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Transport and immunomodulatory effect of *Hericium erinaceus* peptide LPGKVIAS

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Abstract

Hericium erinaceus–derived peptides have been found to exhibit various bioactivities, including immunoregulatory properties. This study investigated the transport, absorption, and potential immunomodulatory activities of a new peptide, Leu-Pro-Gly-Lys-Val-Ile-Ala-Ser (LPGKVIAS), derived from *H. erinaceus*. Transport and absorption of LPGKVIAS were analyzed by near-infrared fluorescence *in vivo* imaging in mice injected with a fluorescence probe–labeled LPGKVIAS. RNA sequencing was used to explore the immunological effects of the peptide on mouse splenocytes. Kyoto Encyclopedia of Genes and Genomes pathway analysis showed that LPGKVAS upregulated differentially expressed genes involved in immune regulation. Notably, activation of the lysosome–phagosome pathway in splenocytes of mice treated with LPGKVIAS was identified as a crucial immune defense mechanism against pathogen infection. Furthermore, reverse transcription–polymerase chain reaction studies validated the gene expression data, supporting the potential application of the peptide LPGKVIAS as an immunomodulator.

Keywords: *Hericium erinaceus* active peptide; fluorescence imaging; transportation; RNA-Seq; immunoregulatory properties.

Introduction

Hericium erinaceus, a traditional edible mushroom, has been found to possess a range of immunomodulatory, anti-inflammatory, antioxidant, and antitumor bioactivities (Chen *et al*., 2017; Chang *et al*., 2021; Xie *et al*., 2022). The active components including polypeptides and polysaccharides extracted from cultured mycelium and fruiting bodies of *H. erinaceus* have been shown to significantly enhance both specific and nonspecific immune responses (Li *et al*., 2020; Wang *et al*., 2020; Liu *et al*., 2022; Gu *et al*., 2024). Peptides isolated from *H. erinaceus* in previous studies have demonstrated promising immunomodulatory activities (Yu *et al*., 2021).

Bioactive peptides, which typically comprise 2–20 amino acid residues with molecular weights less than 3 kDa, are well known for their physiological health benefits that surpass the nutritional value of their precursor proteins (Cui *et al*., 2020; Katayama *et al*., 2021). Recently, bioactive peptides derived from edible mushrooms have garnered significant attention due to their diverse biological activities, including immunomodulation (Li *et al*., 2011, 2021), antihypertensive effects (Lau *et al*., 2013; Wu *et al*., 2019; Zhou *et al*., 2020), and antioxidant properties (Khatun *et al*., 2015; Wu *et al*., 2022a). However, there is limited information available regarding the immunological activities of bioactive peptides from *H. erinaceus*. In a previous study, we isolated an enzymatic hydrolysate, hephaestin (HEPH)-A, from *H. erinaceus* protein. HEPH-A exhibited good immunomodulatory potential by stimulating the proliferation, phagocytic activity, and nitric oxide secretion in macrophages. The bioactive peptide Leu-Pro-Gly-Lys-Val-Ile-Ala-Ser (LPGKVIAS, 784.5 Da) was isolated and identified from HEPH-A using ultrafiltration and multiple chromatographic technologies (Yu *et al*., 2021). However, the *in vivo* absorption and immunomodulatory effects of the peptide remain unclear. To elucidate the immunomodulatory mechanisms of LPGKVIAS, its potential as an immunostimulant was explored.

Preliminary investigations have addressed the molecular structure and biological activity of active peptides, but few studies have investigated their *in vivo* absorption and transport (Katritzky *et al*., 2008; Dean and Palmer, 2014; Bi *et al*., 2022). While some studies have shown that bioactive peptides can traverse the intestinal barrier and enter the bloodstream in their intact form to carry out their biological functions, others are vulnerable to degradation in the gastric microenvironment, which hinders their bioactivity (Xu *et al*., 2019). Many bioactive peptides have the ability to resist intestinal membrane proteases, survive through gastrointestinal tract, and reach their target organs intact. While *in vitro* models like

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Caco-2 cells provide insights into the absorption and transport of active peptides, they are unable to fully replicate the *in vivo* environment, particularly the complex intestinal barrier (D'Opazo *et al*., 2023; Zhang *et al*., 2023). Therefore, it is crucial to carry out *in vivo* studies to gain a better understanding of the actual absorption process of active peptides within the body. Our study investigated the *in vivo* absorption of LPGKVIAS in mice after oral administration. We introduced structural modifications to create an active site without affecting the optical properties of cyanine (Cy) and generated fluorescence probe–labeled LPGKVIAS peptides to assess their transport and absorption in mice (Shindy, 2017; Wang *et al*., 2017; Zhang *et al*., 2021).

The spleen, the body's largest immune organ, plays a crucial role in immune functions, hematopoiesis, and blood filtration (Lewis *et al*., 2019). Various immune cells, including macrophages, dendritic cells, B lymphocytes, and T lymphocytes, respond to inflammation and infections by releasing specific cytokines. Pathogens and apoptotic cells are eliminated through phagocytosis, a process carried out by specialized cells called professional phagocytes, such as macrophages, neutrophils, monocytes, dendritic cells, and osteoclasts (Uribe-Querol and Rosales, 2020). Phagocytosis is vital for the innate defense system, as it involves ingesting and eliminating pathogens. This process involves identifying and engulfing foreign substances into an organelle called the phagosome, which is crucial for the ability of phagocytes to eliminate pathogens (Lee *et al*., 2020a; Watts, 2022). Studies have shown that the phagosome and lysosome pathways contribute to immunity in various animals, including mice, humans (Zheng *et al*., 2024), sea urchins (Chiaramonte *et al*., 2019), and shrimp (Xu *et al*., 2024), making them essential for understanding the immune regulatory mechanisms in animals. Our study involved a transcriptomic analysis of mouse spleen cells treated with LPGKVIAS to identify key mechanisms involved in immune modulation, and changes in gene expression were validated by reverse transcription–polymerase chain reaction (RT-PCR) analysis.

Materials and Methods

Reagents and animals

The peptide structure was determined by mass spectrometry according to our previously described method (Yu *et al*., 2021). The LPGKVIAS oligopeptide was synthesized by GenScript Biotechnology (Nanjing, China) via solid-phase synthesis (purity≥98%).

Male ICR and C57BL/6 mice (20±2 g) were obtained from Henan Skobes Biotechnology (Anyang, China; Certificate number: SCXK (Yu) 2020–0005). The mice were housed in a facility maintained at 23±1 °C with a 12-h light/dark cycle. All the mice were acclimated in the laboratory for one week before the experiment.

Ethical statement

All animal experiments complied with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines and were carried out following the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, EU Directive 2010/63/EU. The China Pharmaceutical University Animal Ethics Committee evaluated and approved the experimental protocol (permission number: 2022-09-003).

Preparation of the fluorescent probe–labeled peptide

The cyanine 7 (Cy7) fluorescent probe–labeled peptide was prepared as described elsewhere (Bouteiller, *et al*., 2007). The synthetic route is illustrated in Figure 1B. The detailed reaction conditions and processes are described in the supporting information. Electrospray ionization mass spectrometry was performed on a Waters QTOF micro mass spectrometer (Waters, Milford, CT, USA).

Near-infrared fluorescence imaging

LPGKVIAS-Cy7 (200 μmol/L) was prepared in dimethyl sulfoxide. The mice were given a dose of 200 μL of LPGKVIAS-Cy7 by intragastric administration after a 24-h fasting period with free access to water. *In vivo* imaging was performed using the IVIS Lumina III small animal optical two-dimensional imaging system (PerkinElmer, Waltham, MA, USA) to monitor the *H. erinaceus* peptides over time (0, 1, 2, 6, 8, 12, 24, and 48 h). For *ex vivo* imaging, the mice were sacrificed, and major organs were harvested at 1, 6, 12, and 24-h post-administration.

