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Combined transcriptomics and TMT-proteomics reveal abnormal complement and coagulation cascades in cow's milk protein allergy

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ABSTRACT

Cow's milk protein allergy (CMPA) is primarily due to the inability of the intestinal mucosa to establish typical immunological tolerance to proteins found in cow's milk, and the specific molecular mechanism is still unclear. In order to investigate molecular alterations in intestinal tissues during CMPA occurrence, this study analyzed the jejunal tissue of β-lactoglobulin (BLG)-sensitized mice through transcriptomics and quantitative tandem mass tag (TMT)-labeled proteomics. A total of 475 differentially expressed genes (256 up-regulated, 219 down-regulated) and 94 differentially expressed proteins (65 up-regulated, 29 down-regulated) were identified. Comparing the KEGG pathways of the two groups, it was found that both were markedly enriched in the signaling pathways of complement and coagulation cascade. Among these, kallikrein B1 (KLKB1) in this pathway is speculated to be pivotal in CMPA. It may potentially enhance the release of bradykinin by activating the kallikrein-kinin system, leading to pro-inflammatory effects and exacerbating intestinal mucosal damage. This study suggests that the pathways of complement and coagulation cascades could be significant in the context of intestinal immunity in CMPA, and KLKB1 may be its potential therapeutic target.

1. Introduction

Cow's milk protein allergy (CMPA) results from an aberrant immune response to proteins in cow's milk, with clinical manifestations mainly consisting of bloody stools, diarrhea, vomiting, rash and other multi-system symptoms. In severe cases, it can lead to the development of enterocolitis syndrome and anaphylactic shock, causing developmental delays in infants and even posing a threat to life [1]. In recent years, there has been a notable rise in the prevalence of CMPA, presenting a significant challenge to public health. Epidemiological data from the World Allergy Organization indicate that the prevalence of milk protein allergy in infants and young children is estimated to be around 2–4.5 % [2], while the prevalence in China has increased from 1.6 % to 5.7 % in the past 20 years [3].

The main pathogenesis of CMPA is that the immune function of infants and young children is not yet mature, and the intestinal mucosa is unable to establish normal immune tolerance to food antigens. When allergies occur, food allergens can activate epithelial cells on the intestinal mucosa to produce epithelial alarms including TSLP, IL-25 and IL-33, induce the initiation and amplification of type 2 immune responses, assist B cells to produce IgE antibodies, and promote the activation and degranulation of mast cells and basophils, thereby triggering type I hypersensitivity reactions, inhibiting the generation of Treg cells in the small intestine, and affecting intestinal homeostasis [4–6]. Even after treatment, intestinal function may remain suboptimal or exhibit disturbances in the intestinal microbiota, functional gastrointestinal disorders, etc. The complexity and heterogeneity of the intestinal environment make the study of the pathogenesis of CMPA and the identification of potential therapeutic targets challenging. While approaches like the study of the gut microbiota and dietary interventions contribute to understanding the biological changes in CMPA, they still fall short of providing sufficiently detailed and comprehensive insights into the disease mechanisms [7,8]. Therefore, it is imperative to delve deeper into the molecular changes in the intestinal environment during the occurrence of CMPA.

Transcriptomics can respond to changes in gene expression in time and space [9]; while proteomics can provide the expression level of gene splicing and post-translational proteins [10]. The integration of both

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methodologies offers a comprehensive perspective on the occurrence and development of diseases, serving as an effective method to explore potential pathogenic mechanisms. Through omics approaches, a better understanding of the molecular changes in allergic-influenced intestinal tissues can be obtained, providing a basis for discovering the pathogenesis and potential regulatory pathways of CMPA.

In this study, we explored the global view of intestinal molecules at the onset of CMPA by combining transcriptomics and proteomics strategies, so as to systematically explore the mechanism of intestinal damage in CMPA, screen potential targets that are expected to be used for its treatment, and provide new intervention strategies for treating and preventing CMPA.

2. Materials and methods

2.1. Materials and reagents

β-Lactoglobulin (BLG) was purchased from Sigma-Aldrich (Cat#L3908); aluminum hydroxide adjuvant was purchased from Biodragon (Cat#KX0210054); IgE, histamine, and cytokines ELISA kits were purchased from Jianglai Biology (Cat#JL12885 (Cat#JL12885, JL45802, JL20266, JL20267, JL20247); BCA kit was acquired from Thermo Scientific (Cat#23225); iTRAQ labeling kit was obtained from Thermo Scientific (Cat#4381663-1KT); goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Zsbio (Cat#ZB-2305, Cat# ZB-2301).

2.2. Animals

SPF-grade female Balb/c mice (5–6 weeks old, weighing 16 ± 1 g) were obtained from Zhejiang Vital River Laboratory Animal Technology Co., Ltd. The animals were accommodated at the Experimental Animal Center of the Advanced Technology Institute of the University of Science and Technology of China. The facility maintained a constant temperature of approximately 23 ± 2 °C. The relative humidity is controlled at 40 %-60 %, and the animal illumination achieves 12 h/12 h alternation of light and dark. This research protocol was approved by the Experimental Animal Management Committee of the Artificial Intelligence Research Institute of Hefei Comprehensive National Science Center (No. 2023-N(A)-017).

2.3. Mouse sensitization and oral challenge

Twenty-four female balb/c mice were acclimated for one week, receiving standard feed without milk protein and access to purified water. After one week, the mice were randomly assigned to either the control group or the BLG group. The BLG group was intraperitoneally injected with 0.2 mL of allergen solution (1 mL of aluminum hydroxide adjuvant + 1 mL of 2 mg/mL BLG) on days 7, 14 and 21 [11]. Mice in the control group received intraperitoneal injections of an equivalent volume of sterile PBS. On day 28, each mouse underwent oral challenge with 50 mg of BLG, followed by assessment of allergic symptoms after 30 min. Subsequently, after blood collection from the eyeballs, the mice were killed by cervical dislocation. Dissection was carried out in a biological safety cabinet, with the thymus and spleen weighed. Mouse jejunum tissue (10 cm) was taken from 15 cm below the pylorus, frozen in liquid nitrogen, ground and mixed thoroughly, and stored in a −80℃ refrigerator for transcriptomics, proteomics and subsequent verification experiments.

2.4. Scoring of allergic symptoms in mice

Systemic allergic symptoms were observed within 1 h after BLG allergen gavage stimulation, and clinical scores were made based on the symptoms [12] (0 to 5 levels): 0 = asymptomatic; 1 = hair standing up, scratching the mouth, ears and head; 2 = increased respiratory rate, decreased activity, and diarrhea; 3 = arched body, difficulty breathing, cyanosis around the mouth, and swelling around the eyes and mouth; 4 = inactivity after the stimulation or tremor and convulsions; 5 = death.

2.5. Determination of mouse immune organ index [13]

After 1 h of oral challenge, the thymus and spleen of the mice were dissected, and adipose tissue was carefully removed. After absorbing the bloodstains with absorbent paper, the weight of the organs was measured. The organ index was calculated as follows: Organ Index = Organ Weight (mg) / Body Weight (g).

