Acta Oceanol. Sin., 2018, Vol. 37, No. 8, P. 46–53
DOI: 10.1007/s13131-018-1227-z
http://www.hyxb.org.cn
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Transcriptomic analysis reveals the effect of the exopolysaccharide of *Psychrobacter* sp. B-3 on gene expression in RAW264.7 macrophage cells

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Received 1 December 2017; accepted 12 April 2018

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Abstract

B-3 exopolysaccharide is extracted from the Antarctic psychrophilic bacterium *Psychrobacter* sp. B-3. We have previously shown that it activates macrophages and affects their immunoregulatory activities. To determine what genes are affected during this process, we detected the genes differentially expressed in cells of RAW264.7 macrophages treated with B-3 exopolysaccharide by transcriptomic analysis. B-3 exopolysaccharide treatment caused differential expression of 420 genes, of which 178 were up-regulated and 242 were down-regulated. These genes were shown to be involved in many aspects of cell function, mainly metabolism and immunity. Genes were enriched in multiple immune-related pathways, and the most significantly enriched genes were involved in antigen processing and presentation pathways. The pathway in which differentially expressed genes were the most significantly enriched was the metabolic pathway; specifically, the expression of many metabolic enzyme genes was altered by B-3 exopolysaccharide treatment. Additionally, the genes involved in metabolisms of amino acids, carbohydrates, lipids and nucleotides, varied to certain degrees. B-3 exopolysaccharide, therefore, appears to directly affect the immune function of RAW264.7 macrophages as an immunostimulant, or to indirectly change intracellular metabolism. This is the first study to determine the effect of an Antarctic psychrophilic bacterial exopolysaccharide on RAW264.7 macrophages. Our findings provide an important reference for research into the regulation of macrophage immune function by different polysaccharides.

Key words: *Psychrobacter* sp. B-3, B-3 exopolysaccharide, RAW264.7 macrophage, differentially expressed genes, immunological pathway, metabolic pathway

Citation: Zhang Pingping, Li Jiang, Yu Leiye, Wei Jingfang, Xu Tong, Sun Guojie. 2018. Transcriptomic analysis reveals the effect of the exopolysaccharide of *Psychrobacter* sp. B-3 on gene expression in RAW264.7 macrophage cells. Acta Oceanologica Sinica, 37(8): 46–53, doi: 10.1007/s13131-018-1227-z

1 Introduction

Polysaccharides from fungi, plants, bacteria, and animals have been shown to exhibit a variety of biological activities, including antitumor (Wasser, 2002; Lemieszek and Rzeski, 2012), immunoregulatory (Wakabayashi et al., 1997; Wasser, 2002; Schepetkin and Quinn, 2006; Won et al., 2011), and antiviral functions (Arena et al., 2009; Wang et al., 2012). The immunoregulatory activity of polysaccharides, such as β-glucans from fungi and higher plants, has been an important topic for research, both *in vitro* and *in vivo*. Studies have shown that polysaccharides affect the production of cytokines and other molecules, thereby activating different immune cells, such as macrophages (Stier et al., 2014) and T cells (Inatsuka et al., 2013; Stier et al., 2014).

Many *in vitro* studies have determined the effects of polysaccharides on macrophage activation (Schepetkin and Quinn, 2006; Tseng et al., 2012; Li et al., 2017), and RAW264.7 macrophages have been widely used as a model system. Most *in vitro* studies regarding macrophage regulation (Leung et al., 2006; Li et al., 2007; Won et al., 2011; Reiber et al., 2017; Lee et al., 2018) have tested the following effects: secretion of cytokines and small molecules, phagocytic ability, differentiation state, and activation of signaling pathways and polysaccharide receptors on cell membranes. However, it is not clear whether polysaccharides solely affect the expression of specific immune-related molecules and signaling pathways in macrophages, or whether other genes are affected. A comprehensive understanding of polysaccharide-induced gene expression changes in activated macrophages would thus contribute to a better understanding of the underlying molecular mechanisms.

In recent years, high-throughput sequencing technology has

Foundation item: The Important National Science & Technology Specific Projects under contract No. 2011ZX8001-003; the National Natural Science Fundation of China under contract No. 40706053; Chinese Polar Environment Comprehensive Investigation & Assessment Programs under contract No. CHINARE2017-01-05.