Spleen collection and cell culture

Ten male purebred C57BL/6 mice were sacrificed, and under aseptic conditions, the spleens were surgically extracted and homogenized with sterile syringes. The spleen cells were suspended in red blood cell lysis buffer and filtered through a 40-μm nylon mesh. The isolated cells were propagated in Dulbecco's Modified Eagle Medium (DMEM; Hyclone, Logan, UT, USA) with 10% fetal bovine serum (Grand Island, NY, USA) at 37°C and 5% CO_2 in a humidified incubator. Exponential-phase cells were seeded in 96-well plates ($2{\times}10^4$ cells/well) for the cell proliferation experiment and in 6-well plates ($5{\times}10^6$ cells/well) for RNA extraction and RT-PCR analysis.

Cell proliferation assay

The experimental design followed that of Yu *et al*. (2021) with slight modifications. Briefly, 96-well plates were seeded with 100 μL of cell suspension and incubated overnight at 37 °C, 5% CO_2, and saturated humidity. The supernatant was discarded and replaced with 100 μL of culture medium containing LPGKVIAS (0, 50, 100, 200, 400, and 600 μmol/L). Each concentration was tested in five replicate wells, with one set of wells serving as the control group without the conditioned medium. After 24 h, 20 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT, 5 mg/mL; Beyotime Biotech, Shanghai, China) was added. The plate was placed in the incubator for another 4 h. Subsequently, 150 μL of dimethyl sulfoxide was added, and the mixture was shaken gently in a constant temperature shaker incubator for 10 min to dissolve the formazan crystals. The absorbance was measured at 490 nm. The experiment was performed three times. Cell viability was calculated as the percent change in absorbance (A490) between the control and treated cells as follows:

$$\text{Cell viability } (\%) = \frac{\text{ODe} - \text{ODo}}{\text{ODc} - \text{ODo}} \times 100\ \%$$

Here, ODe and ODc are the absorbance values of treated and untreated cells, respectively, and ODo is the absorbance value of the cell culture medium.

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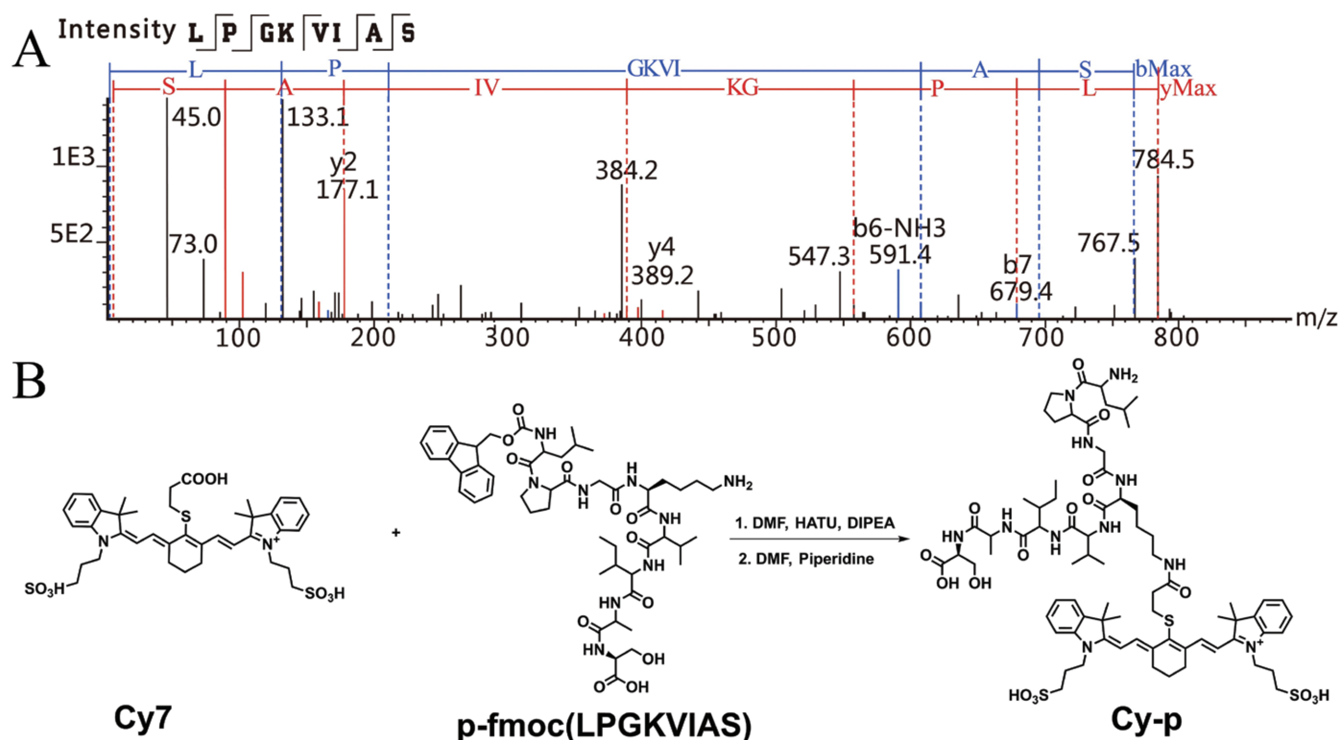

Figure 1. (A) Mass spectrometry of LPGKVIAS and (B) synthesis process of Cy7-LPGKVIAS. DMF: *N,N*-dimethylformamide; HATU: *O*-(7-azabenzotriazol-1-yl)-*N,N,N,N*-tetramethyluranium hexafluorophosphate; DIPEA: *N,N*-diisopropylethylamine.

RNA extraction and unique molecular identifier library creation

Total RNA was isolated from the spleen cells using TRIzol reagent (ThermoFisher, Waltham, MA, USA). A nanophotometer (Implen, Munich, Germany) was used to assess RNA purity and concentration. An Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) was used to measure RNA integrity (as shown in Supplementary Figure). The NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, Lpswich, MA, USA) was used to construct sequencing libraries. Finally, RNA samples were collected and stored at −80 °C.

RNA sequencing and analysis of differentially expressed genes

RNA sequencing was performed by Novogene (Beijing, China). The libraries were sequenced using an Illumina NovaSeq 6000 platform with 150 bp paired-end reads (San Diego, CA, USA). The clean reads were selected by removing the low-quality reads. The paired-end clean reads were mapped to the reference genome using HISAT2 (Kumar *et al*., 2023). Differential gene expression between the control and treatment groups was analyzed using the DEGSeq R package. The threshold for identifying differentially expressed genes (DEGs) was $|\log_2 FC|>0.58$ and $P<0.05$ (Zuo *et al*., 2021). Raw sequencing data were uploaded to the NCBI database (BioProjectID: PRJNA1032465).

Gene ontology and Kyoto encyclopedia of genes and genomes enrichment analyses

We performed gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analyses of the DEGs using clusterProfiler R (Robinson *et al*., 2010).

Quantitative real-time PCR

Five immune-related genes were selected from the DEGs to validate the accuracy of RNA-Seq expression profiles. Complementary DNA (cDNA) was synthesized using a cDNA reverse transcription kit (Vazyme Biotech Co. Ltd., Nanjing, China). The quantitative real-time PCR (qRT-PCR) cycle conditions were as follows: 95 °C for 30 s, 95 °C for 10 s, 60 °C for 30 s, and 40 cycles. Solubility curve analysis was performed from 60 to 95 °C. The primers used are listed in Table 1. Relative gene expression was quantified using the $2^{-\Delta\Delta C_t}$ method (Vats *et al*., 2023).