2.6. Determination of serum total IgE, histamine and cytokine levels

After modeling, the collected whole blood was left at room temperature for 2 h, then centrifuged at 3000 rpm for 15 min, and the resulting supernatant was collected and stored in a −80 °C refrigerator. The concentrations of total IgE, histamine, IL-4, IL-5, and IL-13 in mouse serum were meticulously measured following the guidelines provided by the ELISA kit.

2.7. Intestinal histopathological studies

Separately, 2 cm of jejunum tissue was taken from 15 cm below the pylorus of the mouse, and 2 cm of ileum tissue was taken from 1 cm proximal to the cecum. The intestinal contents were rinsed out using sterile PBS, and put into 10 mL of 4 % formaldehyde to be fixed at room temperature for 24 h, followed by dehydration, paraffin embedding, hematoxylin-eosin staining. Pathological scoring of mouse jejunum and ileum under microscope according to Chiu's [14] scoring criteria (0–5 points): 0 = normal intestinal mucosal villi; 1 = cystic spaces under the epithelium at the top of the villi, accompanied by capillary congestion; 2 = enlarged epithelial interstitial space of the small intestine, moderate edema of the lamina propria, and dilatation of the central celiac ducts; 3 = degeneration and necrosis of the epithelial cells of the intestinal mucosa, marked edema of the lamina propria, and apical detachment of a few villi; 4 = degeneration, necrosis and shedding of the epithelial cell layer, shedding of some villi, exposure of the lamina propria, and dilated and congested capillaries; 5 = shedding of the villi of the small bowel, hemorrhage and ulcer formation. At the same time, five fields of view were randomly selected from each pathological section at 400x using a single-blind method. Two observers counted the number of goblet cells between every 200 columnar epithelial cells in the small intestinal villi, and calculated the average value. If the difference between the two is large, the count will be recalculated by a third researcher.

2.8. Transcriptomic analysis of jejunum tissue

In order to investigate the transcriptional characteristics of small intestinal tissues at the onset of CMPA, we collected 50 mg of jejunum from four mice randomly selected from each of the control and BLG groups, and stored the samples at −80 °C after liquid nitrogen snap-freezing, and then sent the samples to OE Biotech Co., Ltd. (Shanghai, China). The specific operation was as follows: initially, added 1 mL of Trizol reagent to extract total RNA, and used NanoDrop 2000 spectrophotometer to detect the purity and quantification of RNA. The RNA concentration, purity, and integrity were further accurately evaluated using an Agilent 2100 Bioanalyzer. Subsequently, constructed the transcriptome library according to the instructions provided with the VAHTS Universal V5 RNA-seq Library Prep kit, and finally sequenced using the Illumina Novaseq 6000 sequencer [15]. To ensure data quality, the fastp software was employed to remove low-quality raw reads. The cleaned reads were compared with the reference genome using HISAT2 software to calculate the gene expression. The screening criteria for differentially expressed genes (DEGs) were p-value < 0.05 and $|\log2FC| > 1.0$. Finally, the identified DEGs were subjected to Gene Ontology

(GO) annotation analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis by comparing them with the GO database (https://www.geneontology.org/) and KEGG database (https://www.genome.jp/kegg/).

2.9. Tmt-labeled proteomic analysis of jejunal tissue

Firstly, the jejunum tissues from the two groups of mice described in 2.8 were thoroughly ground in liquid nitrogen, and the total protein solution was obtained by adding the sample extraction solution and lysis solution. Strictly followed the instructions of the BCA kit to ascertain whether the protein concentration of the sample meets the superior standards for mass spectrometry. SDS-polyacrylamide gel electrophoresis was employed to identify the expression of each group of proteins. Subsequently, according to the measured protein concentration, 50ug of protein was taken out of each sample, and trypsinization was performed after diluting and adjusting the different groups of samples to the same concentration and volume using lysis solution. The peptide labeling was performed as per the instructions of the TMT kit, and the peptide concentration was measured using a microplate reader with an absorbance of 280 nm; finally, the reversed-phase chromatography separation was performed using the Agilent 1100 HPLC system in a mobile phase with a pH of 10, followed by mass spectrometry analysis [16,17]. The raw data obtained from mass spectrometry were subjected to database retrieval and analysis using Proteome Discoverer 2.4.1.15 (Thermo Fisher Scientific). The significantly differentially expressed proteins (DEPs) were screened and analyzed through R language calculation (selection criteria: Foldchange $\geq$ 1.2 or Foldchange $\leq$ 0.83 and p-value $<$ 0.05). The GO database was selected to conduct functional cluster analysis of all DEPs, and the KEGG pathway database was used to conduct enrichment analysis of the signaling pathways involved in DEPs. The sequencing was performed by Shanghai OE Biotech Co., Ltd.

2.10. Integrative analysis of transcriptome and proteome

We performed correlation analysis on the obtained DEGs and DEPs and mapped them to the KEGG Pathway database to select signaling pathways common to both groups. However, due to the complexity of the Pathway, it is difficult to understand the interactions between the covarying signaling pathways. The KEGG Markup Language (KGML) file, a sub-library of the KEGG database, contains both the relationships of the graphical objects in the KEGG pathway as well as the immediate homologous genes in the KEGG genes database information, which can be a good solution to this problem [18,19]. In order to explore the connection between the signaling pathways shared by the two omics, we used Cytoscape (version 3.5.1) software to visualize the connection between the pathways of the differential genes (proteins) of the two omics obtained in KGML and drew it as Network of relationships.

2.11. Real-Time polymerase chain reaction (qPCR)

To further verify the precision of the transcriptomic data, 12 differentially expressed genes were selected for qPCR analysis. The jejunal tissues were ground into powdered form under liquid nitrogen, and 1 mL of Trizol reagent (Life Technologies, Waltham, MA, USA) was employed for total RNA extraction. Reverse transcription to prepare cDNA was performed using the PrimeScript™ RT Kit (TaKaRa, Otsu, Shiga, Japan) and reverse transcription primers. Subsequently, PCR amplification was conducted with specific upstream and downstream primers for each gene, including KLKB1, KNG, PCK1, SLC13A2, SGK1, FOXO3, CYP27A1, PPARG, FKBP5, TSC22D3, ANGPT14, and PDK4, with β-actin as the reference gene. The relative expression of each target gene was calculated by $2^{-\triangle\triangle Ct}$. The PCR primers were designed and synthesized by Sangon Biotech, and their sequences are shown in Table 1.

Table 1
Sequences of primers.

