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Immunomodulatory effect of thymopentin on lymphocytes from supramammary lymph nodes of dairy cows

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ABSTRACT

Previous study showed that injection of thymopentin (TP 5) in the area of supramammary lymph nodes (SMLN) had therapeutic effect on the intramammary infection (IMI) in cows. This study was to explore the underlying mechanisms by investigating the immunomodulatory effect of TP 5 on SMLN lymphocytes. Lymphocyte proliferation, cell cycle distribution and cytokine mRNA expression were determined by MTT, FCM and RT-qPCR, respectively. Laser scanning confocal microscope (LSCM) was used to observe the binding between TP 5 and SMLN lymphocytes. Moreover, RNA-sequencing (RNA-seq) was performed to observe the difference between the lymphocytes with and without TP 5 treatment. The results showed that TP 5 significantly promoted lymphocyte proliferation, accelerated cell cycle progression, and enhanced mRNA expression of IL-17A and IL-17F. Laser scanning confocal microscopic analysis revealed the binding of TP 5 to the surface of SMLN lymphocytes. A total of 1094 genes were identified as differentially expressed genes (DEGs) using RNA-seq with 692 up- and 402 down-regulated genes. 48 significantly enriched GO terms were identified by RNA-seq. In KEGG analysis, 1/3 of DEGs were enriched in the immune system pathway, including IL-17 signaling pathway, cytokine-cytokine receptor interaction, Th1 and Th2 cell differentiation, T cell receptor signaling pathway, Th17 cell differentiation. Among them, IL-17 signaling pathway was the most prominent. This study suggested that the therapeutic benefit of TP 5 in the treatment of bovine mastitis might be attributed to its immunomodulatory activity in SMLN lymphocytes.

1. Introduction

Mastitis, inflammation of mammary gland, is a common disease in dairy cows and mainly results from intramammary infection (IMI) by pathogenic bacteria such as *Staphylococci aureus*, *Streptococci uberis* and *Escherichia coli* (*E. coli*) [1]. The disease is estimated to affect 30% of dairy cows and account for $35 billion economic losses annually [2,3]. Antibiotic therapy is the main approach to mastitis treatment [4]. However, this approach has caused public concern because it has the potential to cause residues in milk and its products which are harmful to human health [5]. Therefore, searching for alternative methods to treat bovine mastitis and reduce the use of antibiotics in mastitis treatment has become a valuable topic [2,6–10].

The mammary gland has been reported susceptible to IMI when the udder defense mechanism is weak and enhancement of the udder defense will benefit the mammary gland to eliminate the invading pathogens [11]. The supramammary lymph nodes (SMLNs) as the peripheral immune organs are subcutaneously located at the posterosuperior portion of the udder in dairy cows. The lymph node has been reported to play an important role in regulation of the mammary immunity [12]. We previously found that injection of thymopentin (TP 5) in the area of SMLNs has a therapeutic effect on bovine mastitis by exhibiting reduction in somatic cell count (SCC) and IMI [13]. TP 5 was found in the study of the thymus for its role in lymphocyte differentiation, maturation and immune activity [14,15]. In the 1970s, thymopeptide (TP) was isolated from bovine thymus for the first time, which was identified as a single peptide chain of 49 amino acids [16,17]. A few years later, TP 5 was synthesized that represented the 32–36 residues of TP (Fig. 1). Because of its similar biological activity, TP 5 was considered to be an effective fragment of TP [18]. TP 5 has been found to correct imbalances in the immune system without any obvious side effects [19,20]. At present, TP 5 is widely used in the treatment of immune-related diseases, such as immunodeficiency disorders, autoimmune diseases and cancer, as well as inflammation [21,22].

Although TP 5 is widely used in the treatment of immune deficiency in humans and animals, no reports have been found regarding the mechanism behind its therapeutic effect on mastitis in dairy cows. The

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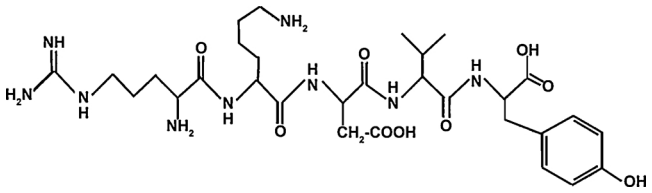

Fig. 1. The structure of thymopentin (TP 5).

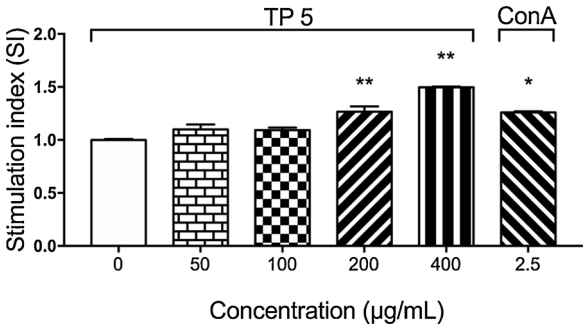

Fig. 2. Lymphocytes proliferative responses to TP 5. SMLN lymphocytes (5×10^6 cells/well) were cultured for 4 h with MTT after 44 h incubation with gradual doses of TP 5 and ConA and then mixed with 150 μL dimethyl sulfoxide (DMSO) per well just before measurement. Result was evaluated at 570 nm using a microplate reader. Values represent mean ± SE (n = 3). * $p < 0.05$, ** $p < 0.01$, compared with control group.

present study was designed to investigate the immunomodulatory effect of TP 5 on SMLNs by MTT, Flow Cytometry (FCM) and RT-qPCR. Laser scanning confocal microscope (LSCM) was employed to observe the binding effect between TP 5 and SMLN lymphocytes. The possible molecular mechanisms and potential signal transduction pathways were explored by RNA-sequencing (RNA-seq).