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been used to study the entire genome (Zhu et al., 2014; Han et al., 2011). As a form of deep sequencing technology, RNA sequencing has been widely applied to research at the transcriptional level (Wang et al., 2009; Landolt et al., 2016). For example, transcriptomic analysis has been used for research into quantitative gene expression regarding the biological processes of cells and tissues.

B-3 exopolysaccharide was extracted from an Antarctic psychrophilic bacterium, *Psychrobacter* sp. B-3, which was isolated from ice samples collected at the Uruguay Station (62°11'50.52"S, 58°55'50.4"W) during the 24th Chinese National Antarctic Research Expedition. We previously showed that B-3 exopolysaccharide is composed of two monosaccharides, including mannose and glucose, and has a molecular weight of 5 400 Da. An immunomodulation assay showed that B-3 exopolysaccharide activates RAW264.7 macrophages and affects their immunomodulatory activity, for example, by increasing the production of tumor necrosis factor-α and nitric oxide (NO) (Yu et al., 2016). Based on our previous work, the present study used transcriptomic analysis to analyze the effects of B-3 exopolysaccharide on the gene expression of RAW264.7 cells. This is the first time that the effect of an exopolysaccharide from an Antarctic bacterium on RAW264.7 macrophage global gene expression has been determined. These results will provide an important reference for in-depth and comprehensive studies regarding activated macrophages and the changes to their immunomodulatory activity that are induced by polysaccharides from various sources.

2 Materials and methods

2.1 *Sample handling*

RAW264.7 cells were purchased from KeyGEN BioTECH (Nanjing, China), and cultured in DMEM medium (Gibco, Waltham, MA, USA) containing 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified incubator with 5% CO_2. Cells were seeded into six-well plates at a density of 2×10^6 cell/mL in 1 mL medium per well. Next, 3.3 μL of 3 mg/mL Polymyxin B (20 μg) was added to each well and incubated for 30 min. Then, 1 mL of diluted B-3 exopolysaccharide was added to two wells at a final concentration of 2 μg/mL, while 1 mL of complete medium was added to each of two other wells as the control group. The plates were incubated for 24 h because we previously showed that 0.02–20 μg/mL B-3 exopolysaccharide enhanced cell proliferation, phagocytosis, and NO production after stimulation for 24 h or 48 h (Yu et al., 2016).

2.2 *RNA extraction and quality determination*

Total RNA of four samples was extracted using an ultrapure RNA extraction kit (Kangwei Shiji Biological Technology Co., Ltd., Beijing, China) and stored at –80°C for later use. Before freezing, a sample was separated via 1% agarose gel electrophoresis at 180 V for 16 min. Concentration was measured by a NanoDrop spectrophotometer; integrity and purity were tested using the Agilent 2100 bioanalyzer and NanoDrop spectrophotometer, respectively.

2.3 *cDNA library construction and sequencing*

Oligo (dT) magnetic beads were used for mRNA enrichment. mRNA was broken into short pieces and used as a template. First-strand cDNA was synthesized from this using random hexamer primers; other components of PCR amplification were added for second-strand cDNA synthesis. Double-stranded cDNA was purified by AMPure XP beads, then ends were repaired, a poly-A tail was added, and sequencing joints were connected. AMPure XP beads were used to select the correct fragment sizes, and PCR was performed to enrich the cDNA library.

The Qubit2.0 fluorometer, Agilent 2100 bioanalyzer, and quantitative PCR were used respectively for initial quantitative detection, insert size detection, and accurate concentration quantification (effective concentration of the library >2 nmol/L). The SE50 strategy was used for sequencing. Sequencing and subsequent data analysis were performed by Novogene (Beijing, China).

2.4 *Data analysis*

The Illumina HiSeq™2500 sequencing platform was used for high-throughput sequencing. The original raw image data file was converted by base calling into sequenced reads, using CASAVA base recognition. Clean reads were obtained by removing belt joints and low-quality reads, and then used for subsequent analysis. Filtered clean sequences were aligned with the reference sequence using TopHat software for genome positioning analysis. The positioning number (total mapped reads) and clean reads percentage were obtained. Reads per kilobase of transcript per million mapped reads (RPKM), which considers both the depth and length of the gene sequence, was used to estimate the gene expression level (Mortazavi et al., 2008).