Statistical analysis

Data are reported as means±standard deviation (SD). GraphPad Prism 9 (La Jolla, CA, USA) was used for data visualization. SPSS software (IBM, Armonk, NY, USA) was used for statistical analysis, and $P<0.05$ denoted a significant difference.

Results

Synthesis and characterization of the fluorescent probe–labeled peptide

Previously, a novel bioactive peptide (LPGKVIAS; molecular weight (Mw) 784.5 Da) was identified in our investigation (Figure 1A). To track LPGKVIAS transport in mice, we generated a Cy7-fused peptide (Cy7-p), which was purified by reversed-phase high-performance liquid chromatography to >98%. Electrospray ionization mass spectrometry (ESI-MS) demonstrated an mass-to-charge ratio (*m/z*) of 765.76, which was consistent with the calculated *m/z* of 765.86 for $C_{75}H_{109}N_{11}O_{17}S_{32}^{-}$, confirming the successful conjugation of Cy7 to LPGKVIAS (Figure 2).

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Tissue distribution of fluorescent probe–labeled LPGKVIAS *in vivo*

Cy7 is an exceptionally selective and sensitive near-infrared fluorescence (NIRF) probe used for imaging mice (Feng *et al.*, 2020; Huang *et al.*, 2021; Wu *et al.*, 2022b). Therefore, animal experiments were designed and carried out as shown in Figure 3A. In this study, LPGKVIAS was labeled with Cy7 for NIRF imaging to examine its distribution and absorption *in vivo*. Figure 3B shows the tissue distribution images of Cy7-p after oral administration in mice. High signal intensity indicated that a significant portion of Cy7-p reached the gastrointestinal tract. Moreover, *in vitro* optical imaging of the gastrointestinal tract was performed to determine the absorption properties of Cy7-p. To characterize the distribution of Cy7-p, vital organs were photographed *ex vivo*. At 1 h post-administration, Cy7-p fluorescence was prominently distributed throughout the small intestine (Figure 3C). Over time, the fluorescence intensity gradually decreased, likely due to absorption and excretion. At 24 h, fluorescence persisted in the small intestine, indicating sustained retention of the active peptide LPGKVIAS. Furthermore, a distinct Cy7-p fluorescence signal was detected in the lungs, suggesting absorption into the systemic circulation. These findings support the ability of Cy7-p to traverse the gastrointestinal barrier and enter systemic circulation.

Table 1. Primer sequences

Gene	Primer sequence (5'-3')
CXCL3	Forward: CCAGAGAGGCTGTTCAGAAGTTCAC Reverse: GTCGGAAGTGGTTGGGGTCTTTG
STCK	Forward: TCCAGCCAGCCAGCCCATC Reverse: CATGCCCACTCCCTTCCAAAGC
CD14	Forward: GTGTCGTGGGCAACAAGGGATG Reverse: CTAAGGTGGAGAGGGCAGGGAAG
MARCO	Forward: TGAAAGGAAGCAAGGGTGACACAG Reverse: TTCTGCCTACAAGACCTGGGACTC
C1qb	Forward: GGCTCTGGGCTCTGGGAATC Reverse: CTAGAAGCAGCAGTAACAGGTGTG
β-actin	Forward: ATCACTATTGGCAACGAGCG Reverse: TCAGCAATGCCTGGGTACAT

Effects of LPGKVIAS on splenic cell proliferation

Splenic cell proliferation *in vitro* is a reliable indicator of whether a substance can stimulate immune cells and influence immunity (Hou *et al.*, 2012). The proliferation of spleen cells isolated from LPGKVIAS-treated mice (at 24 h) was evaluated by MTT assay (Figure 4), which revealed that LPGKVIAS significantly stimulated cell proliferation compared with the control group. Over a concentration range of 0 to 600 μmol/L, the most significant induction of splenic cell proliferation was observed at 50 μmol/L, confirming the potent immunostimulatory effect of LPGKVIAS. Thus, 50 μmol/L LPGKVIAS was selected for further experiments.

Transcriptome profile of mouse splenic cells

To ensure data accuracy and consistency, the original dataset was filtered to eliminate nonmethod-determined base information and low-quality reads. The preprocessing results of the sequencing data are presented in Table 2. After filtering, 20 875 773 reads were retained, with a minimum of 20 036 290 reads containing unique molecular identifiers. In addition, raw data filtering resulted in a minimum of 6.26 gigabases, with over 91.94% having quality values exceeding 30. The minimum percentage of G and C in the clean data was 49.4%. These combined indicators demonstrate that the sequencing data passed quality control standards and are suitable for further bioinformatics analysis. Principal component analysis of the transcriptome profile revealed significant differences between the spleen cells of the mice treated with LPGKVIAS and the control group (Figure 5).

Identification of DEGs

RNA-Seq analysis was conducted to compare the gene expression profiles of the spleen cells of the mice treated with LPGKVIAS with those of the untreated mice. The results revealed 954 DEGs with 726 upregulated and 228 downregulated in the LPGKVIAS-treated group compared

Figure 2. ESI mass spectrum of the fluorogenic substrate of Cy7-p.

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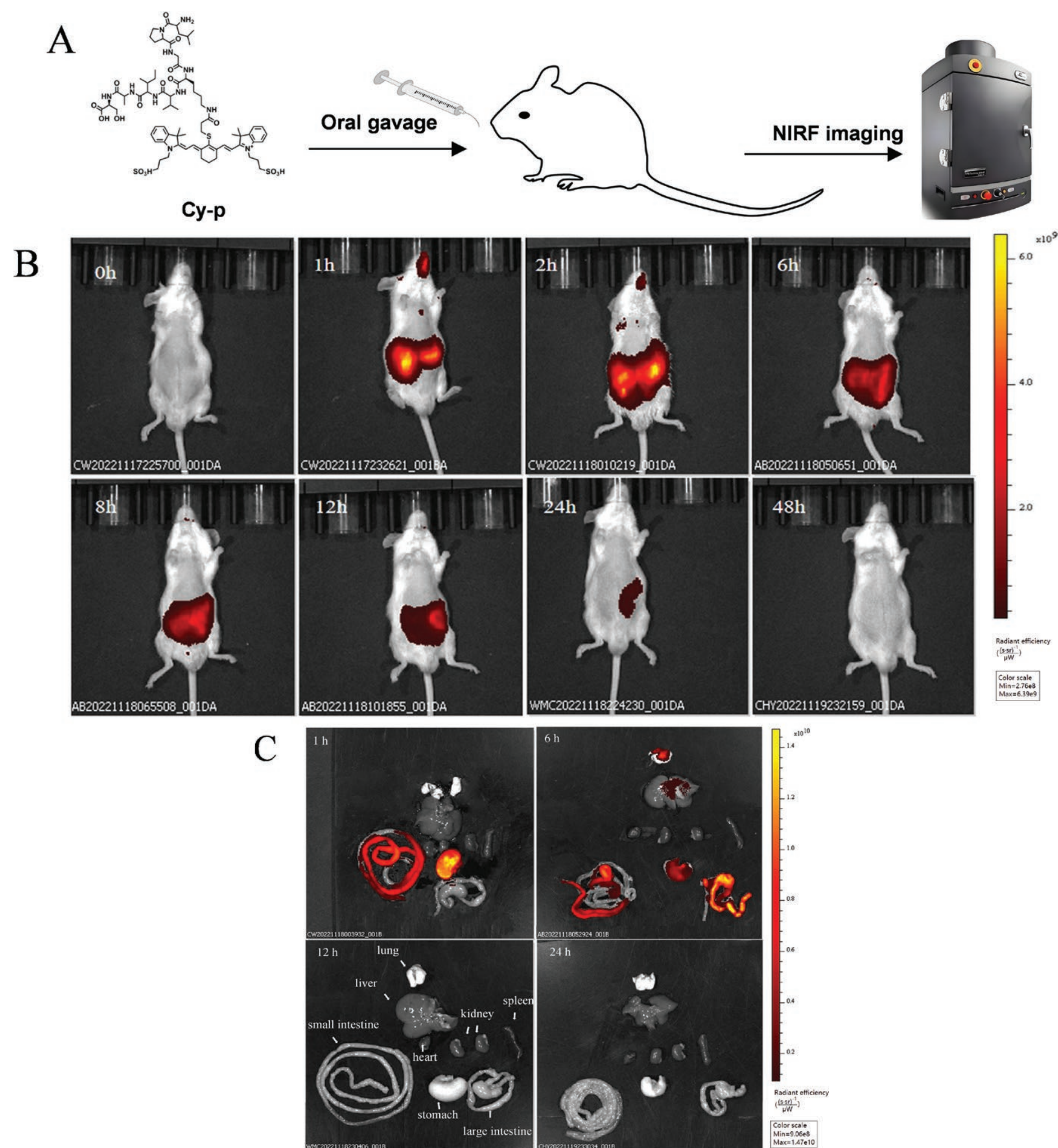