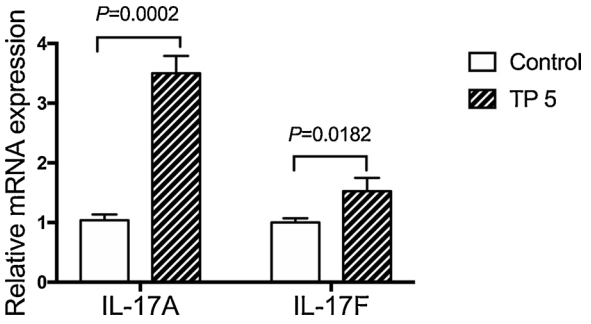

Fig. 4. mRNA expression of cytokines associated with mastitis (IL-17A and IL-17F). The SMLN lymphocytes (5×10^6 cells/well) were treated with 0, 200 μg/mL TP 5 for 48 h. Production of cytokine mRNA was analyzed by RT-qPCR. Values represent mean ± SE (n = 3).

2. Materials and methods

2.1. Chemicals

TP 5 was purchased from SL pharmaceutical Co., Ltd. (Beijing, China). Endotoxin levels in the TP 5 solution were confirmed free of endotoxin with an Endotoxin-Specific Tachypleus Amebocyte Lysate (Xiamen Bioendo Technology Co., Ltd., Xiamen, China). FITC-labeled TP 5 was purchased from Chinese Peptide Co. (Zhejiang, China). Concanavalin A (ConA) and MTT were purchased from Sigma Chemical Co. (St Louis, MO, USA). RPMI 1640 was purchased from GENOM Inc. (Hangzhou, China). Fetal bovine serum (FBS) was obtained from Gibco

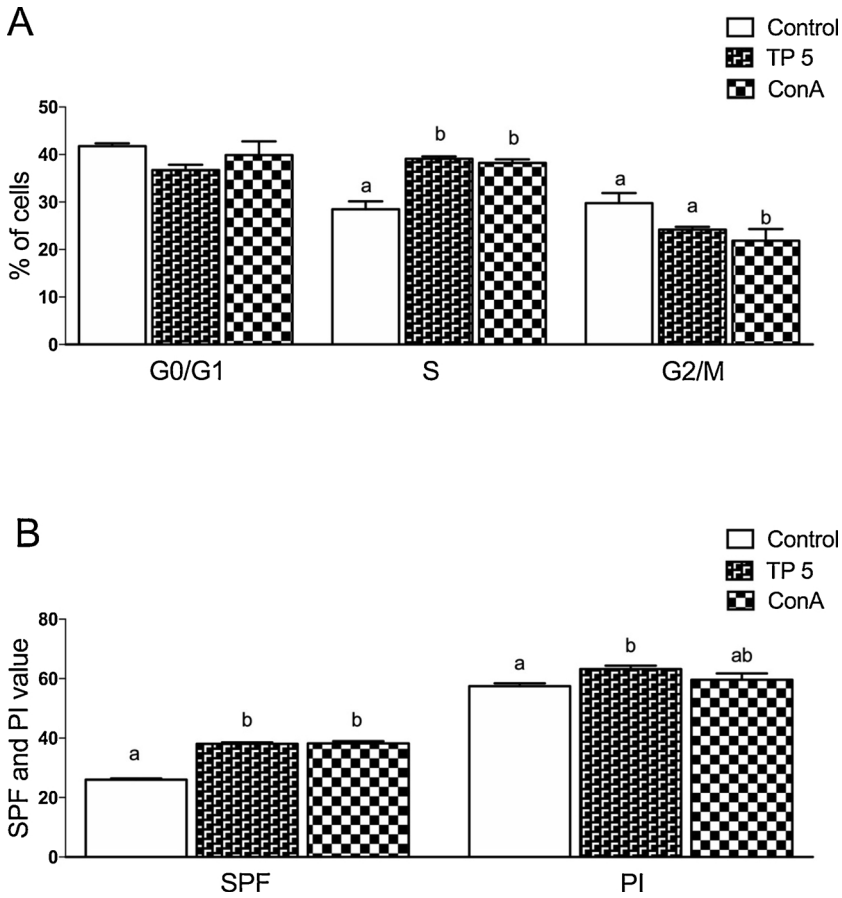

Fig. 3. (A) Effect of TP 5 on SMLN lymphocytes cell cycle; (B) SPF and PI values of SMLN lymphocytes stimulated with TP 5. SMLN lymphocytes (5×10^6 cells/well) were treated with 0, 200 μg/mL TP 5 or 2.5 μg/mL ConA for 48 h. Then the lymphocytes were washed and fixed overnight. After labelling with propidium iodide, the samples were analyzed by FCM. Values represent mean ± SE (n = 3). Bars with different letters differ significantly ($p < 0.05$).