We adopted DESeq (Anders and Huber, 2010) for the analysis of differentially expressed genes. The screening standard for differentially expressed genes was $p_{adj}<0.05$. Gene Ontology (GO) and Kyoko Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed for differentially expressed genes.

2.5 *Gene function annotation*

The following databases were used for gene function annotation: Ensembl (http://www.asia.ensembl.org), GO (http://www.geneontology.org), and KEGG (http://www. genome.jp/kegg/pathway.html).

2.6 *Real-time PCR*

RAW264.7 cells were stimulated by 2 μg/mL B-3 exopolysaccharide for 24 h. A total of 3 μg RNA per sample was reverse transcribed into cDNA using Invitrogen MMLV retrovirus kits, following manufacturer's instructions. Quantitative PCR was performed using the IQ5 real-time multiple fluorescent quantitative PCR system (Bio-Rad, Hercules, CA, USA). Primer sequences are listed in Table A1. Reaction mixtures (15 μL) contained 4.5 μL double-distilled water, 7.5 μL 2X QuantiFast SYBR Green PCR Master Mix (Qiagen, Beijing, China), 1 μL upstream and downstream primers (6 μmol/L), and 1 μL cDNA. PCR conditions were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, and 60°C for 30 s. Each experiment was performed in triplicate. GAPDH was chosen as a reference gene. The $2^{-\triangle\triangle Ct}$ method was used for relative quantitation.

2.7 *Statistical analysis*

SPSS17.0 software was used for statistical analysis, and significant differences between means were analyzed using Student's *t*-test. Values of $p<0.05$ were regarded as statistically significant.

3 Results and discussion

3.1 *Evaluation and analysis of sequencing data*

To study the effect of B-3 exopolysaccharide on RAW264.7 cell gene expression, cells were treated for 24 h with 2 μg/mL B-3 exopolysaccharide. Sequencing results are summarized in Table 1.

Table 1. Sequencing data output quality list

Sample name	Raw reads	Clean reads	Clean bases	Error rate/%	Q20/%	Q30/%	GC content/%
MRCN2	11564799	11558830	0.58G	0.01	98.48	97.05	50.64
MRCN3	12618276	12611092	0.63G	0.01	98.50	97.08	50.79
MRCP2	13197215	13189345	0.66G	0.01	98.53	97.15	51.27
MRCP3	11969746	11962711	0.6G	0.01	98.52	97.13	50.82

Note: MRCN2, MRCN3, MRCP2 and MRCP3 indicate the samples of Control group 1, Control group 2, Experimental group 1 and Experimental group 2, respectively.

The original data received from the control and experimental groups were 11564799, 12618276 and 13197215, 11969746, respectively, including clean sequences of 11558830, 12611092 and 13189345, 12611092, respectively. Each accounted for 99.9% of the original data, and the sequencing error rate was only 0.01%. The percentages of total sequence positioning to the genome for MRCN2, MRCN3, MRCP2, and MRCP3 were 94.35%, 94.22%, 93.68%, and 94.35%, respectively, while sequences with the sole position comprised 81.22%, 81.22% and 80.8%, 80.96%, respectively. Only a small number of sequences were found in multiple alignments. Comparing the distribution of reads with the genome, most sequences matched to exons, followed by introns and intergenic regions. Clean reads were used for subsequent GO and KEGG analyses of gene expression.

3.2 *Quantitative analysis of gene expression and differentially expressed genes*

RPKM was used to evaluate gene expression levels (Mortazavi et al., 2008) via the standard RPKM>1. HTSeq software was used to analyze gene expression levels of various samples via the union model. A Venn diagram of gene expression was drawn to depict the quantitative results of gene expression, as shown in Fig. 1. A total of 209 genes were only expressed in the B-3 exopolysaccharide-treated group (MRCP), while 214 were only expressed in the control group (MRCN); 10 567 genes were expressed in both groups. Differentially expressed genes were screened using the standard of genetic variations, p_{adj}<0.05, as a filter. A total of 420 genes were differentially expressed between MRCP and MRCN, of which 178 were up-regulated while 242 were down-regulated, as shown in Fig. 2. These genes were involved in diverse functions within the cell.