Figure 3. *In vivo* biodistribution profile of Cy7-p orally administered in healthy mice. (A) Schematic representation of Cy-p visualization. (B) Fluorescence intensity images of mice whole body from 0 to 48 h. (C) *Ex vivo* imaging of major organs (heart, liver, spleen, lung, kidney, intestine, and stomach) from 0 to 48 h.

with the untreated control group (Figure 6A). This suggests that LPGKVIAS treatment had a significant effect on the gene transcriptional profiles of mouse spleen cells. The volcano plots depicted the specific expression and distribution of these DEGs, indicating that the differences between genes were notably significant (Figure 6B). This suggests that LPGKVIAS had a significant influence on the functions associated with these DEGs in spleen cells.

GO enrichment analysis

The functional roles of the DEGs were elucidated by GO pathway analysis ($P<0.05$). The top 30 enriched GO terms are depicted in Figure 7. The upregulated DEGs involved in biological processes were predominantly enriched in pathways related to inflammatory response, positive regulation of locomotion, response to interferon-gamma, positive regulation of cell motility, positive regulation of cell migration,

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collagen metabolic process, regulation of inflammatory response, regulation of defense response, regulation of cytokine production, and positive regulation of defense response. Upregulated cellular component DEGs were enriched in the lysosome, lytic vacuole, vacuole, vacuolar membrane, late endosome, lytic vacuole membrane, lysosomal membrane, collagen trimer, phagocytic vesicle, and cytoplasmic vesicle membrane. Upregulated molecular function DEGs were predominantly associated with receptor ligand activity, signaling receptor regulator activity, signaling receptor activator activity, integrin binding, platelet-derived growth factor binding, immunoglobulin binding, oxidoreductase activity, immunoglobulin E binding, cytokine activity, and growth factor binding. These findings indicate that LPGKVIAS may play an immunoregulatory role by modulating gene expression in various immune-related processes within mouse spleen cells.

KEGG pathway enrichment analysis

We performed KEGG pathway enrichment analysis to explore the functional implications and pathways of the DEGs (Bayerlová *et al.*, 2015; Xing *et al.*, 2016). As shown in Figure 8A, the pathways in the LPGKVIAS vs. comparison group were categorized into environmental information processing, metabolism, organismal systems, and cellular processes. The DEGs were predominantly enriched in signaling molecules and interaction, the immune system, and several major metabolic pathways. In addition, Figure 8B shows the distribution of DEGs in major significantly upregulated pathways in the LPGKVIAS versus comparison group. These pathways included lysosomes, phagosomes, rheumatoid arthritis, fluid shear stress and atherosclerosis, tuberculosis, complement and coagulation cascades, viral protein interactions with cytokines and cytokine receptors, hematopoietic cell lineage, cytokine–cytokine receptor interactions, and oxidative phosphorylation, thus shedding light on the potential mechanisms underlying the immune response.

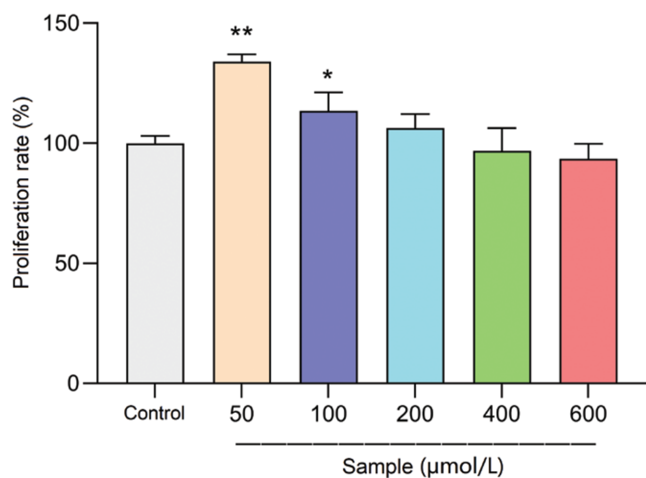

Figure 4. Effect of *Hericium erinaceus* active peptides LPGKVIAS on the proliferation rate of mouse spleen cells. * $P<0.05$ and ** $P<0.01$.

qRT-PCR analysis

In our study, the upregulated DEGs were predominantly involved in immune-related pathways, including lysosomes, phagosomes, complement and coagulation cascades, viral protein interactions with cytokines and cytokine receptors, and cytokine–cytokine receptor interactions. Table 3 presents the key DEGs associated with these pathways. To confirm the findings from transcriptome sequencing, we conducted qRT-PCR analysis on five selected DEGs linked to phagosomes, lysosomes, signaling receptor regulator activity, and inflammatory response (Figure 9). These DEGs were recombinant cathepsin (CTS) K, recombinant macrophage receptor with collagenous structure (MARCO), cluster of differentiation 14 (CD14), complement C1q subcomponent subunit b (C1qb), and CXC chemokine ligand 3 (CXCL3). The results showed significant differences ($P<0.05$) in the expression levels of these five DEGs. The qRT-PCR results corroborated the transcriptome sequencing results, providing additional validation of the transcriptome results.

Discussion

Our research group has focused on screening compounds derived from edible mushrooms to enhance immune response, which is an important research direction in immunology. Through enzymatic hydrolysis, we isolated the bioactive LPGKVIAS peptide from *H. erinaceus* and confirmed its ability to promote the proliferation of immune cells and enhance immune activity (Yu *et al.*, 2021). However, the specific cells involved and the mechanism of action remain unknown, and there is limited research on the absorption of LPGKVIAS *in vivo*. Therefore, our study aimed to investigate the mechanism and effect of LPGKVIAS on immune cells of the mouse spleen at the molecular level. For this study, we labeled LPGKVIAS with Cy7 and used NIRF to preliminarily explore its absorption *in vivo*. We also performed transcriptome analysis of LPGKVIAS-treated mouse

Table 2. Sequencing data quality assessment of mouse spleen cells

Sample	Clean_reads	Umi_reads	Clean_bases	Q30	GC
Control-1	24 676 456	23 754 571	7.40G	93.06%	49.61%
Control-2	20 875 773	20 036 290	6.26G	92.39%	49.40%
Control-3	24 767 579	23 758 596	7.43G	91.94%	49.51%
LPGKVIAS-1	21 359 722	20 560 476	6.41G	93.46%	49.99%
LPGKVIAS-2	29 738 913	28 494 175	8.92G	92.09%	49.75%
LPGKVIAS-3	28 201 714	27 073 087	8.46G	92.22%	50.52%