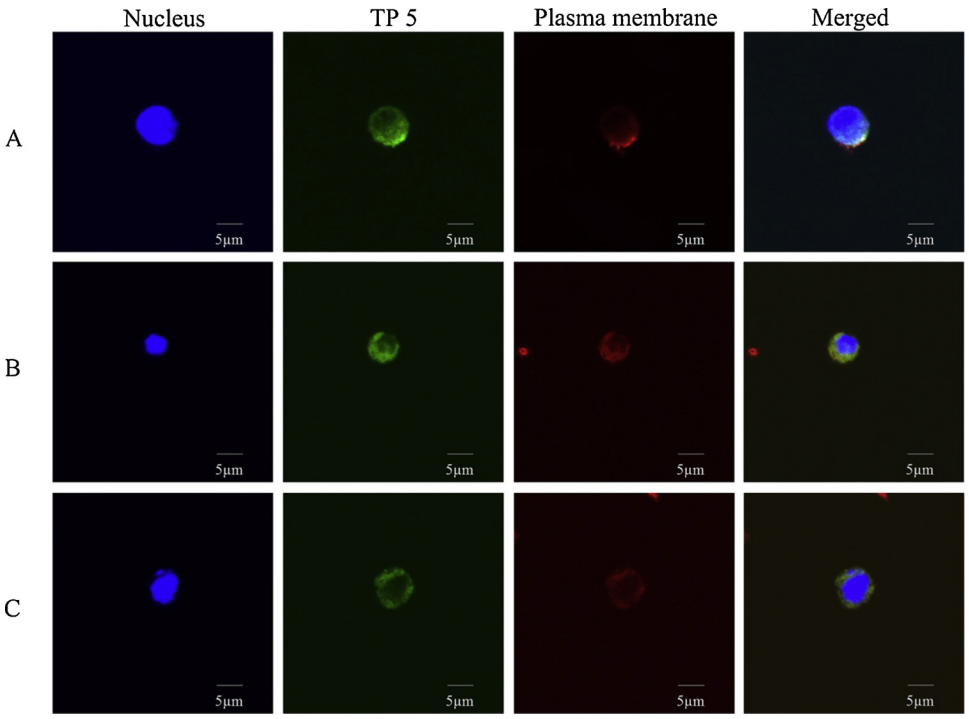

Fig. 5. Confocal microscopic observation of the binding of TP 5 to SMLN lymphocytes. (A), (B) and (C) were represented 3 different lymphocytes, respectively. The SMLN lymphocytes were incubated with 200 μL of FITC-labeled TP 5 for 2 h and then washed with PBS. After incubated with DAPI for 15 min to stain the cell nuclei, cell membrane was stained with Dil for 10 min. Images were captured at 600× magnification.

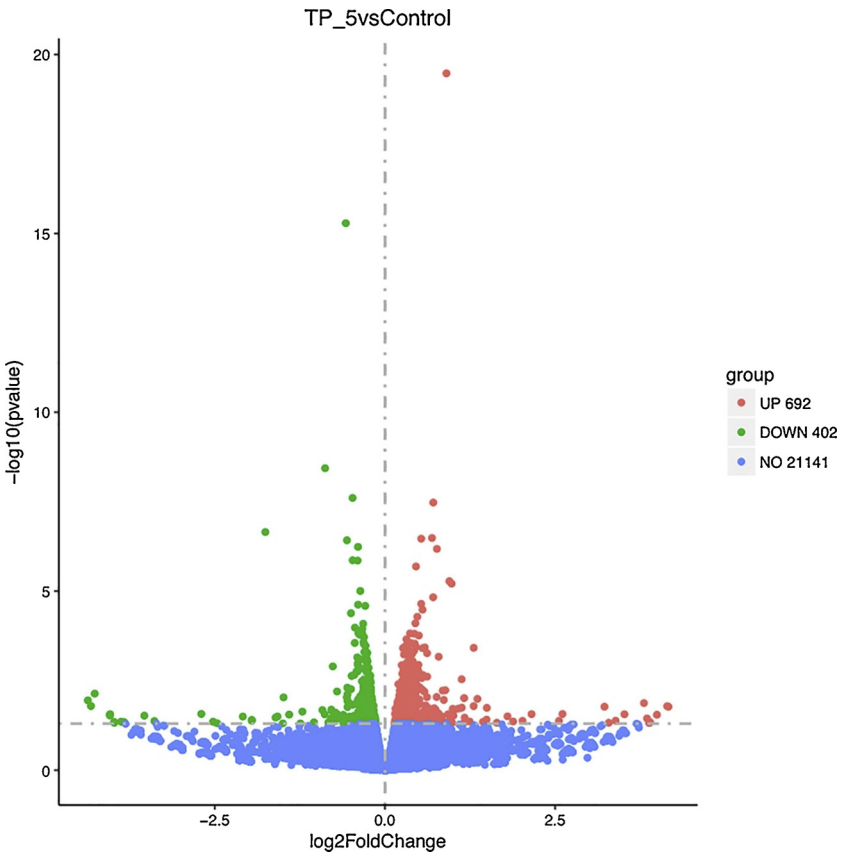

Fig. 6. Volcano plot for DEGs. The X-axis represents the log2FoldChange of all the genes. The Y-axis represents −log10 (*p*-value). Red represents up-regulated DEGs. Green represents down-regulated DEGs. Blue represents no-DEGs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Waltham, MA, USA). RNA-Quick Purification Kit was purchased from ES Science Biotech (Shanghai, China). iScript™ cDNA Synthesis kit and RT Master Mix and SYBR® Premix Ex TaqTM II (Tli RNaseH Plus) were obtained from Bio-Rad (USA). Cell Cycle and Apoptosis Analysis Kit was purchased from Multi Sciences Biotech Co., Ltd. (Hangzhou, China). Dil perchlorate and DAPI were purchased from Meilun Biotechnology Co., Ltd. (Dalian, China). Antifade Mounting Medium was obtained from Beyotime Biotechnology (Shanghai, China).