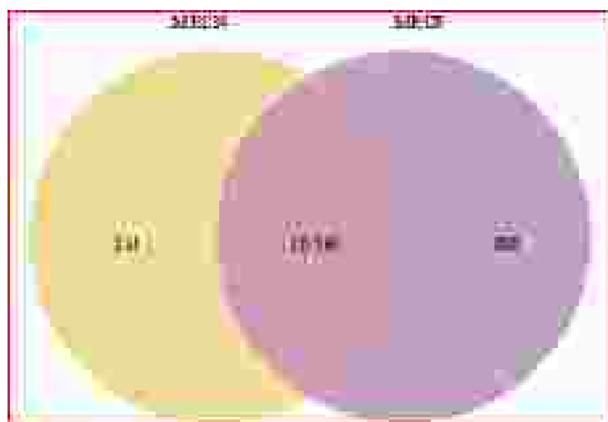

Fig. 1. Venn diagram of gene expression. The sum of the numbers in each large circle represents the total number of genes expressed in this group. The overlap shows the number of genes expressed in both groups. Reads per kilobase of transcript per million mapped reads (RPKM) greater than 1 was the standard of gene expression. MRCN represents control group and MRCP B-3 exopolysaccharide-treated group.

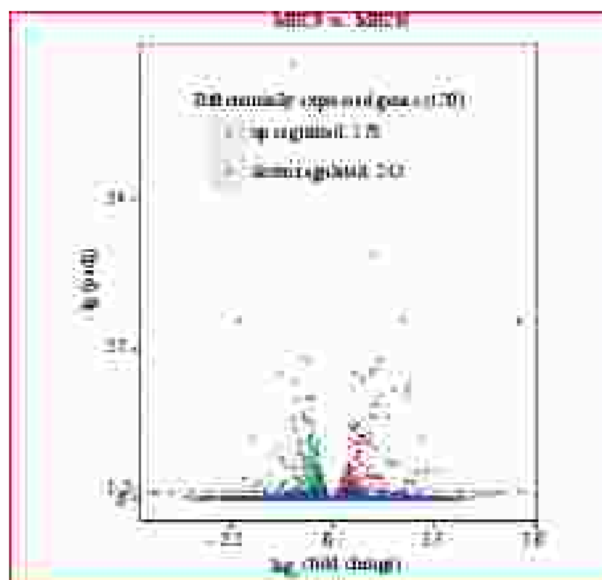

Fig. 2. Volcano figure of differentially expressed genes. Significantly differentially expressed genes are indicated by red dots (up-regulated) and green dots (down-regulated). Genes with no significant difference in expression between the two groups are indicated by blue dots. Abscissae represent multiple changes of genes expressed in different samples; ordinates represent statistically significant differences of changes in gene expression. MRCN represents control group and MRCP B-3 exopolysaccharide-treated group.

3.3 *Real-time quantitative PCR validation*

To verify our preliminary results that B-3 polysaccharide treatment changed the expression of an extensive proportion of RAW264.7 macrophage genes involved in different biochemical pathways, we selected four differentially expressed genes that are representative of different biochemical pathways for quantitative PCR analysis: *Acy1* (encoding aminoacylase 1), *Nos2*, *Pgm5* (encoding phosphoglucomutase 5), and *Pik3c2b* (encoding phosphoinositide-3-kinase, class 2, beta polypeptide). *Acy1* and *Pgm5* are mainly involved in substance metabolism, while *Nos2* and *Pik3c2b* have roles in immune-related pathways. Our quantitative PCR results were consistent with differential expression findings. *Nos2* and *Acy1* were upregulated, while *Pgm5* and *Pik3c2b* were downregulated after treatment with B-3 exopolysaccharide, as shown in Fig. 3.

3.4 *GO enrichment analysis*

GO is the international standard classification system of gene function that is mainly divided into the three categories of Cellular Component, Molecular Function, and Biological Process. GO