Gene name	Forward primer (5′→3′)	Reverse primer (5′→3′)
KLKB1	ACACGAAGGAACAAGGTGAA	ATCTCCCTTACAAGCGTCTG
KNG	CTAAGTGGTGAGGCAGAGAG	GAACTGAGGGACTGTTTTGC
PCK1	CCTGCTCCAGCTTTGAGATA	TCTTATTTGCCCCTAGCCTG
SLC13A2	CCAGATCTCTTTGCCCTGTA	CACCCGGACTCTACTGAAAA
SGK1	AAACCCTCCGACTTTCACTT	GGAAAGGGTGCTTCACATTC
FOXO3	GAAGGGAGGAGGAGGAATGT	CTTCCCTTCAGGAACGAGG
CYP27A1	AAAGCCCGAGTTCTGTCTG	CCCGAGAAGCCAAGAGTTTA
FKBP5	GAAGCTCCGAGAGTACAACA	GATTGACTGCCAACACCTTC
TSC22D3	CTGCTGGGCAAGTCTCTC	CTAGCCTTTTGGGACTCCAG
ANGPT14	TTTAGGTGCAACCGTGAAAC	GGCTCAGACTTAGACTTGCT
PDK4	AACCGCATTTCTACTCGGAT	CACACTCAAAGGCATCTTGG
PPARG	GGGCTGAGAAGTCACGTT	ACAGAGCTGATTCCGAAGTT
β-actin	AGTGTGACGTTGACATCCGT	TGCTAGGAGCCAGAGCAGTA

2.12. Western blotting analysis

Mouse intestine tissues were lysed using RIPA lysis buffer to extract total protein, followed by protein quantification. Following SDS-PAGE separation, the protein bands were transferred to PVDF membrane, rinsed in Western washing solution for 5 min, then 5 % skimmed milk powder was added, and the membrane was closed at room temperature for 2 h on a shaker. The membrane was placed in anti-KLKB1 antibody (1:2000), anti-BK antibody (1:1000), anti-Itgam antibody (1:1000), anti-serpinb2 antibody (1:2000), anti-EPX antibody (1:1000), anti-Hp antibody (1:1000), and anti-ALOX15 antibody (1:1000) solutions, respectively, incubated overnight at 4 °C, and washed with PBST 3 times, each time for 10 min. Subsequently, horseradish peroxidase (HRP)-labeled secondary antibody was added, incubated at room temperature for 1.2 h, and washed three times with PBST for 10 min each time. Protein detection was carried out following the instructions provided with the ECL chemiluminescence kit (Thermo, Waltham, MA, USA). GAPDH was used as the reference protein, and Image J software (USA) was employed for quantification and analysis of the developed bands.

2.13. Statistical analysis

Data analysis and graphing were performed using GraphPad Prism 10.1 (GraphPad Software, San Diego, CA, USA). The data in this study are presented as mean ± standard error of the mean (SEM). If the samples met the assumptions of normality and homogeneity of variances, statistical significance analysis was conducted using an independents sample *t*-test. If the samples followed a normal distribution but exhibited inhomogeneous variances, the Welch approximation *t*-test was employed. The data come from at least three replicate experiments. A p-value less than 0.05 is considered statistically significant.

3. Results

3.1. Total IgE content, allergic symptom scores and immune organ index of mice

When food allergy occurs, there is a significant increase in the total IgE content of children. This elevation can be used as one of the criteria for evaluating whether the body is experiencing an allergic reaction [20]. As shown in Fig. 1A, the serum total IgE content in the BLG group mice significantly increased to 535.8 ± 56.32 ng/mL, which was markedly higher than the control group's level of 80.47 ± 6.83 ng/mL. This result indicates the successful establishment of the BLG-induced CMPA mouse model. The spleen and thymus, as primary immune organs, play a crucial role in the body's immune response. The immune organ index can roughly judge the physiological functional status of the body and can be used as an indicator to evaluate the sensitization effect. As depicted in Fig. 1B, after oral stimulation with BLG, the mice in the

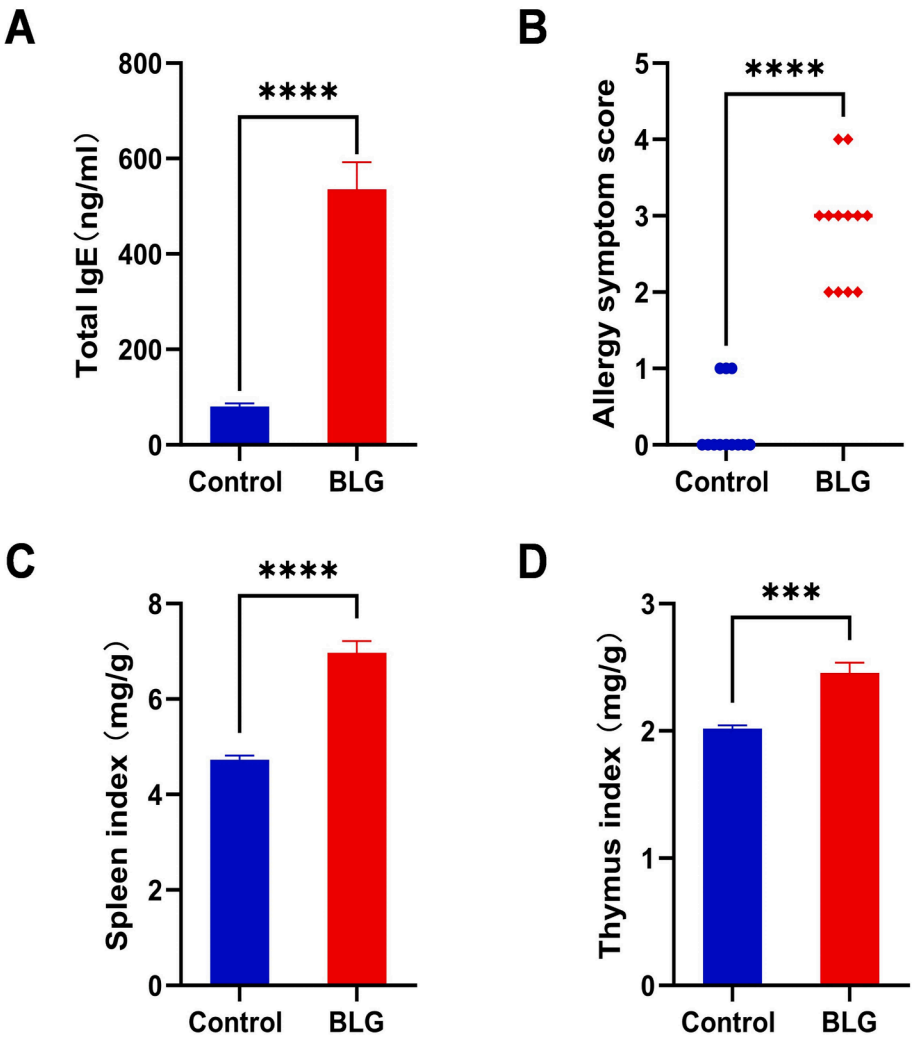

Fig. 1. Establishment of the mouse BLG model. (A) Total IgE content in mouse serum; (B) Allergy symptom scores in mice; (C) Spleen index; (D) Thymus index; data are expressed as mean ± SEM; n = 12 mice/group; *** $p < 0.001$; **** $p < 0.0001$.