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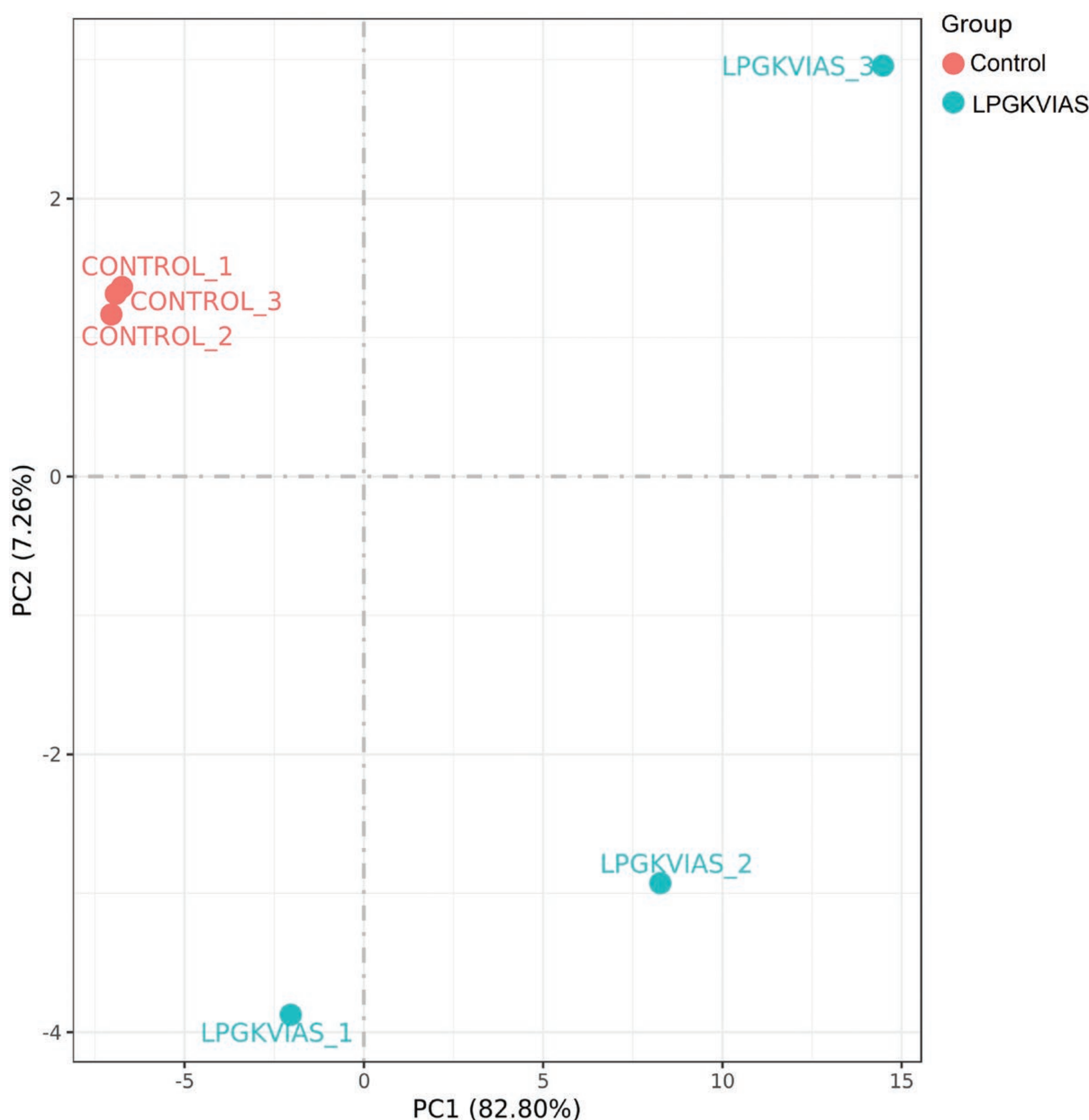

Figure 5. Principal component analysis.

splenic cells and identified the lysosome–phagosome immune regulation pathway.

Oral real-time biodistribution of Cy7-p

The structural integrity of active peptides must be maintained through transport to the site of function to demonstrate physiological bioactivity. Therefore, it is important to study the absorption characteristics and changes in immune activity when applying bioactive peptides. In this study, fluorescence probe–labeled LPGKVIAS (Cy7-p) was synthesized and administered to mice, and *in vivo* NIRF imaging was performed to observe peptide transport and absorption. At 24-h post-administration, fluorescence was present throughout the small intestine, indicating that the active LPGKVIAS peptide can remain in the small intestine for an extended period. A clear Cy7-p fluorescence signal was also detected in the lungs, indicating that Cy7-p was absorbed into the systemic circulation. Previous reports have indicated that peptides containing 4–9 amino acids can be absorbed intact via the paracellular pathway and transcytosis, primarily due to the presence of proline residues and hydrophobic amino acids such as Ala, Leu, Ile, Val, and Phe, which make them resistant to enzymatic hydrolysis and facilitate their passage through the small intestinal epithelium (Daniel, 2004; Brandsch *et al.*, 2008; Wang *et al.*, 2023a). Consistent with these findings, LPGKVIAS comprises hydrophobic amino acids like Val, Ala, Ile, and Leu, enabling its complete absorption by the intestines. However, a study by Wang *et al.* (2015) suggested that long-chain bioactive peptides (2–9 peptides) are more susceptible to hydrolysis, resulting in lower peptide transport through the cell membrane. This may explain the relatively weak fluorescence intensity in some of the observed mouse organs. Furthermore, fluorescent labeling of active peptides with Cy7 enabled the investigation of their transport and absorption *in vivo*. There is still limited research on the *in vivo* transporters for bioactive peptides. In the future, the precise mechanism of LPGKVIAS absorption will need to be further explored.

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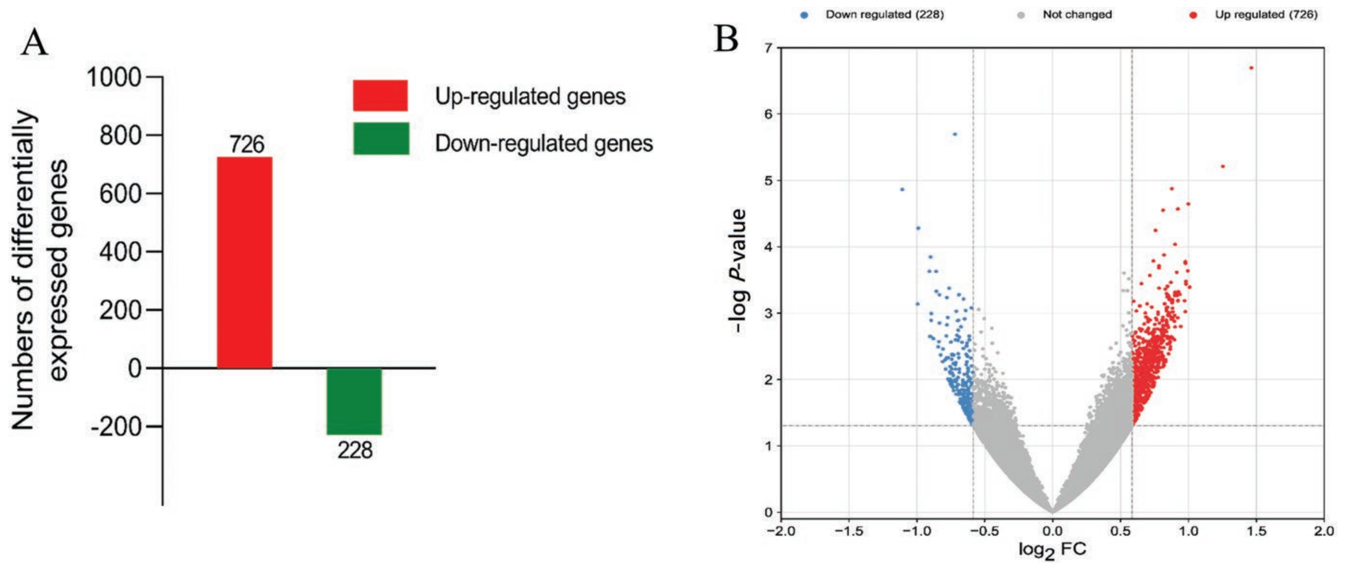

Figure 6. Numbers of DEGs (A) and volcano map distribution (B) of DEGs in the LPGKVIAS/control group.