2.2. Isolation and culture of lymphocytes

Isolation of SMLN lymphocytes was as described previously [23]

Table 1
KEGG pathways of DEGs.

Pathway	*p*-value	*p*-adjust
IL-17 signaling pathway	3.78×10^{-7}	1.09×10^{-4}
NF-kappa B signaling pathway	3.49×10^{-6}	4.86×10^{-4}
Epstein-Barr virus infection	5.08×10^{-6}	4.86×10^{-4}
Influenza A	1.65×10^{-5}	1.19×10^{-3}
Hepatitis C	4.57×10^{-5}	2.62×10^{-3}
Herpes simplex infection	8.11×10^{-5}	3.79×10^{-3}
Steroid biosynthesis	9.24×10^{-5}	3.79×10^{-3}
NOD-like receptor signaling pathway	1.18×10^{-4}	4.23×10^{-3}
Measles	1.35×10^{-4}	4.31×10^{-3}
TNF signaling pathway	1.65×10^{-4}	4.73×10^{-3}
Adipocytokine signaling pathway	5.46×10^{-4}	1.42×10^{-2}
Apoptosis	6.49×10^{-4}	1.55×10^{-2}
Osteoclast differentiation	9.18×10^{-4}	2.03×10^{-2}
Biosynthesis of amino acids	1.04×10^{-3}	2.12×10^{-2}
Chronic myeloid leukemia	1.31×10^{-3}	2.50×10^{-2}
Cytokine-cytokine receptor interaction	1.98×10^{-3}	3.40×10^{-2}
MAPK signaling pathway	2.01×10^{-3}	3.40×10^{-2}
Human cytomegalovirus infection	2.25×10^{-3}	3.59×10^{-2}
Th1 and Th2 cell differentiation	2.70×10^{-3}	4.09×10^{-2}
Toll-like receptor signaling pathway	3.00×10^{-3}	4.31×10^{-2}
Cytosolic DNA-sensing pathway	3.19×10^{-3}	4.34×10^{-2}
C-type lectin receptor signaling pathway	3.33×10^{-3}	4.34×10^{-2}
T cell receptor signaling pathway	3.68×10^{-3}	4.59×10^{-2}
Salmonella infection	3.95×10^{-3}	4.72×10^{-2}
Th17 cell differentiation	4.21×10^{-3}	4.83×10^{-2}

with some modifications. Briefly, SMLN was aseptically removed from a slaughtered cow and transferred to the laboratory for further processing. A piece of tissue was dissected in the SMLN and placed in Hank's Balanced Salt Solution (HBSS). Tissue fragments of 5 *g* were washed with HBSS and carefully minced with surgical scissors, then filtered through a fine steel mesh net to obtain a homogeneous cell suspension. The suspension was transferred to a new tube and centrifuged at 500 rpm for 10 min. The pelleted cells were washed 3 times in PBS and resuspended in RPMI 1640 complete medium (RPMI 1640 medium supplemented with 100 UI/mL penicillin, 100 μg/mL streptomycin and 10% heat inactivated FBS). Cell suspension was adjusted to 5×10^7 cells/mL with a haemocytometer by trypan blue dye exclusion with viability above 95%.

The animal procedure was approved by the Institutional Animal Care and Use Committee at Zhejiang University and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.3. Lymphocyte proliferation assay

The SMLN lymphocytes (5×10^6 cells/well) were cultured in triplicate in RPMI-1640 medium supplemented with 10% FBS at 37 °C in a humid atmosphere with 5% CO_2 in the presence of ConA (2.5 μg/mL) or TP 5 (0, 50, 100, 200, 400 μg/mL). After treatment for 44 h, 20 μL of MTT solution (5 mg/mL) was added to every well followed by incubation for another 4 h. Then the plate was centrifuged at 1500 rpm for 5 min and the untransformed MTT was carefully removed by pipetting. To each well, a total of 150 μL DMSO was added and the absorbance was evaluated in a microplate reader (Multiskan FC, Thermo, USA) at 570 nm. The stimulation index (SI) was calculated based on the following formula: SI = absorbance value for mitogen cultures divided by the absorbance value for non-stimulated cultures.

2.4. FCM analyses of cell cycle distribution

SMLN lymphocytes were collected and resuspended in RPMI 1640 complete medium, then 5×10^6 cells/well were seeded into a 24-wells plate in triplicate and treated with 0, 200 μg/mL TP 5 or 2.5 μg/mL ConA. After incubation for 48 h, lymphocytes were collected, washed twice with PBS and fixed in cold 75% ethanol solution at 4 °C overnight. The fixed lymphocytes were washed twice with PBS and labeled with propidium iodide in the presence of RNaseA at 37 °C for 30 min following the instructions of Cell Cycle and Apoptosis Analysis Kit (Meilun, Dalian, China). The samples were then analyzed by FCM. The percentages of cells in each phase were determined using ModFit LT 3.1 cell cycle analysis software (Becton Dickinson, NJ, USA). S-phase fraction (SPF) and proliferation index (PI) were calculated on the basis of the following equations : SPF = S/(G0/G1 + S + G2/M) × 100% ; PI =(S + G2/M)/(G0/G1 + S + G2/M) × 100%.