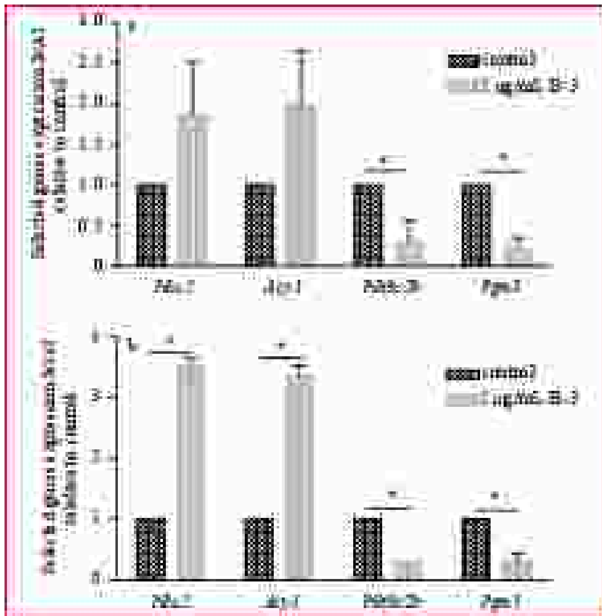

Fig. 3. Histogram of selected differentially expressed genes, constructed using GraphPad Prism 6. a. Quantitative PCR results using the $2^{-\triangle\triangle Ct}$ method and b. differential gene expression. Gene reads per kilobase of transcript per million mapped reads (RPKM) ratios of experimental groups relative to control groups. Student's *t*-test was used for statistical analysis with SPSS17.0 software. * $p<0.05$.

enrichment analysis of differentially expressed genes identified in the present study was performed via GOseq software (Young et al., 2010), which annotated 319 differentially expressed genes. We selected the 30 most significantly enriched GO terms, as shown in Fig. 4. Among them, the immune response (GO: 0006955) and immune system process (GO: 0002376) were the most significantly enriched within the Biological Process category. Eleven genes in these two GO terms were differentially expressed, of which six were up-regulated and five were down-regulated. Dephosphorylation (GO: 0016311) was another significantly enriched GO term within Biological Process, while catalytic activity (GO: 0003824) was the most enriched GO term within Molecular Function. A total of 154 genes in this GO term were differentially expressed, of which 60 were up-regulated and 94 were down-regulated. Enzyme activities (GO: 0030234) was another significantly enriched GO term within Molecular Function, which revealed 16 GO differentially expressed genes (seven up-regulated and nine down-regulated), as well as GTP combination (GO: 0005525), which showed 16 GO differentially expressed genes (six up-regulated and 10 down-regulated), and amidine base nucleotide (GO: 0032561), which revealed 16 GO differentially expressed genes (six up-regulated and 10 down-regulated). Cell migration regulation, cell adhesion regulation, phosphatase activity, nicotinamide adenine dinucleotide (NAD) layer synthesis, metabolism, protein complex, and tumor necrosis factor receptor were also significantly enriched GO terms.

To determine which catalytic activities of enzymes were involved in the GO term catalytic activity (0003824), this term was further characterized. The differentially expressed gene enrichment numbers of this GO term are listed in Table 2. The three most enriched categories were hydrolase activity, transferase activity, and oxidoreductase activity. As examples, *Nos2* encodes an oxidoreductase, *Acy1* encodes a hydrolase, and *Pik3c2b* encodes a hydrolase/transferase. Genes with enzyme catalytic activity involved in this GO term participated in the synthesis, processing, and metabolism of a variety of cellular substances. This suggests that B-3 exopolysaccharide greatly changed the metabolism of RAW264.7 cells. This may be because B-3 exopoly-