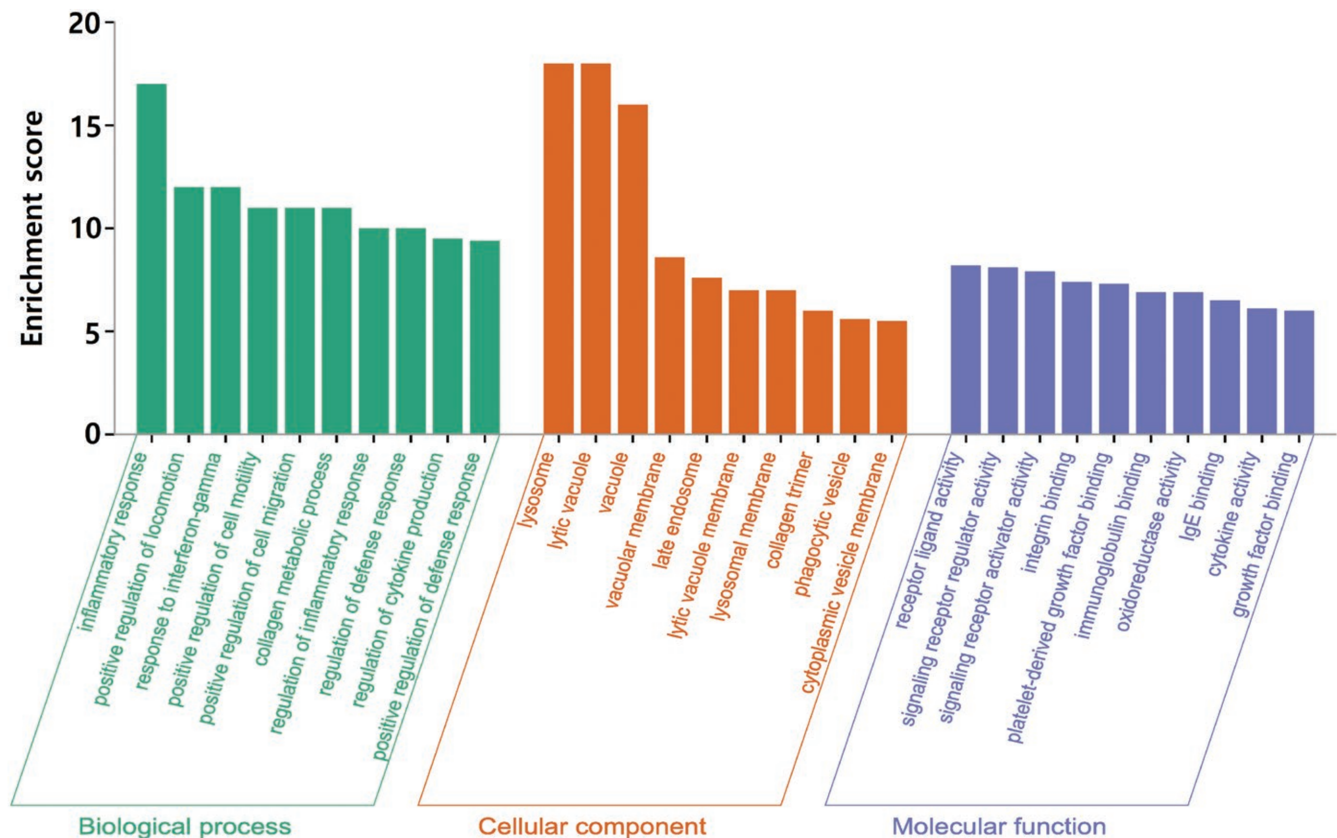

Figure 7. GO enrichment analysis of upregulated DEGs in the LPGKVIAS/control group.

Effects of LPGKVIAS on the immune mechanism of the mouse spleen

The spleen cell transcriptome, crucial for the immune system, was prepared and analyzed to compare the control and LPGKVIAS groups. GO and KEGG pathway enrichment analyses of the LPGKVIAS group revealed significant enrichment in the lysosome–phagosome pathway, cytokine–cytokine receptor interactions, and complement and coagulation cascade signaling pathways. This finding suggests that the immune mechanism effect of mouse spleen cells may be associated with these pathways.

Activation of the lysosome–phagosome pathway

Phagocytes are the most abundant immune cells in the mouse spleen, and phagocytosis plays a crucial role in mouse innate

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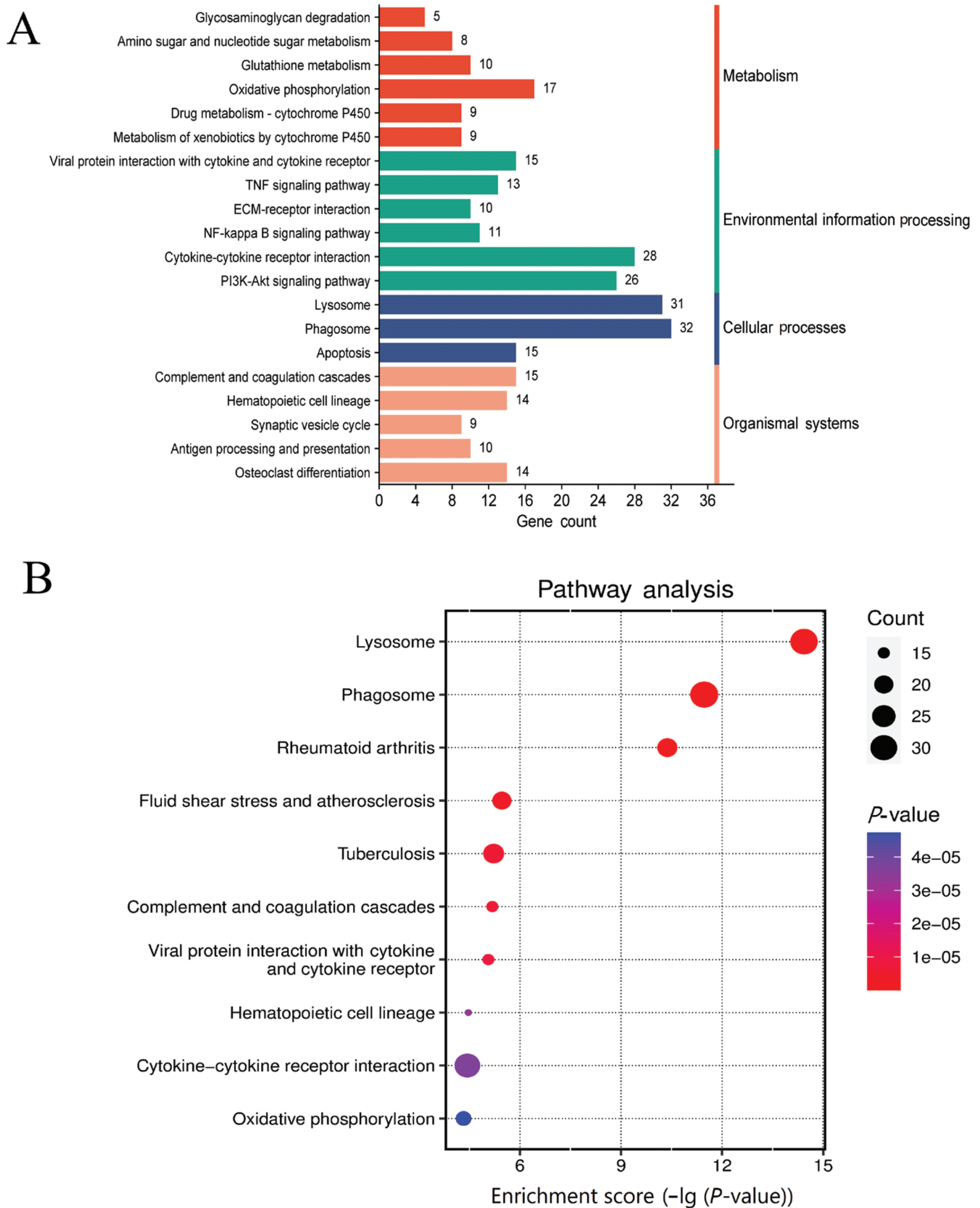