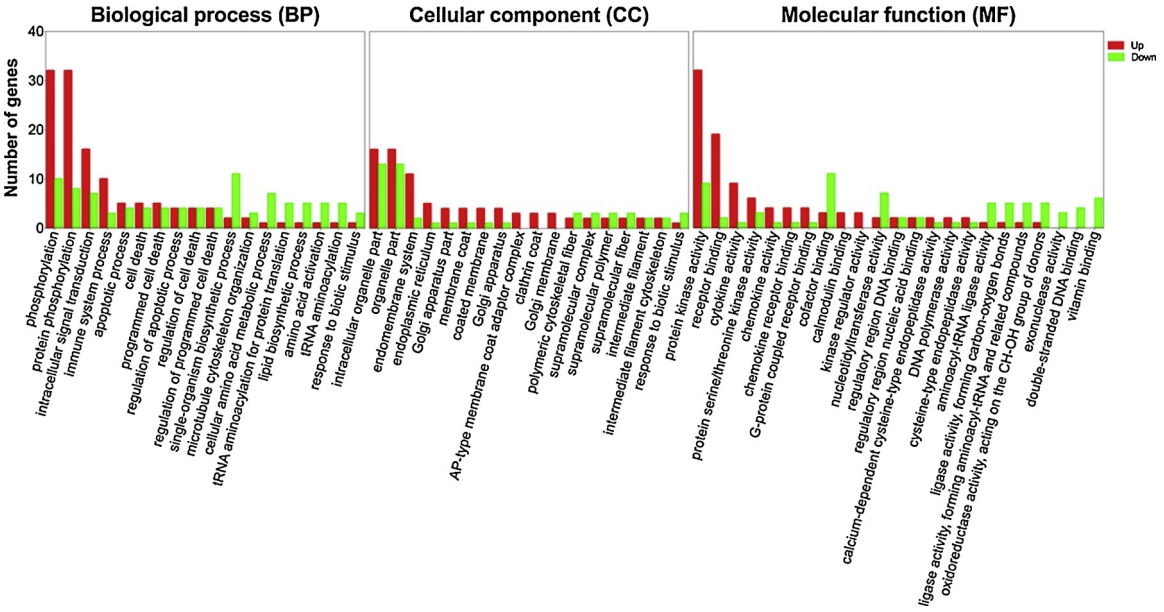

Fig. 7. The column diagrams for GO analysis of DEGs. The X-axis represents the functions of GO analysis. The Y-axis represents the numbers of DEGs. Red represents up-regulated DEGs. Green represents down-regulated DEGs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

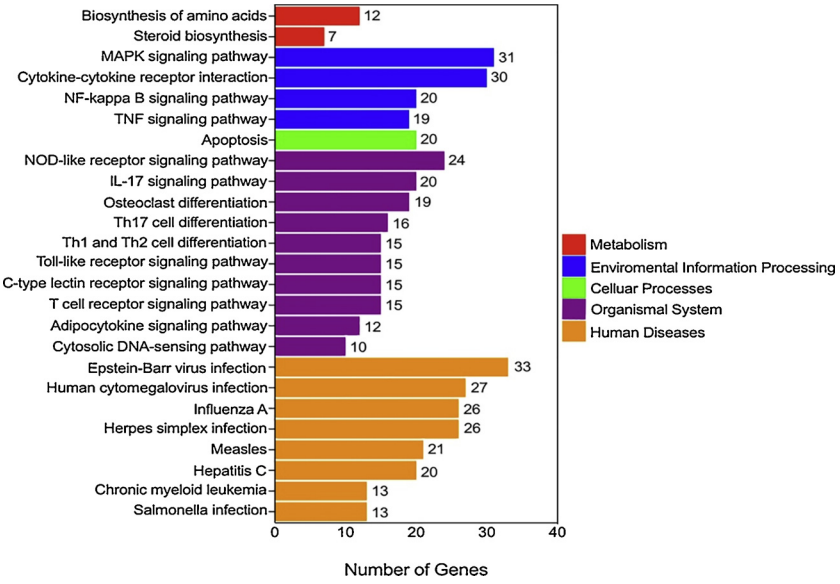

Fig. 8. The column diagrams for KEGG analysis of DEGs. The X-axis represents the numbers of DEGs. The Y-axis represents the functions of pathways. Each color represents the appropriate biological process.