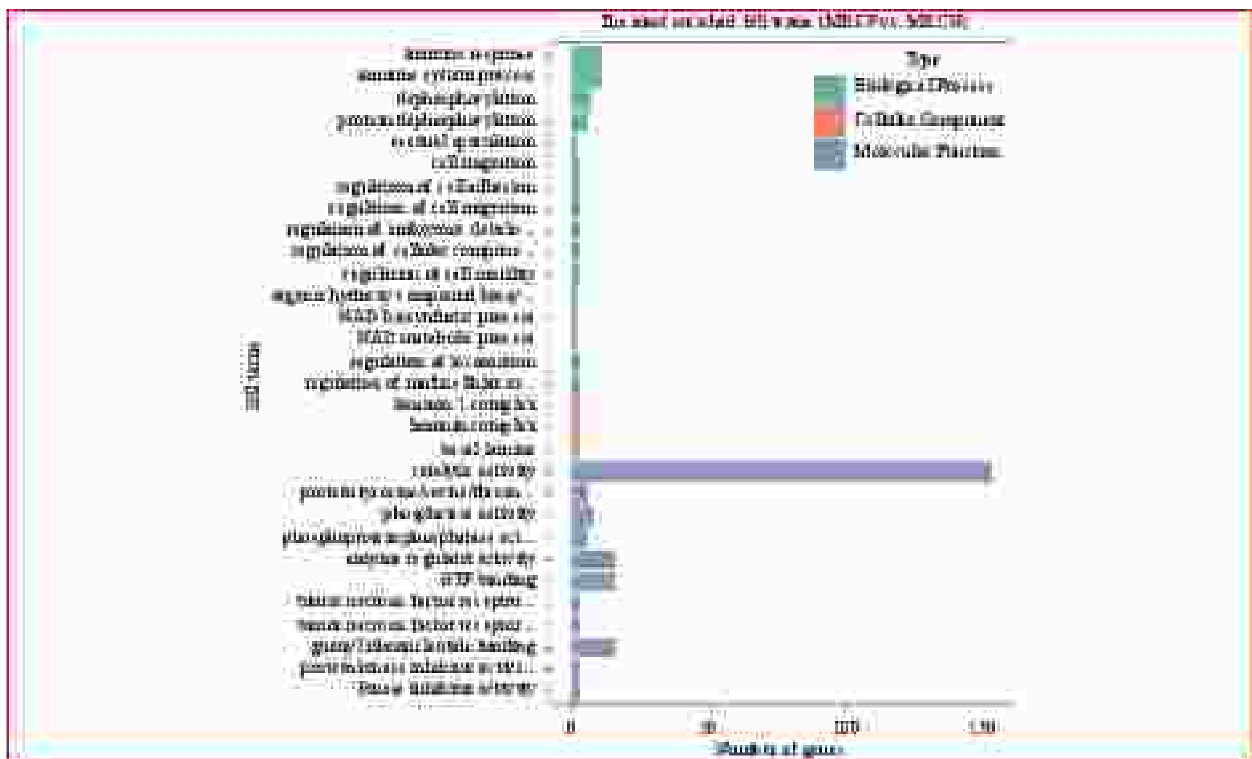

Fig. 4. Histogram of GO enrichment. MRCN represents control group and MRCP B-3 exopolysaccharide-treated group.

Table 2. Enzyme catalytic activity involved in GO enrichment

GO: accession	Description	DEG_item (up:down)
GO: 0003824	catalytic activity	154 (60:94)
GO: 0008987	quinolinate synthetase A activity	1 (1:0)
GO: 0009975	cyclase activity	1 (0:1)
GO: 0016491	oxidoreductase activity	29 (11:18)
GO: 0016740	transferase activity	49 (15:34)
GO: 0016787	hydrolase activity	66 (27:39)
GO: 0016829	lyase activity	7 (4:3)
GO: 0016853	isomerase activity	7 (2:5)
GO: 0016874	ligase activity	7 (4:3)

Note: DEG_item represents the number of differentially expressed genes related to the GO term, up up-regulated genes, and down down-regulated genes.

saccharide was regarded as an immunostimulant or source of energy, so its ingestion by the cells induced downstream signaling. However, further studies are needed to confirm this.

3.5 *KEGG enrichment analysis*

KEGG mainly analyzes signaling pathways of differentially expressed genes (Kanehisa et al., 2008). In this study, the differentially expressed genes were enriched in 224 KEGG pathways, of which the most significantly enriched was the antigen processing and presentation pathway (mmu04612). Several genes were up-regulated in this pathway, including *H2-D1* (encoding histocompatibility 2, D region locus 1), *H2-T24* (encoding histocompatibility 2, T region locus 24), *H2-T23* (encoding histocompatibility 2, T region locus 23), *li* (encoding CD74), *Tapbp* (encoding TAP binding protein), *B2m* (encoding beta-2 microglobulin), and *Calr* (encoding calreticulin). These are critical genes of major histocompatibility complex (MHC) Class I or Class II pathways (Vyas et al., 2008; Blum et al., 2013). This indicates that stimulation by B-3 exopolysaccharide enhanced the antigen processing and presentation abilities of macrophages through the MHC Class I and Class II pathways. Other immune-related pathways were also enriched (Table 3), which play an important role in initial and adaptive immunity. This shows that B-3 exopolysaccharide enhanced the immune reactivity of RAW264.7 cells. Moreover, several infection pathways were also significantly enriched, such as those involving hepatitis C (mmu05160) and measles (mmu05162).