Figure 8. KEGG enrichment analysis of DEGs in the LPGKVIAS/control group. (A) KEGG pathway classification and (B) KEGG enrichment top 10.

immunity. Phagocytosis is the process by which phagocytes identify and engulf foreign substances into an organelle called the phagosome, which is essential for the phagocytes' ability to eliminate pathogens (Brown, 2024). The relationship between phagosomes and immunity has been widely studied. Lou *et al*. (2017) demonstrated that sorting nexin 10 (SNX10) enhances the bacterial killing ability of macrophages by promoting phagosome maturation. Similarly, Lee *et al*. (2020a)

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Table 3. Differentially expressed immune genes

KEGG signaling pathway	Key gene
Lysosome	CTSK, CTSD, CTSB, ATP6V0C, CTSZ, LAMP1
Phagosome	MARCO, CD14, C1ra, ATP6V0C, Fcgr3, ATPase H+ transporting V1 subunit G1 (ATP6V1G1), Fcgr4
Complement and coagulation cascades	C1qb, C1qc, C1qa
Viral protein interaction with cytokine and cytokine receptor	CXCL3, CXCL2, CCL4, TNF, IL-18
Cytokine–cytokine receptor interaction	CXCL3, TNFSF12, CXCL2, CCL4, TNF, IL-18

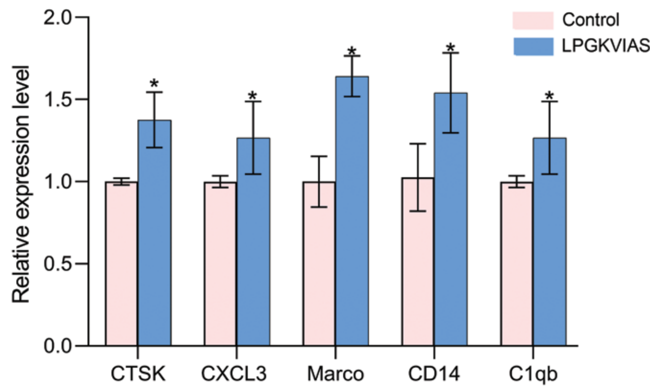

Figure 9. qRT-PCR validation of DEGs. The data are shown as means±SD, *P<0.05.

reported that lysophosphatidylcholine promotes bactericidal activity through a nuclear factor-kappa B (NF-κB)-dependent pathway in murine macrophages during *Salmonella* infection. Phagocytic cells express multiple receptors on their membrane that promote phagocytosis, such as MARCO, Fc receptors for IgG (Fc gamma R), and complement receptor 3 (CR3) (da Silveira *et al*., 2024). In our study, the upregulation of CD14, MARCO, and Fc gamma R (Fcgr3 and Fcgr2), associated with the phagosome pathway, indicates the activation of phagocytosis in the spleen cells of mice in response to LPGKVIAS. The MARCO protein on phagocytic cells functions as a plasma membrane receptor for microorganisms or environmental particles. Knocking down the MARCO gene inhibits macrophage phagocytic function, suggesting that MARCO plays a functional role in immunity (Hirano and Kanno, 2015; Yamada *et al*., 2019; Xing *et al*., 2021). In addition, CD14 is a receptor for bacterial lipopolysaccharide and is responsible for phagocyte uptake of pathogens, environmental particles, and apoptotic cells (Zanoni *et al*., 2011; Zizzo and Cohen, 2018). Fc gamma R (Fcgr3 and Fcgr2) expressed on macrophages and natural killer cells are important mediators of opsonophagocytosis and serve as a link between innate and adaptive immunity (Huber *et al*., 2001). Therefore, we deduced that LPGKVIAS might activate phagosome signaling by upregulating the expression of genes related to the phagocytosis receptor, thereby enhancing the ability to eliminate pathogens.

In addition to the upregulation of the phagosome pathway, we observed activation of the lysosome pathway in murine splenic cells. Lysosomes, which serve as intracellular degradation centers and signaling hubs, play an important role in cellular homeostasis and pathogen defense and degradation (Mahapatra *et al*., 2021; Yang and Wang, 2021). They harbor a diverse array of hydrolytic enzymes that are commonly used as markers of lysosomal immune function (Gao *et al*., 2015). Notably, CTS represents the most plentiful lysosomal proteases, primarily localized in acidic lysosomes. They are involved in various intracellular functions, including protein degradation, energy metabolism, and immune response (Petruzzella *et al*., 2024).

Previous human studies have shown that CTS inhibition significantly reduces antigen presentation by *Mycobacterium tuberculosis*–infected macrophages (Khan *et al*., 2022). In this study, CTSK, CTSD, CTSB, CTSZ, and CTS in the lysosomal pathway of mouse splenocytes were significantly upregulated by LPGKVIAS. Studies have shown that CTS is a key mediator of phagosomal proteolysis and collagenolysis in macrophages (Fabrik *et al*., 2023).

Moreover, lysosomal membrane proteins (adenosine triphosphatase (ATPase) H+ transporting V0 subunit c (ATP6V0C)) and lysosome-associated membrane protein 1 (LAMP1) are upregulated in the lysosomal pathway. LAMP1 is commonly used as a lysosome marker and is transported to phagosomes during maturation. The upregulation of LAMP1 by LPGKVIAS showed that LAMP1 mediates cytotoxic activity and could facilitate Golgi apparatus–driven secretion of a large number of lysosomes (Mauvezin *et al*., 2015). The transcript levels of ATP6V0C were significantly upregulated by LPGKVIAS.

Vacuolar-type ATPase (V-ATPase) is key in maintaining lysosomal homeostasis by establishing and sustaining an acidic environment essential for lysosomal function. Acidification is critical for the hybrid organelles formed by the fusion of lysosomes and endosomes (Pu and Qi, 2024). For instance, in zebrafish models, the knockdown of the V0 A1 subunit of V-ATPase prevents the fusion of phagosomes and lysosomes in microglia (Peri and Nüsslein-Volhard, 2008). Moreover, inhibiting the macrophage-specific V0 subunit D2 in mouse macrophages reduces phagosome and lysosome fusion and *Salmonella* clearance (Xia *et al*., 2019). In summary, LPGKVIAS treatment primarily activated the transport of lysosomal enzymes and endocytosis in the lysosome pathway to combat pathogen infection by significantly enhancing the expression of CTSK, CTSD, CTSB, CTSZ, ATP6V0C, and LAMP1 genes.