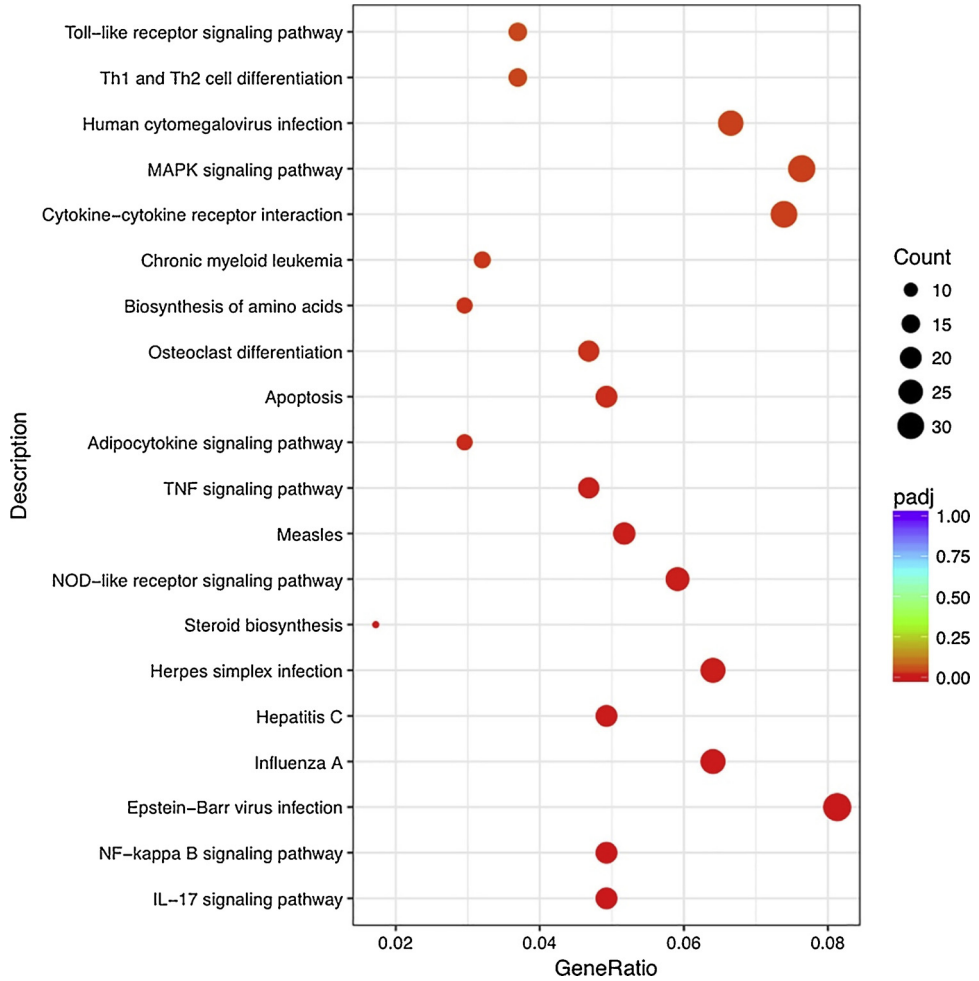

Fig. 9. The diagram for the enrichment degree of pathways. The X-axis represents the value of gene ratio (the ratio of annotated DEGs to all genes of enriched pathway). The Y-axis represents the enrichment term of pathway. The size of the dot represents the number of DEGs. The color represents adjust *p*-value.

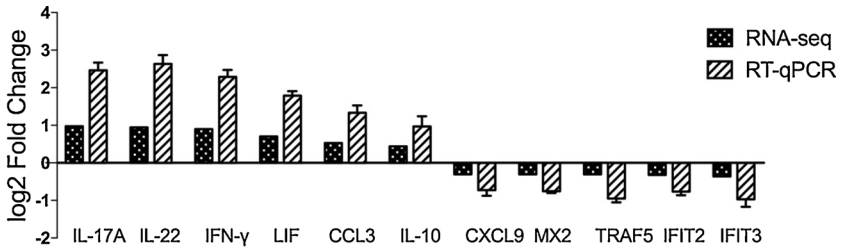

Fig. 10. log2FoldChange of relative mRNA expression level by RNA-seq and RT-qPCR. 11 DEGs (5 up-regulated DEGs and 6 down-regulated DEGs) were selected to validate the transcriptome sequencing results using RT-qPCR. Values represent mean ± SE (n = 3).

2.5. Quantification of target genes by RT-qPCR

SMLN lymphocytes were seeded into a 24-well plate at 5×10^6 cells/well in triplicate and treated with 0 or 200 μg/mL TP 5. After incubation for 48 h, lymphocytes were collected by centrifugation at 1500 rpm for 5 min. Total RNA was extracted using RNA-Quick Purification Kit (ES Science, China) and converted into cDNA with PrimeScript™ RT Master Mix by a T100™ thermal cycler (Bio-Rad Laboratories, Inc., USA). The specific primers satisfying the criteria of NCBI/Primer-BLAST were synthesized by Sangon Biotech with the following sequences: β-actin, 5′-TCACCAACTGGGACGACA-3′ and 5′-GCATACAGGGACAGCACA-3′; IL-17A, 5′-TGTCTACAGTGAACTGGA AGGAAC-3′ and 5′−CCACCAGACTCAGAAGCAGTAG-3′; IL-17F, 5′-TGACATTGTGGCTCACCCTC-3′ and 5′−CCCTCACAATGTGGTCC TCC-3′. Quantitative PCR was conducted using SYBR®Premix Ex TaqTM II (Tli RNaseH Plus) on CFX-96 Touch™Real-Time Detection System (Bio-Rad, USA) and determined employing the comparative Ct method ($2^{-\Delta\Delta Ct}$). All samples were analyzed in triplicate.

2.6. Confocal microscope analysis of membrane binding

SMLN lymphocytes were incubated with 200 μL of FITC-labeled TP 5 for 2 h at 37 °C in the presence of 5% CO_2. Then SMLN lymphocytes were washed with PBS and incubated with DAPI for 15 min at room temperature to stain the cell nuclei, and then cell membrane was stained with Dil perchlorate (Meilun Biotechnology Co., Ltd., Dalian, China) for 10 min. Lymphocytes were washed 3 times and preserved in Antifade Mounting Medium (Beyotime Biotechnology, China) for confocal microscopy.