BLG group showed increased respiratory rate, reduced activity, lethargy, and even no response after stimulation. Concurrently, Fig. 1C and D illustrate that the spleen and thymus indexes of the BLG group were 6.97 ± 0.24 mg/g and 2.46 ± 0.08 mg/g respectively, which were also both significantly different from those of the control group. Thus, it can be observed that mice in the BLG group displayed pronounced allergic symptoms and exhibited a discernible impact on the immune organs of the mice.

3.2. Serum histamine and Th2 cytokine levels in BLG mice

When an allergic reaction occurs in the body, the inflammatory mediator histamine released by mast cells significantly increases, attracting eosinophils and stimulating the release of Th2 cell cytokines, thereby exacerbating the allergic response, serving as indicators for evaluating acute allergic reactions [21]. In Fig. 2A, the histamine content in the BLG group was significantly elevated at 455.5 ± 64.78 ng/mL, which was significantly increased compared with the control group's content of 200.4 ± 12.47 ng/mL ($P < 0.05$); In Fig. 2B–D, the concentrations of Th2 cell-secreted cytokines, namely IL-4, IL-5, and IL-13, in the serum of the BLG group were significantly elevated compared to those in the control group.

3.3. Intestinal histopathologic analysis

As shown in Fig. 2E and F, the jejunum and ileum's intestinal mucosa in the control group exhibited a well-defined structure, characterized by neatly arranged and tightly packed intestinal villi. The intervillous stroma of the villi showed no signs of edema, and there was no infiltration of inflammatory cells. In contrast, the BLG group displayed disorganized intestinal villi with evident injuries, breaks, or missing sections. The cup cells showed noticeable damage, accompanied by a substantial infiltration of inflammatory cells. These findings suggest that BLG-induced food allergy triggers an inflammatory response throughout the small intestine.

3.4. Effect of CMPA on the transcription level of small intestinal tissue

Our transcriptome analysis results indicated that the tissue samples passed the RNA purity and integrity tests. A total of 475 DEGs were identified with more than 2-fold change and $P < 0.05$, among which 256 were up-regulated and 219 were down-regulated. The volcano plot depicting DEGs is illustrated in Fig. 3A, while the clustered heatmap of these genes is featured in Fig. 3B. In order to better explore the potential biological processes of CMPA, GO enrichment analysis revealed that DEGs are predominantly associated with biological processes such as coagulation, sodium ion transport, response to bacteria, circadian regulation of gene expression, and regulation of heart rate through

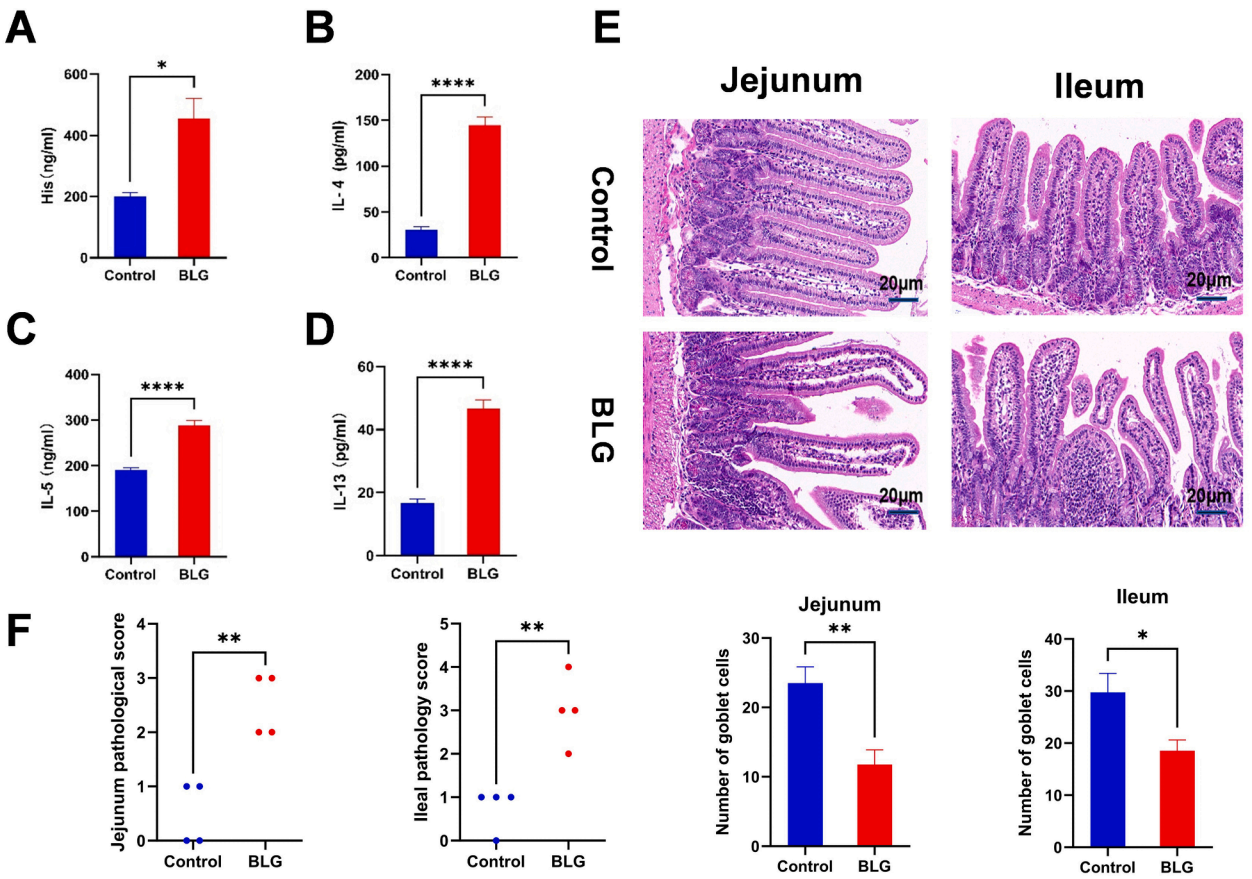

Fig. 2. Serum inflammatory factors and intestinal pathological changes in BLG mice. (A-D) ELISA method detected the levels of histamine (His), IL-4, IL-5, and IL-13 in serum; n = 6 mice/group; data are expressed as mean ± SEM; * $p < 0.05$; **** $p < 0.0001$. (E) HE staining to observe the pathological changes in the jejunum and ileum of BLG-sensitized mice (400X, Scale: 20 μm). (F) Jejunum and ileum pathological scores and goblet cell counts, n = 4 mice/group.

cardiac conduction. The molecular functions associated with DEGs include transmembrane transporter activity, acyl-CoA oxidase activity, extracellular glutamate-gated ion channel activity, and aromatase activity. Furthermore, the cellular components enriched with DEGs involve GABA receptor complexes, aromatic compound receptor complexes, extracellular regions, and synapses. Fig. 3C shows the GO enrichment analysis of the top30 DEGs. In the bubble chart of the top 20 KEGG pathway enrichment analysis shown in Fig. 3D, it is evident that DEGs are predominantly enriched in pathways such as the PPAR signaling pathway, complement and coagulation cascade, asthma, linoleic acid metabolism, and arachidonic acid metabolism.

