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Transcriptome analysis of bovine lymphocytes stimulated by *Atractylodis macrocephalae Koidz.* polysaccharides *in vitro*

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ABSTRACT

Our previous studies demonstrated that total polysaccharides isolated from *Atractylodis macrocephalae Koidz.* (*RAMPtp*) had a therapeutic effect on bovine subclinical mastitis and an immunomodulatory effect on the lymphocytes from the supramammary lymph node (SMLN) in cows. To investigate the potential molecules involved in the immunomodulation, the present study identified 243 differentially expressed genes (DEGs) in *RAMPtp*-stimulated SMLN lymphocytes by RNA-seq. GO/KEGG enrichment analyses identified 47 significantly enriched gene ontology (GO) terms and 9 canonical pathways. These findings indicated that *RAMPtp* might stimulate lymphocytes to modulate productions of cytokines and chemokines through multiple compounds, targets and pathways. In particular, the Jak-STAT signaling pathway might be primarily involved in the immunomodulation. The therapeutic effect of *RAMPtp* on bovine subclinical mastitis may be attributed to activation of SMLN lymphocytes by injection of the polysaccharides in the area of SMLN in cows.

1. Introduction

Lymph nodes are the important organs where innate responses lead to acquired immunity (von Andrian and Mempel, 2003). Advances in the current knowledge of the role of the lymphatic system in pathological change and immunity have driven the increasing recognition that lymph node-targeted delivery has the significant potential to transform disease treatment and vaccination (Thomas et al., 2014; Trevaskis et al., 2015). We have previously shown that subcutaneous injection of *RAMPtp*, a Chinese herbal extract with immunostimulatory effects (Xie et al., 2012; Xie et al., 2013), in the area of SMLN had therapeutic value in cow mastitis (Xu et al., 2015). Lymphocytes in the ipsi lateral SMLN in mastitic cows have been demonstrated to migrate into the mammary gland in response to bacterial infection (Kehrli and Harp, 2001; Soltys and Quinn, 1999). Based on this observation, we hypothesized that the therapeutic effect of *RAMPtp* on cow mastitis might be due to the improvement of mammary gland immunity resulting from the *RAMPtp*-mediated SMLN lymphocytes activation, which is supported by the promoted lymphocytes proliferative response, accelerated cell cycle progression and up-/down-regulated cytokine mRNA expression *in vitro* (Xu et al., 2017). However, the underlying molecular mechanism remained obscure. In the present study, transcriptome sequencing (Mortazavi et al., 2008) was performed to investigate the effect of *RAMPtp* on gene expression in SMLN lymphocytes and to explore the potential signal transduction pathways.

2. Materials and methods

2.1. Animals and ethics statement

Three Holstein cows in their first to third lactation were used in this investigation. They were clinically healthy and showed no mammary disorder. The animal procedure was approved by the Institutional Animal Care and Use Committee at Zhejiang University (ZJU20170268) and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. RAMPtp

RAMPtp with purity of 95.66% was obtained by water extraction and ethanol precipitation in our laboratory (Xu et al., 2017). It was composed of glucose, mannose, rhamnose, arabinose and galactose, with mass percentages of 60.67%, 14.99%, 10.61%, 8.83% and 4.90%, respectively, connected by 1, 3-linked β-D Galp and 1, 6-linked β-D

Abbreviations: RAMPtp, total polysaccharides from *Atractylodis macrocephalae Koidz.*; SMLN, supramammary lymph node; IFN, interferon; IFIT, interferon-induced protein; ISG, interferon-stimulated gene; OAS, 2′-5′-oligoadenylate synthetase; RSAD, radical S-adenosyl methionine domain containing; IL, interleukin; CCL, CC chemokine ligand; CXCL, C-X-C motif chemokine ligand; PPBP, pro-platelet basic protein

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Galp residues, and was amorphous in structure.

2.3. Lymphocytes culture, RAMPtp stimulation and RNA extraction

The procedure for isolation of SMLN lymphocytes was as per the method described previously (Xu et al., 2017). Then the freshly prepared lymphocytes were seeded at 5×10^6 cells/ml in a complete RPMI 1640 medium (RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat inactivated FBS) and treated without or with 100 μg/ml of *RAMPtp* for 6 h (selected according to the preliminary experiment results) at 37 °C in 5% CO_2 atmosphere. Both treatments in each cow were carried out in triplicate. After that, cells with the same treatment in each cow were pooled and RNA was isolated with RNAiso™ Plus (Takara, Dalian, China).

2.4. cDNA library construction and RNA-seq

The quality and quantity of RNA samples were assessed by Nanodrop 2000 Spectrophotometer (Thermo, USA) and Agilent 2100 Bioanalyzer (Agilent, USA). RNA samples with high purity (OD260/280 between 1.8 and 2.2) and high integrity (RIN > 7.5) were used for library construction with the Illumina TruSeq™ RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). The libraries were sequenced using Illumina HiSeq platform with a PE150 strategy. Raw Data was processed with Perl scripts to ensure the quality of data. The adopted filtering criteria are as follows: (1) Remove the adaptor-contaminated reads. Reads containing more than 5 adapter-contaminated bases were regarded as adaptor-contaminated reads and filtered out; (2) Remove the low-quality reads. Reads with the number of low quality bases (phred quality value less than 19) accounting for more than 15% of total bases are regarded as low-quality reads; (3) Remove reads with number of N bases accounting for more than 5%; (4) Remove reads with number of N bases accounting for more than 5%. As for paired-end sequencing data, both reads would be filtered out if any read of the paired-end reads are adaptor-contaminated. Statistics analysis was carried out on filtered data to assess its quality and quantity, including Q30, data quantity and base content statistics, etc. Reads were aligned to the bovine reference genome UMD3.1 (http://www.ncbi.nlm.nih.gov/genome/?term=bos+taurus) (Zimin et al., 2009). Bowtie/Bowtie2 (Langmead and Salzberg, 2012) was used for building the genome index, and filtered data was mapped to the reference genome using TopHat v2.0.12 (Trapnell et al., 2009; Trapnell et al., 2012).