2.7. RNA-seq Analysis and validation

Library preparation and sequencing were performed at Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). Total RNA sample libraries were extracted using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA), according to the standard manufacturer's instructions. In brief, poly-T oligo-attached magnetic beads were used to extract mRNA from total RNA. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). cDNA was then synthesized from RNA. First strand cDNA was synthesized with random hexamer primer and Reverse Transcriptase. In the second strand of cDNA synthesis, DNA Polymerase I and RNase H were used. After the library was constructed, the fragmented library was enriched by PCR amplification, and then the library was selected according to the size of the fragment. The size of the library was 250 to 300 bp in length. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer to generate RNA-seq libraries ready for sequencing. Before sequencing, RNA-seq libraries were qualified on the Agilent Bioanalyzer 2100 system. Quantification of libraries for clustering was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer's instructions. Sequencing was performed using the Next-Generation Sequencing (NGS) technology based on an Illumina Hiseq platform and 150 bp paired-end reads were generated. For quality control, clean reads were obtained by removing reads containing adapter, reads containing poly-N and low-quality reads from raw reads. All the downstream analyses were based on the clean data with high quality. HTSeq v0.6.0 was used to count the read counts for each gene in the ensemble annotation. For differentially expressed genes (DEGs) analysis, DESeq2 R package (1.16.1) was selected to detect the differential expression between the TP 5 group and Control group (n = 3) with an adjusted $p < 0.05$. ClusterProfiler R package was used to perform Gene Ontology (GO) enrichment analysis and to test the statistical enrichment of DEGs in Kyoto encyclopedia of genes and genomes (KEGG) pathways. GO terms and KEGG terms with corrected $p < 0.05$ were considered significantly enriched by DEGs.

11 DEGs (5 up-regulated DEGs and 6 down-regulated DEGs) were selected to validate the transcriptome sequencing results using RT-qPCR as previously described (*2.6*).

2.8. Statistical analysis

Data were presented as means ± SE and were analyzed with the GraphPad Prism 7.0 software (GraphPad, San Diego, CA, USA). All statistical analyses were conducted with Student's *t*-test or one-way analysis of variance (ANOVA). In the tests of lymphocyte proliferation assay, cell cycle and RT-qPCR, $p < 0.05$ were considered to indicate statistically significant differences between the control and other groups.

3. Results

3.1. The effect of TP 5 on cell proliferation of SMLN lymphocytes

To investigate the effect of TP 5 on T lymphocyte activation, we used MTT assay and found SMLN lymphocytes were significantly more when the cells were incubated with TP 5 at the concentration of 200 and 400 μg/mL than control group (Fig. 2).

3.2. Effect of TP 5 on SMLN lymphocytes cell cycle

As shown in the Fig. 3A, in G0/G1 phase, there was no significant difference among groups. In S phase, the percentage of cells in TP 5 group and ConA group was significantly higher than that in control group. There was no difference between TP 5 group and ConA group. In G2/M phase, the percentage of cells in ConA group was significantly lower than that in control group and TP 5 group. As illustrated in Fig. 3B, SPF and PI results showed that TP 5 had similar activity to ConA in promotion of SMLN lymphocyte proliferation.

3.3. Effects of TP 5 on mRNA expression of cytokines associated with mastitis

As illustrated in Fig. 4, 2 cytokines related to cow mastitis were screened for RT-qPCR by referring to the related literature of cow

mastitis and the results of transcriptome sequencing. The results showed that the mRNA expressions of IL-17A and IL-17F were significantly increased.

3.4. Binding sites of TP 5 on SMLN lymphocyte

Fig. 5 showed that FITC-labeled TP 5 was on the surface of SMLN lymphocytes, suggesting that TP 5 may bind to the receptors on the cell membrane and exert its immunomodulatory activities.

3.5. RNA-seq and bioinformatic analysis

To further insights into the underlying molecular mechanisms involve in the immunomodulatory activity of TP 5 on SMLN lymphocytes, RNA-seq was performed. DEGs analysis showed 1094 genes were differentially expressed in a significant manner (adjusted p value < 0.05) in TP 5 stimulated SMLN lymphocytes, of which the up and down regulated genes were 692 and 402, respectively (Fig. 6). The known DEGs of $|\log2FoldChange| > 2$ in TP 5 group compared with Control group were listed in Table 1. To determine the functional genes which were chiefly involved in responses to TP 5 stimulation, GO enrichment analysis was performed. A total of 48 significant enriched GO terms were identified (Fig. 7). Among them, 18 GO terms were enriched in biological process, the top 5 were shown as phosphorylation, protein phosphorylation, intracellular signal transduction, immune system process and apoptotic process, respectively. 17 GO terms were enriched in cellular component, the top 5 were shown as intracellular organelle part, organelle, endomembrane system, endoplasmic reticulum and Golgi apparatus part, respectively. 23 GO terms were enriched in molecular function, the top 5 were shown as protein kinase activity, receptor binding, cytokine activity, cofactor binding and nucleotidyltransferase activity, respectively. To further understand the biological function of DEGs, KEGG pathway analysis was carried out. KEGG pathway enrichment analysis identified significant enrichment of DEGs involved in 25 pathways (Table 1), which were classified in 5 classifications by the function (Fig. 8). The analysis also demonstrated significant enrichment of functions associated with immune system pathway (Fig. 9).

To certify the RNA-seq results, 11 DEGs that were up or down regulated in TP 5 stimulated SMLN lymphocytes were selected and measured gene expression by RT-qPCR. The result showed that the tendency of RT-qPCR and RNA-seq were same (Fig. 10).