3.5. *Effect of CMPA on protein levels in small intestinal tissue*

Our proteomic analysis results indicated that the number of effective spectra was 151207, 85,322 peptides were identified, 8454 proteins were finally identified, and a total of 94 DEPs were screened, among which 65 DEPs were up-regulated, and 29 DEPs were down-regulated. Fig. 4A and B shows the volcano plot and heat map of DEPs, respectively. Fig. 4C shows the GO analysis of DEPs of top30. It can be seen from the figure that the biological processes in which the DEPs are mainly involved include the positive regulation of B cell activation, lipid oxidation, phagocytosis, immune response, etc., and are involved in many metabolic processes, suggesting that CMPA is involved in multiple metabolic pathways; secondly, DEPs are mainly involved in the molecular functions including serine-type endopeptidase activity, oxidoreductase activity, binding of the complement component C3b, superoxide producing NAD(P)H oxidase activity, etc., which are mainly related to enzyme binding activation. Lastly, cellular compositions in which DEPs are primarily involved include the IgG immunoglobulin complex, the immunoglobulin complex, and the NADPH oxidase complex. As can be seen from the TOP20 bubble plot of KEGG enrichment analysis in Fig. 4D, it can be seen that DEPs are highly enriched in signaling pathways such as complement and coagulation cascade, Fcγr-mediated phagocytosis, phagosomes, and neutrophil extracellular trap formation.

3.6. *Integrated analysis of transcriptomics and proteomics*

Integrated analysis of transcriptomics and proteomics revealed a total of five co-up-regulated DEGs (or DEPs), namely Retnlg, Itih3, Pck1, Prg2, and Slc13a2; 2 showed opposite trends, while 92 exhibited unique trends (Fig. 5A). By analyzing the pathways to which the differential genes and differential proteins of the two histologies mapped, we observed that the key signaling pathways of the two groups mainly focus on four types of signaling pathways: complement and coagulation cascade, asthma, estrogen signaling pathway, and Staphylococcus aureus infection (Fig. 5B). Further KGML (KEGG Markup Language) network analysis of the differentially mapped pathways revealed that complement and coagulation cascades are at the core of the network (Fig. 5C). Therefore, it can be inferred that the complement and coagulation cascade signaling pathways may play a crucial reuse in the immune mechanism of CMPA. Previous studies have found that the development of food allergy can inhibit the function of complement C3b through the complement and coagulation cascade pathway, promote the production of complement C3a and C5a, and exacerbate the allergic reactions [22–24], further confirming that this pathway has an important role in CMPA. The differential gene kallikrein B1 (KLKB1), as a key protein in this pathway, may aggravate intestinal damage in the occurrence and development of CMPA. Studies have found that KLKB1 can promote bradykinin (BK) to act on B1R and B2R receptors on the

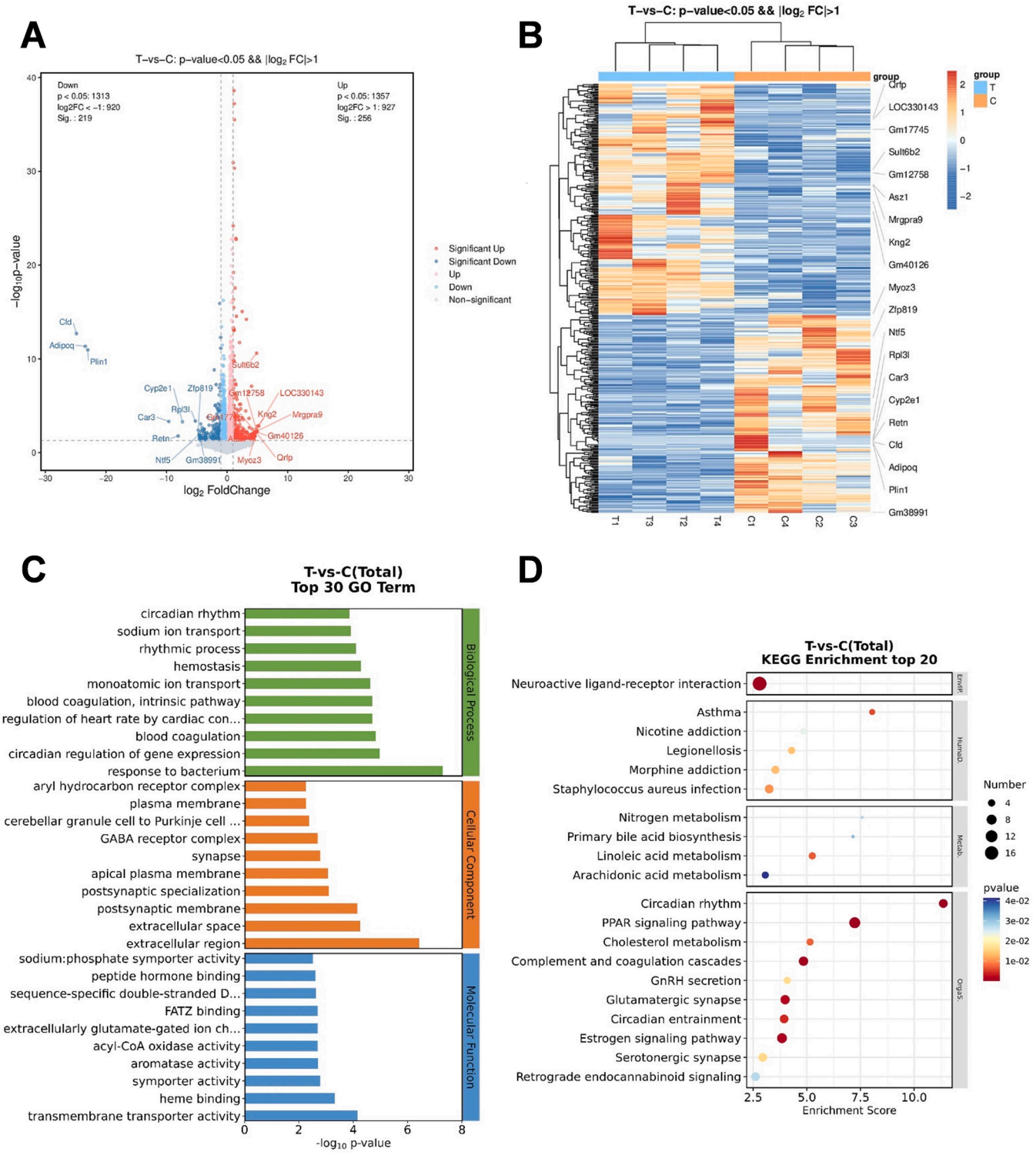

Fig. 3. Transcriptomic analysis of differentially expressed genes (DEGs) in the jejunum of BLG-sensitized mice. (A-B) Volcano plots and heat maps showing DEGs between the two groups. (C) GO enrichment analysis top30 histogram of DEGs. (D) KEGG enrichment analysis top20 bubble plot of DEGs between two groups. n = 4 mice/group;

surface of intestinal tissue through the kallikrein-kinin system, thereby releasing inflammatory factors such as leukotrienes and nitric oxide, causing damage to the intestinal mucosa [25,26]. Through Fig. 6A and Fig. 7B, we also verified that KLKB1 is highly expressed in the CMPA small intestine, suggesting that it may play a crucial role in intestinal damage in CMPA.

