, which might induce cell division. A total of 12,498,414 and 11,840,624 bp paired-end reads were obtained for the control and BSF-1 groups, respectively, and they corresponded to a total size of 12.5 G bp and 11.8 G bp, respectively, after the low-quality reads and adapter sequences were removed. Approximately 81.83% of the total number of genes (8257) were expressed (RPKM ≥1) and more than 1366 genes were highly expressed (RPKM >60) in the BSF-1 group. A GO enrichment analysis generated 13,042 assignments to cellular components, 13,094 assignments to biological processes, and 13,135 assignments to molecular functions. A KEGG pathway enrichment analysis showed that the MAPK signaling pathways are significantly enriched for DEGs between the two cell groups. An analysis of transcriptome resources enabled us to examine gene expression profiles, verify differential gene expression, and select candidate signaling pathways as the mechanisms of the immunomodulatory activity of BSF-1. Based on the experimental data and the results from our previous work, we believe that the significant antitumor activities of BSF-1 in vivo may involve the MAPK signaling pathway. Our results provide a foundation for understanding the molecular mechanisms underlying the antitumor activity and immune activity of polysaccharides.

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Conflicts of interest

There are no conflicts of interest.

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