2.5. Differential expression and quantification analysis

Fragments Count for each gene in each sample was counted by HTSeq v0.6.0 (Anders et al., 2015), and FPKM (Fragments Per Kilobase Per Million Mapped Fragments) was then calculated to estimate the expression level of genes in each sample. For analysis of DEGs, DESeq/DESeq2 (Love et al., 2014) was employed to detect the differential expression between the two groups using a model based on the negative binomial distribution. The *P* value was assigned to each gene and adjusted as *q* value by the Benjamini and Hochberg method (Anders et al., 2015) for controlling the false discovery rate (FDR). Genes with $q < 0.05$ and $|\log_2\text{FoldChange}| \geq 1$ were defined as DEGs.

2.6. GO/KEGG enrichment analyses

The GO (Gene Ontology, http://geneontology.org) enrichment of DEGs was conducted by the hypergeometric test, in which *P* value was calculated and adjusted as *q* value. GO terms with $q < 0.05$ were considered to be significantly enriched (Zheng and Wang, 2008). For pathway enrichment analysis, all DEGs were assigned to terms in KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.kegg.jp) database and searched for significantly enriched KEGG terms with the same analytic approach.

2.7. Real time quantitative PCR (RT-qPCR) validation

Total RNA was converted into cDNA with PrimeScript™RT Master Mix (Takara, Dalian, China) by a T100™ thermal cycler (Bio-Rad Laboratories, Inc., USA). RT-qPCR was performed for a total of 10 DEGs using SYBR®Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Dalian, China) on ABI7300 (PE Applied Biosystems, USA) and determined employing the comparative C_T method ($2_T^{-\triangle\triangle C}$) (Livak and Schmittgen, 2001). *ACTB* was employed as the reference gene after comparing the expression stability of three candidated house-keeping genes. All samples were analyzed in triplicate. Sequences of primers used for RT-qPCR were provided in Supplementary material: Table S1.

3. Results and discussion

3.1. Reads mapping

All 6 constructed libraries (named Control1, Control2, Control3, RAMPtp1, RAMPtp2, RAMPtp3) were sequenced with a mean of 51.97 million raw reads (range: 50.69 million to 54.51 million reads) per individual library. Deconvolution and filtering of raw reads yielded a mean of 46.08 million clean reads (range: 44.63 million to 47.53 million reads). Subsequent alignment of the filtered reads to the *B. taurus* reference genome yielded a mean of 36.26 million reads (78.67%) that mapped to unique locations in the bovine genome. These results indicated a high proportion of mapped reads and a high uniformity among samples for both the number of mapping reads and the mapping rates (Supplementary material: Table S2).

3.2. Differential expression due to RAMPtp stimulation

We performed hierarchical clustering as well as the volcano plot for the visualization of differential expression (Fig. 1). A total of 243 genes were identified as DEGs in the comparison Control *vs.* RAMPtp, of which 189 genes were up-regulated, whereas 54 genes were down-regulated, which might have contributed to the activation of SMLN lymphocytes following *RAMPtp* treatment *in vitro*. DEGs identified in biological replicates clustered together, indicating good reproducibility of treatments (Fig. 1B). The up- and down-regulated DEGs were listed in Supplementary material: Table S3. It should be particularly noted that 8 genes (including *MX1*, *IFIT1*, *IFIT2*, *RSAD2*, *ISG15*, *OAS2*, *IFIT3* and *IFIT27*) encoding interferon (*IFN*)-induced proteins were significantly up-regulated, accounting for 40% of the top 20 up-regulated genes. IFNs are a group of immunocompetent proteins with functions of activation and regulation of immune cells. By interacting with specific receptors, IFNs activate signal transducer and activator of transcription (STAT) complexes (Villarino et al., 2015). These results suggested that immunomodulatory activities of *RAMPtp* may include the Jak-STAT signaling pathway.

3.3. GO and KEGG enrichment analyses of DEGs

GO provides a structured standard vocabulary for describing the function of gene products and is divided into three orthogonal ontologies, biological process, molecular function, and cellular component (Schlicker et al., 2006). To determine the functional genes which were chiefly involved in responses to *RAMPtp* stimulation, we performed GO enrichment analysis associated to DEGs. The *q* value distribution of the enriched GO terms was presented in Fig. 2, suggesting that *RAMPtp* might exert its immunomodulatory effect by influencing the binding of ligands to receptors and productions of cytokines and chemokines.

KEGG is a database resource containing a collection of pathway maps representing our knowledge on the molecular interaction and reaction networks (Kanehisa et al., 2017). In the present study, the pathway analysis was carried out by using the KEGG pathway database to further understand the biological function of the DEGs. Our analysis