3.7. Validation of qPCR and Western blotting

To validate the accuracy of the two omics datasets, we selected 12 up-regulated DEGs, designed primers and used qPCR to assess alterations in gene expression between the control and allergic groups, as shown in Fig. 6. Additionally, 7 proteins were selected for Western Blot validation, as depicted in Fig. 7. The results showed that except for

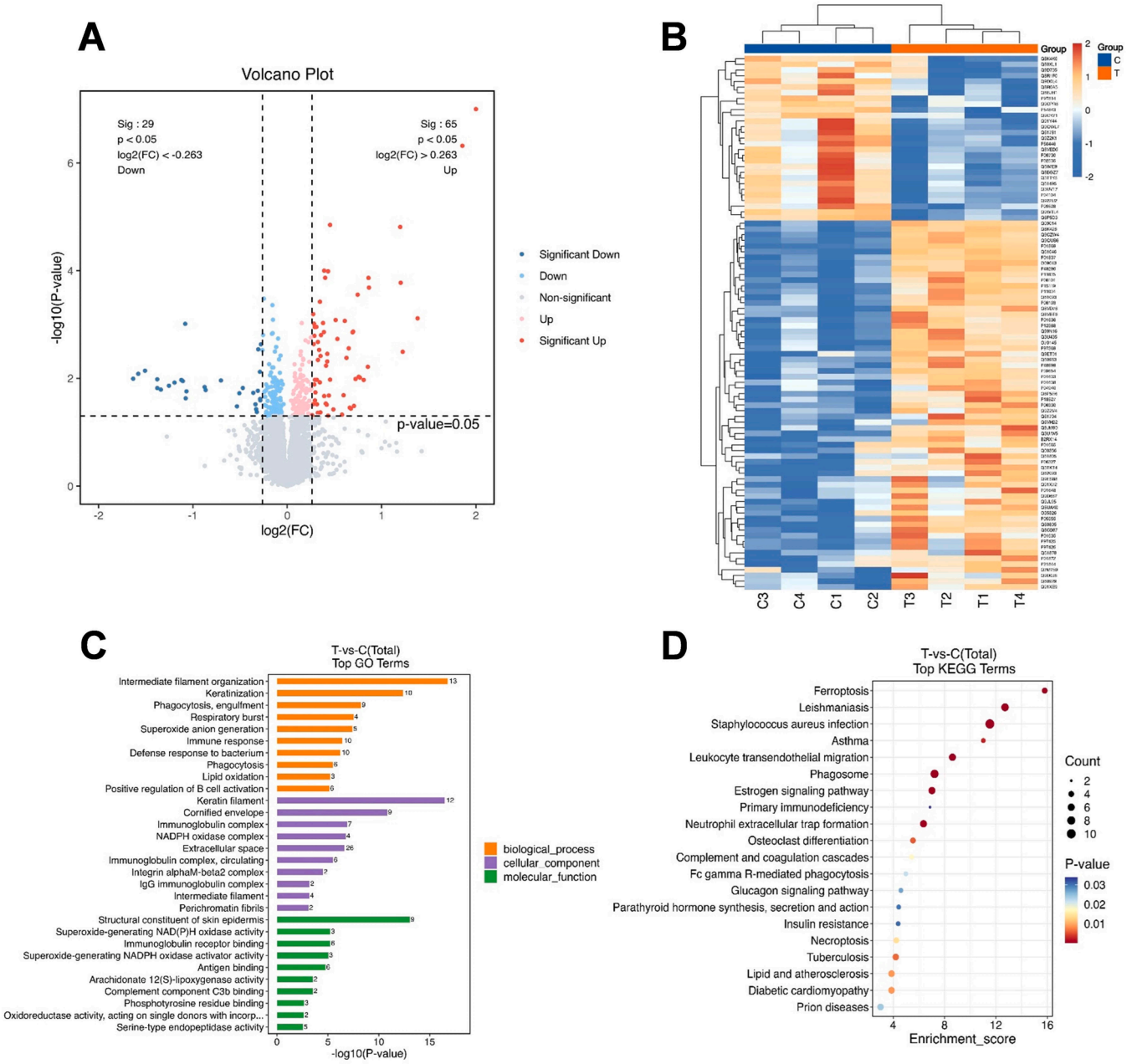

Fig. 4. Proteomic analysis of differentially expressed proteins (DEPs) in the jejunum of BLG-sensitized mice. (A-B) Volcano plots and heat maps showing DEPs between the two groups. (C) GO enrichment analysis top30 histogram of DEPs between the two groups. (D) KEGG enrichment analysis top20 bubble plot of DEPs between two groups. n = 4 mice/group;

KLKB1, which had no obvious difference in proteomics and Western Blot had significant differences, the gene expression trends of other genes were basically consistent with the omics sequencing results, except that there were certain differences in gene expression amplitude, which indicated that the results of histological sequencing were credible.

4. Discussion

CMPA is mainly caused by the intestinal barrier misidentifying milk proteins as invaders of the human body and failing to establish normal immune tolerance, thus inducing allergic reactions [27,28]. The intestinal barrier comprises elements such as intestinal epithelial cells, the immune system, and the gut microbiota. The intricate interplay of these components makes the specific pathogenic mechanisms of CMPA still unclear. Transcriptomics can comprehensively study the overall expression of genes in specific tissues of the organism at a specific period [29]; while proteomics can provide complete expression information of proteins in tissues and their dynamic changes [30]. The combination of the two can provide an in-depth understanding of the intrinsic connection between genes and proteins, revealing the complex pathophysiological mechanisms of diseases from a holistic aspect and breaking the limitations of traditional disease research. Currently, there are few omics studies on the intrinsic mechanism of BLG-induced CMPA leading to intestinal barrier dysfunction. Therefore, this study employed an integrated approach combining transcriptomics and proteomics to explore the pathogenic mechanisms of CMPA.