Among the 224 enriched KEGG pathways, the most enriched was the metabolic pathway (mmu01100), involving 43 differentially expressed genes. This was consistent with the most enriched GO term of catalytic activity (0003824) in the GO results, as enzymes with various catalytic activities are keys to cell metabolism. These metabolic pathways involve a variety of substances, including amino acids, carbohydrates, lipids, vitamins, and nucleotides. Other enriched pathways included signaling pathways, cellular adhesion and migration, material synthesis, and transport. Differentially expressed genes induced by B-3 exopolysaccharide stimulation are therefore involved in multiple functions, including immunity, metabolism, disease, and signaling. The 20 most significantly enriched pathway entries are shown in Fig. 5.

KEGG enrichment analysis indicated that B-3 exopolysaccharide affected multiple immune-related pathways of macrophages, such as antigen processing and presentation, the Toll-like receptor signaling pathway, and leukocyte transendothelial migration. The Jak-STAT signaling pathway and NF-kappa B signaling pathway were also significantly enriched by B-3 stimulation. These two pathways affect macrophage proliferation, differentiation, and immune regulation (Rawlings et al., 2004; Hayden and Ghosh, 2011).

Among all differentially expressed genes, 154 were involved in the catalytic activity (0003824) GO term, and 43 were involved in metabolic pathways (mmu01100) of the KEGG analysis. This indicated that B-3 exopolysaccharide has a significant effect on RAW264.7 cell metabolism. Macrophage metabolism is known to be closely associated with immune function (Ghesquière et al.,

Table 3. Immune-related signaling pathways in KEGG enrichment

Term	ID	Input number	Background number
Antigen processing and presentation	mmu04612	10	81
Fc gamma R-mediated phagocytosis	mmu04666	9	88
Natural killer cell mediated cytotoxicity	mmu04650	10	119
B cell receptor signaling pathway	mmu04662	7	73
T cell receptor signaling pathway	mmu04660	8	105
Cytosolic DNA-sensing pathway	mmu04623	5	63
RIG-I-like receptor signaling pathway	mmu04622	5	68
Toll-like receptor signaling pathway	mmu04620	6	101
Fc epsilon RI signaling pathway	mmu04664	4	70
Leukocyte transendothelial migration	mmu04670	6	121
Chemokine signaling pathway	mmu04062	8	196
Complement and coagulation cascades	mmu04610	3	77
NOD-like receptor signaling pathway	mmu04621	2	58
Intestinal immune network for IgA production	mmu04672	1	42

Note: ID represents unique serial number of pathway in KEGG database; input number number of differentially expressed genes related to this pathway, and background number total number of genes related to this pathway.