4. Discussion

When bacteria break through the physical barrier such as teat canal, and invade the sterile mammary gland, rapid and appropriate host defense is needed to resist bacterial colonization and subsequent pathological changes, so as to control the further development of mastitis. Compared with the healthy individuals, cows with severe negative energy balance (SNEB) have lower innate immunity to resist mastitis, which is characterized by fewer circulating white blood cells, especially lymphocytes [24]. In the mammary gland of dairy cows, lymph is drained into SMLN through lymphatic vessels. Lymphocytes in SMLN mediate adaptive immune response, and regulate the immune response of mammary gland by inducing and regulating the activation, proliferation and differentiation of immune cells. In our study, TP 5 promoted lymphocyte proliferation, increased the proportion of S phase cells, SPF and PI index. Similar results have been also found in other studies. For examples, the rat homologue of the human thymopoietin protein family β (hTMPOβ) called lamina-associated polypeptide 2 (LAP2) was involved in the regulation of the nuclear lamina growth during cell cycle [25]. TP 5 was found to inhibit the growth of human promyelocyte leukemia cell line (HL-60) cells to G0/G1 phase and inhibits cell proliferation [19]. Thus, TP 5 may regulate the cellular immunity at least by accelerating lymphocyte mitosis and DNA synthesis.

Cytokines, as a small molecule substance with wide biological activity, play a bridge role in regulating the interaction between innate immune cells and adaptive immune cells, and are a direct way to participate in the immune response [26]. Recombinant cytokines can be used to treat mastitis, for example, recombinant bovine IFN-γ enhances the activity of T lymphocytes, macrophages and neutrophils [27]. Recombinant bovine IL-1β and IL-2 can enhance the natural defensive mechanisms of mammary gland [28]. When cow mastitis occurs, inflammatory cytokines secreted by immune cells can induce antimicrobial response through epithelial cells and recruit neutrophils and dendritic cells to the infected sites. The mammary gland infected with *E.* coli secretes a large number of leukocytes and inflammatory cytokines such as IL-1, IL-6, TNF-α, IL-10, IL-17A, IL-22 or IFN-γ that contribute to the mammary defense against *E.* coli infection [29,30]. In the late stage of mastitis, pro-inflammatory cytokines produced by activated immune cells are inhibited by anti-inflammatory cytokines to control host tissue damage and the development of immunological diseases [31]. IL-17 family cytokines are involved in acute and chronic inflammation and play an important role in resisting mastitis infection in dairy cows [32]. IL-17A and IL-17F are similar in cell origin and function. They are mainly secreted by Th17 cells. However, it has been found that non-Th17 cells, such as lymphocytes, can also secrete IL-17 [33–36]. In our study, we found that the expression of IL-17A and IL-17F increased significantly when lymphocytes were treated with TP 5, suggesting that TP 5 may modulate SMLN lymphocytes by activating IL-17 signal pathway.

RNA-seq is a technology that has been extensively employed to understand mammalian transcriptome architecture and function [37]. This technology was thus performed to investigate the effect of TP 5 on gene expression by SMLN lymphocytes and to explore the potential signaling pathways in the present study. Among the 1094 DEGs, it should be particularly noted that 6 genes (including IFIT3, IFIT2, MX1, MX2, IFIT5 and IFIH1) encoding interferon-induced proteins were significantly down-regulated.

Cows with SNEB were reported to have less resistance to pathogenic invasion and long period for uterine recovery with high expression of interferon-induced genes such as IFIH, MX1 and MX2 [24]. Down-regulation of these genes found in this study in SMLN lymphocytes indicated that TP 5 might alleviate the SNEB to help animals to eliminate invading pathogens. As another representative DEG in TP 5-activated lymphocytes, SOCS3 (with N-terminal domain, central SH2 domain and C-terminal domain) plays a central role in neutrophil mobilization, T cell development and inflammatory gene transcription by a negative feedback in JAK-STAT signaling pathway [38,39]. The increased expression of SOCS3 observed in TP 5-treated cells might benefit the neutrophil migration to the mammary gland and phagocytosis of pathogens [31,40]. Moreover, GO enrichment analysis of the DEGs revealed that TP 5 might act as an immunoregulatory agent by affecting the activity of protein kinase, receptor binding and cytokine activity, which was consistent with the increased cytokine expression by RT-qPCR and the binding interaction between TP 5 and lymphocytes. KEGG is a knowledge database for systematic analysis of gene functions, linking genomic information with higher order functional information [41]. In KEGG analysis, cytokine-cytokine receptor interaction, MAPK signaling pathway, NF-κB signaling pathway and IL-17 signaling pathway were predominantly enriched, which were in accordance with the increased expression of IL-17A and IL-17F that were able to activate the cascades of NF-κB and MAPK [42,43]. Therefore, TP 5 might regulate SMLN lymphocytes through multiple signaling pathways via interacting with its functional receptors.

5. Conclusion

In the present study, TP 5 significantly promoted lymphocyte proliferation, accelerated cell cycle progression, and enhanced mRNA expression of IL-17A and IL-17F. Laser scanning confocal microscopic

analysis revealed the binding of TP 5 to the surface of SMLN lymphocytes. A total of 1094 genes were identified as DEGs using RNA-seq with 692 up- and 402 down-regulated genes. 48 significantly enriched GO terms were identified by RNA-seq. In KEGG analysis, 1/3 of DEGs were enriched in the immune system pathway, including IL-17 signaling pathway, cytokine-cytokine receptor interaction, Th1 and Th2 cell differentiation, T cell receptor signaling pathway, Th17 cell differentiation. Among them, IL-17 signaling pathway was the most prominent. Therefore, the therapeutic benefit of TP 5 in bovine mastitis might be attributed to its immunomodulatory activity in SMLN lymphocytes.

Declaration of Competing Interest

The author declare that they have no conflict of interest.

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