When CMPA occurs, the differentiation of initial T cells in the body tends to shift towards Th2 cells, promoting the secretion of type 2 cytokines IL-4, IL-5, and IL-13. Simultaneously, a substantial number of effector cells, including eosinophils and mast cells, are recruited,

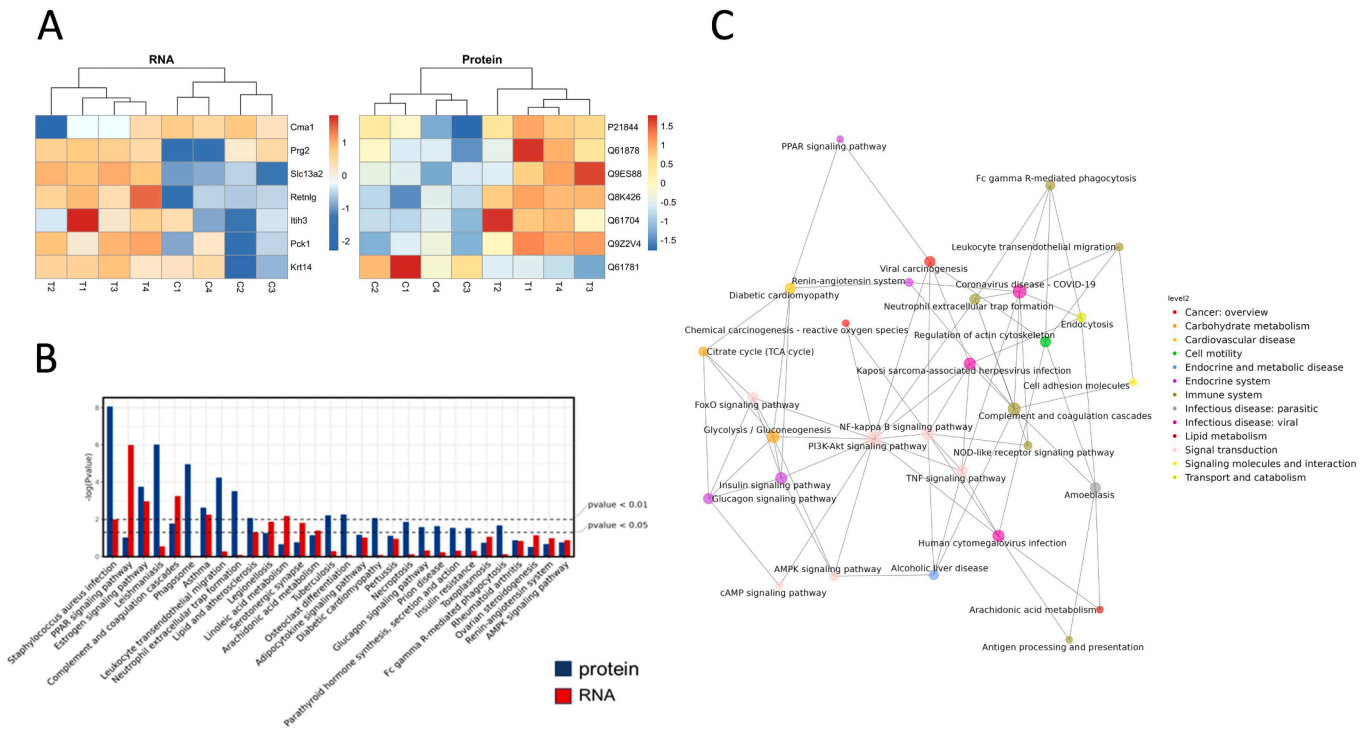

Fig. 5. Joint analysis. (A) Sample clustering heatmap. (B) Differentials co-mapping pathway histogram. (C) KGML interaction network graph: node names are pathway names and node size indicates connectivity size. n = 4 mice/group.

releasing various pro-inflammatory mediators such as histamine and leukotrienes [1,21,31,32]. Among them, IL-4 can regulate T and B lymphocytes, activate eosinophils, and induce IgE production by B cells, causing an imbalance in Th1/Th2 responses, and aggravating allergic reactions [33]. Targeting Th2 has become an effective method for treating allergies. At the same time, immune organs are also susceptible to allergic diseases, and their organ indices can roughly evaluate the allergic status of the body. Since this study investigated the mechanism of intestinal immune injury during the occurrence of CMPA, in order to avoid adjuvant-triggered intestinal immune responses, we induced CMPA production in mice by the intraperitoneal route. In the oral challenge experiment, mice in the BLG group exhibited reduced activity, increased respiratory rate, and even seizures, with no response to stimuli. At the same time, compared with the control group, the BLG group showed a significant increase in serum IgE, histamine, and type 2 cytokines levels. Additionally, the immune organ indices (thymus and spleen) also demonstrated a notable elevation. In addition, the pathology of jejunum and ileum in BLG group mice showed significant swelling and detachment of intestinal mucosal villi and infiltration of inflammatory cells, which also suggested a significant inflammatory response in the small intestine. All of the above experiments indicated that this CMPA model construction was successful.

In this study, a total of 475 DEGs and 94 DEPs were discovered by transcriptomics and proteomics. We focused on investigating the signaling pathways and potential targets associated with the pathological processes of CMPA. The expression levels of selected candidates, such as KLKB1, KNG, PCK1, SLC13A2, SGK1, CYP27A1, FKBP5, TSC22D3, PDK4, and ITGAM, were validated through qPCR or western blotting. By comparing the KEGG results of DEGs and DEPs, we observed that the key signaling pathways of both groups focus on signaling pathways such as complement and coagulation cascade, asthma, estrogen signaling pathway, and Staphylococcus aureus infection. Our further in-depth analysis found that DEGs and DEPs exhibited significant enrichment in the complement and coagulation cascade pathways. Thus, this pathway appears to play a crucial role in the immune mechanism of CMPA.

The complement and coagulation systems are important components of the body's enzymatic cascade reactions. The complement system serves as a vital link connecting innate and adaptive immunity [34], while the coagulation system can convert soluble fibrinogen into insoluble fibrin through a series of protease cascade reactions [35]. The complement and coagulation systems collectively constitute the primary defense mechanism against the intrusion of foreign allergens and are pivotal in the context of food allergy. The complement system is a multi-regulatory factor-controlled cascade system, with receptors for various complement active mediators expressed on various allergy effector cells such as mast cells, macrophages, and eosinophils [36,37]. C3 assumes a central position within the complement system, engaging in diverse pathways such as the classical, alternative, and lectin pathways. When food allergy occurs, C3 can recognize and label exogenous allergens, activate and release biologically active mediators with immunomodulatory effects, and take part in the immune response to allergens in the body. Studies have found that under the stimulation of immune complexes formed by allergens, C3b, the hydrolyzate of C3, and its downstream factors can promote the body's recognition and elimination of immune complexes and reduce allergic reactions. In the study of wheat allergy, it has been observed that wheat allergy can downregulate the expression of CFHR3 through the complement and coagulation cascade pathways, inhibiting the functionality of complement C3b [22]. This inhibition prevents the clearance of immune complexes by the body, thereby exacerbating allergic reactions. Simultaneously, it has also been found that C3a and C5a produced by the degradation of C3 and C5 can interact with cell surface receptors such as mast cells and dendritic cells of the lamina propria, promoting the migration, adhesion and degranulation of mast cells, and releasing Inflammatory mediators such as histamine has pro-inflammatory effects, aggravate allergic reactions, and show a positive correlation with the severity of allergic responses [23,38,39]; it can be seen that complement and coagulation cascade pathways are crucial in the initiation and progression of food allergy.