After internalizing pathogens via phagocytosis, phagosomes sequentially bind with early endosomes and late endosomes, ultimately fusing with lysosomes to generate phagolysosomes (Figure 10) (Naish *et al*., 2023). Recent studies have shown a significant increase in the fusion of phagosomes and lysosomes, involving genes related to CTS, V-ATPase, and LAMP1, which aligns with our experimental findings (Lancaster *et al*., 2021; Anes *et al*., 2022; Gómez-Mellado *et al*., 2022). As phagosomes mature, early

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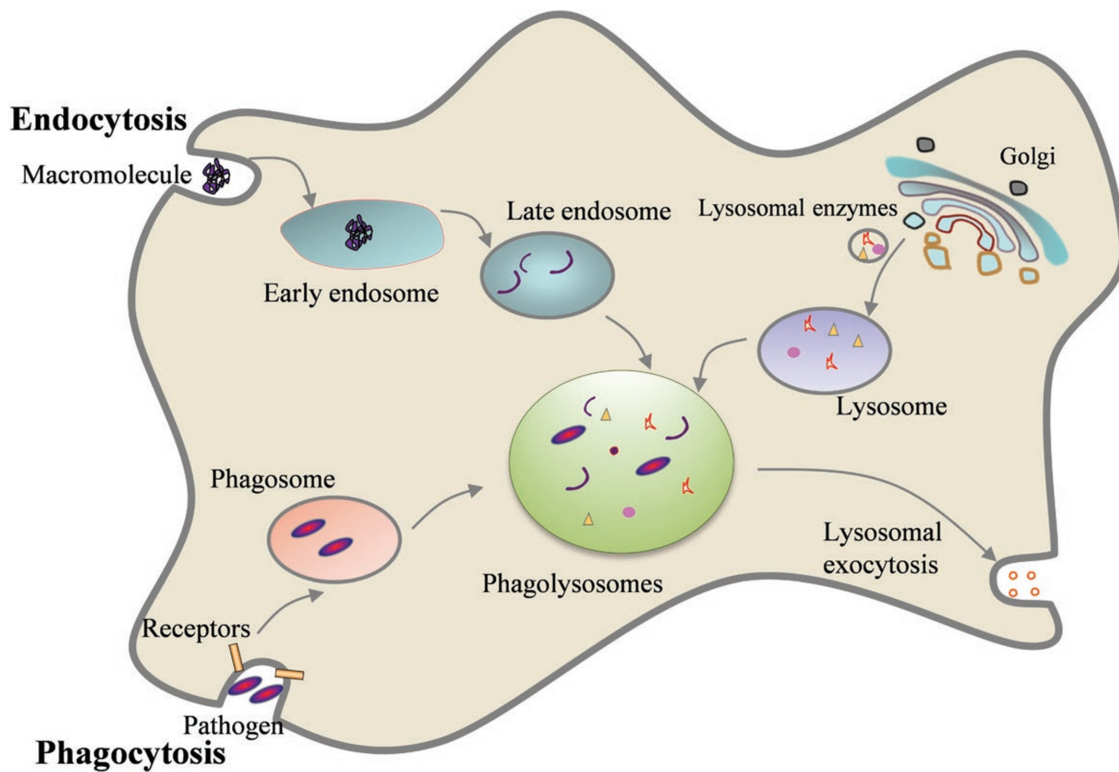

Figure 10. Diagram of the mechanism of the lysosome–phagosome pathway in cells.

phagosomes gradually become more acidic, creating an environment for degrading pathogens that includes V-ATPase, acid hydrolase, and acid protease. Ultimately, the phagosome fuses with the lysosome, forming a phagolysosome capable of degrading its contents using various hydrolases (Jaumouille and Grinstein 2017; Lee *et al.*, 2020b). Phagocytic lysosomes are crucial for eliminating pathogens and presenting antigens to lymphocytes to initiate an adaptive immune response. In our research, the upregulated DEGs were enriched in the phagosome and lysosome pathways. This discovery suggests that LPGKVIAS can regulate mouse immunity through the phagosomal and lysosomal pathways. Other immune studies have produced comparable findings (Alloatti *et al.*, 2015; Zhang *et al.*, 2022; Wang *et al.*, 2023b).

Complement and coagulation cascade signaling pathway

The complement system is crucial in the innate immune response to pathogenic infection (Reis *et al.*, 2019). Complement component 1q (C1q) is a key recognition molecule in this system that is capable of identifying pathogens and triggering mechanisms to eliminate them through phagocytosis and inflammatory responses (van de Bovenkamp *et al.*, 2021; Cao *et al.*, 2023). For example, C1qa gene expression was upregulated in mice infected with *Streptococcus pneumoniae*, and significantly reduced resistance to bacterial infection was observed in C1qa-silenced mice (Brown *et al.*, 2002). Recent research has also demonstrated the importance of classical C1-dependent phagocytosis in clearing apoptotic cells, with C1q acting as a bridge between apoptotic cells and phagocytes to facilitate their removal (Galvan *et al.*, 2012). In addition, because C1q is primarily produced by antigen-presenting cells, it likely plays a critical role in antigen presentation (Ghebrehiwet *et al.*, 2017). Our study revealed increased expression of C1qb in response to LPGKVIAS treatment, suggesting that LPGKVIAS may enhance host phagocytosis or activate the complement system by upregulating the expression of C1q-related genes, ultimately bolstering the host's defense mechanisms.

Cytokine–cytokine receptor interactions

Cytokines, secreted by various immune cells, act as immune response modifiers and can be categorized into chemokines, tumor necrosis factors (TNFs), interleukins (ILs), and interferons. This study revealed that multiple immune-related DEGs are involved in the cytokine–receptor interactions, including chemokine (C-X-C motif) ligand (CXCL3 and CXCL2), C–C motif chemokine ligand 4 (CCL4), IL-18, and TNF. Chemokines regulate the proliferation, differentiation, and migration of immune cells, thereby playing crucial roles in the immune system (Zimmerman *et al.*, 2020). Previous research in mice has demonstrated the response of CXCd to pathogenic infection (Griffith *et al.*, 2014). In this study, we observed upregulation of CXCL2 and CXCL3 in the spleen cells of mice treated with LPGKVIAS, indicating pathogen-induced chemokine regulation. CCL4 can boost immunity by recruiting immune cells with phagocytic ability (Hsu *et al.*, 2013). In addition, gene expression levels of IL-18 and TNF were increased in the cytokine–cytokine receptor interaction pathway. Similarly, Landy *et al.* (2024) suggested that gene expression levels of IL-18 and TNF were increased in response to pathogen infection to adapt to autoimmunity. Our research also demonstrated cytokine expression during LPGKVIAS treatment, suggesting the involvement of these immune-related genes in the response of mouse spleen cells to pathogen infection.

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Conclusions

In this study, we successfully constructed an active, biocompatible peptide probe, Cy7-LPGKVIAS, suitable for NIRF imaging in mice. Transcriptome analysis of splenic cells from LPGKVIAS-treated mice revealed upregulated DEGs enriched in the lysosome–phagosome pathway. Activating this pathway in the splenocytes of LPGKVIAS-treated mice is an important immune defense mechanism against pathogen infection. Our analysis of the immunomodulatory mechanism of LPGKVIAS was limited to mouse spleen cells. The specific target genes of LPGKVIAS need to be explored in future studies, in which gene knockout and other methods should be used to characterize its immune-regulating functions fully. Live animal experiments with active peptides should also be performed.

Supplementary Material

Supplementary material is available at *Food Quality and Safety* online.

Author Contributions

Ronggui Sun: Conceptualization, methodology, investigation, validation, data curation, visualization, writing original draft, and review and editing. Hui Xu: Methodology and review and editing. Minhao Xie: Investigation and validation. Jianhui Liu: Review and editing. Qiuhui Hu: Validation. Anxiang Su: Software. Alfred Mugambi Mariga: Language editing and proofing. Wenjian Yang: Conceptualization, supervision, review and editing, and project administration.

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Conflict of Interest

The authors declare no conflict of interest.

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