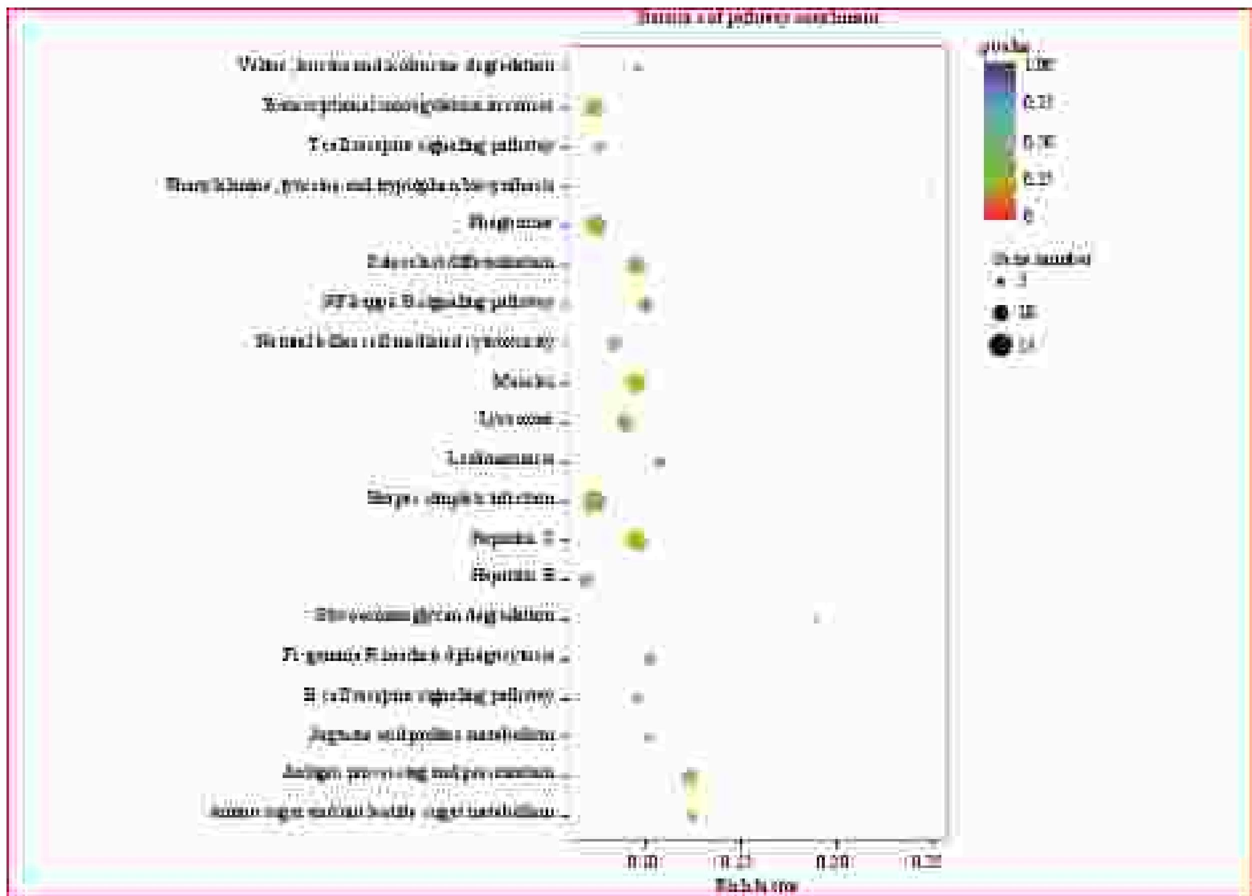

Fig. 5. Scatter diagram of differentially expressed genes in KEGG enrichment. The longitudinal axis represents pathways and the horizontal axis rich factor. The sizes of the points indicate the number of differentially expressed genes in this pathway and the colors of the points correspond to different *q* value ranges.

2014; Kelly and O'Neill, 2015). For example, metabolites and metabolic enzymes regulate immune cell plasticity by affecting gene transcription, signaling pathways, or epigenetics (Ghesquière et al., 2014). Nos2 is a type of oxidoreductase and inducible NO synthase that catalyzes substrates to generate NO, while Acy1 is a hydrolase that catalyzes the hydrolysis of acylated L-amino acids into L-amino acids and an acyl group (https://en.wikipedia.org/wiki/ACY1). Acy1 also participates in the synthesis of L-arginine, which, together with NADPH, is a precursor molecule of NO (Aktan, 2004). NO and Nos2 play complex roles in innate and adaptive immunity (Bogdan, 2015), and immune cell metabolic activity is known to be closely linked to its immune function.

Our sequencing and quantitative PCR results showed that B-3 exopolysaccharide stimulation upregulated *Nos2* and *Acy1* in RAW264.7 cells. The resulting increased NO synthesis would further adjust the immune reactivity of RAW264.7 cells.

4 Conclusions

B-3 exopolysaccharide stimulation was found to alter the expression of a large array of genes in RAW264.7 macrophages. It also dramatically changed the metabolism of various substances and affected multiple signaling pathways, especially immune-related pathways. These results reveal that changes in metabolic activity caused by B-3 exopolysaccharide are closely linked with its immune function. This study provides a reference for research into the regulation of macrophage immune function by polysaccharides.

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Appendix:

Table A1. Genes primers sequences

Gene ID	Gene	Forward primer (5′ to 3′)	Revere primer (5′ to 3′)
ENSMUSG00000023262	*Acy1*	AAGGATTCCGAGGGCTAC	TCGTCAGGCACAAAGGTC
ENSMUSG00000020826	*Nos2*	CACGGACGAGACGGATAG	CATGCAAGGAAGGGAACT
ENSMUSG00000026447	*Pik3c2b*	GCAACACCTGGCAATAAC	ACGCAGCAACTTCCTCAT
ENSMUSG00000041731	*Pgm5*	CCCAGGAAGAATACCAGC	CCCAGGAAGAATACCAGC