Further analysis of DEGs and DEPs in the complement and coagulation cascade pathways revealed that KLKB1 may be a key gene in the occurrence and development of CMPA. KLKB1 is a serine protease

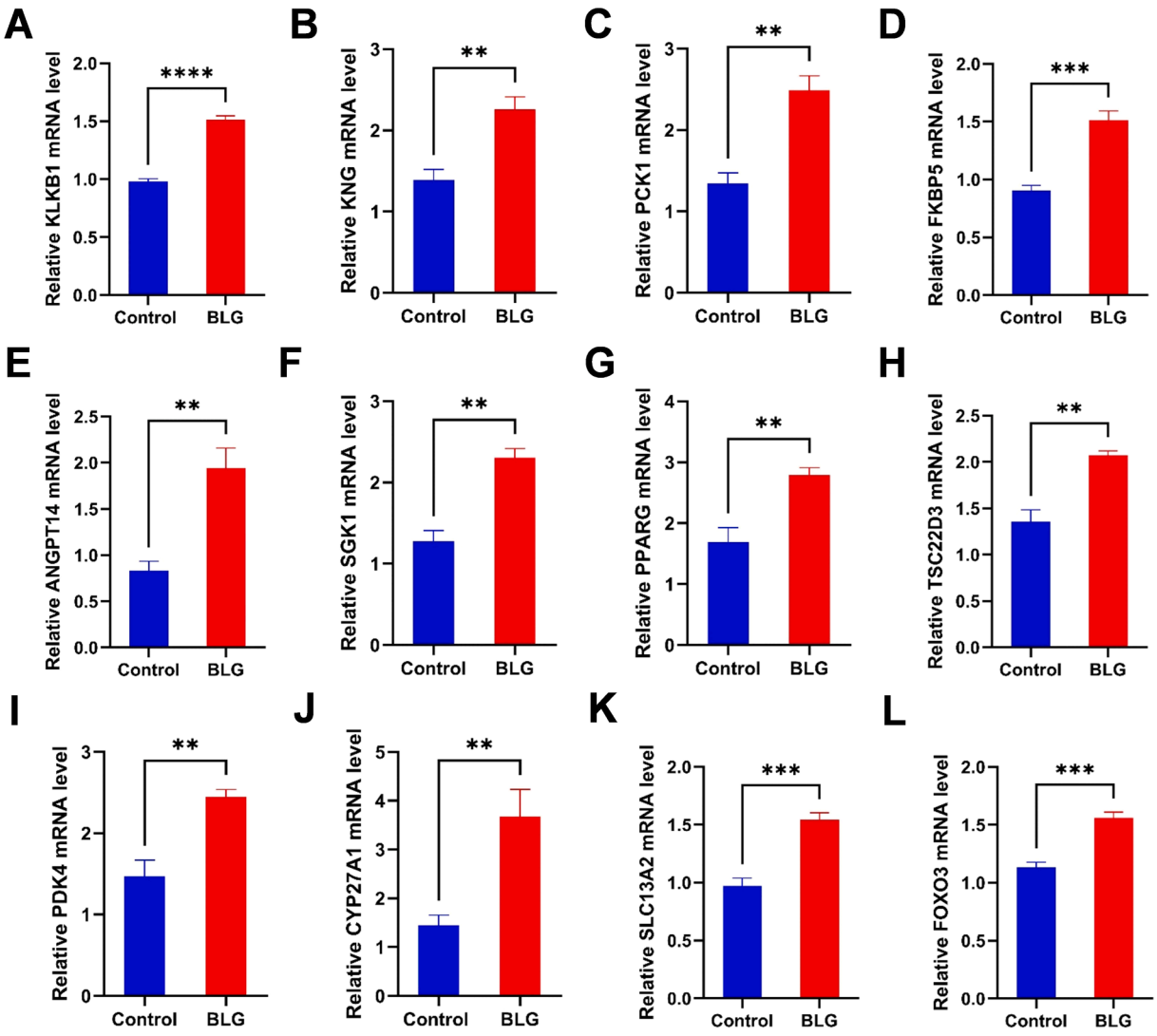

Fig. 6. qPCR verification of transcriptomic sequencing results. (A-L) The mRNA expression of kallikrein B1 (KLKB1), kininogen (KNG), phosphoenolpyruvate Carboxykinase 1 (PCK1), FK506-binding protein 5 (FKBP5), Angiopoietin-like protein 4 (ANGPTl4), Serum and glucocorticoid regulated kinase 1 (SGK1), peroxisome proliferator activated receptor gammaperoxisome proliferator activated receptor gamma (PPARG), TSC22 domain family member 3 (TSC22D3), pyruvate dehydrogenase kinase 4 (PDK4), cytochrome P450 family 27 subfamily A member 1 (CYP27A1), Solute Carrier Family 13 Member 2 (SLC13A2), and Forkhead box O transcription factors 3 (FOXO3) in mouse jejunal tissue were evaluated through the qPCR. data are expressed as mean ± SEM; n = 4 mice/group; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

belonging to the kallikrein family, and it is widely distributed in blood and various tissues such as the intestine, heart, lungs, kidneys, participating in various physiological and pathological processes including fibrinolysis, coagulation cascade reactions and inflammatory responses [40–42]. Normally, the expression level of KLKB1 in intestinal tissue is low [43]. This study found through qPCR and Western blotting that compared with normal mice, the BLG group could significantly increase the gene and protein expression levels of KLKB1 in small intestinal tissue. Highly expressed KLKB1 can be activated into kallikrein, directly regulating the conversion of its downstream gene kininogen (KNG) into BK, which acts on G protein-coupled receptors (B1R, B2R) on tissues, thereby releasing inflammatory factors such as nitric oxide and leukotrienes, increasing capillary permeability, exerting a pro-inflammatory effect, and aggravating tissue damage [42,44–47]. In this study, we confirmed bradykinin protein overexpression by Western Blotting. This shows that KLKB1 may have a significant role in the progression of CMPA by promoting the conversion of KNG to BK and aggravating intestinal barrier damage through the kallikrein-kinin system.

In summary, our study suggests that the complement and coagulation cascade pathways may have a notable impact on the development of CMPA. Notably, KLKB1 is highly expressed in intestinal tissues and may be further involved in the pathogenesis of CMPA by promoting bradykinin production and exacerbating intestinal tissue damage. Therefore, KLKB1 may be a potential biomarker for CMPA, providing new insights into the treatment of CMPA.

We declare that we have no financial or personal relationships with other individuals or organizations that could inappropriately influence our work. The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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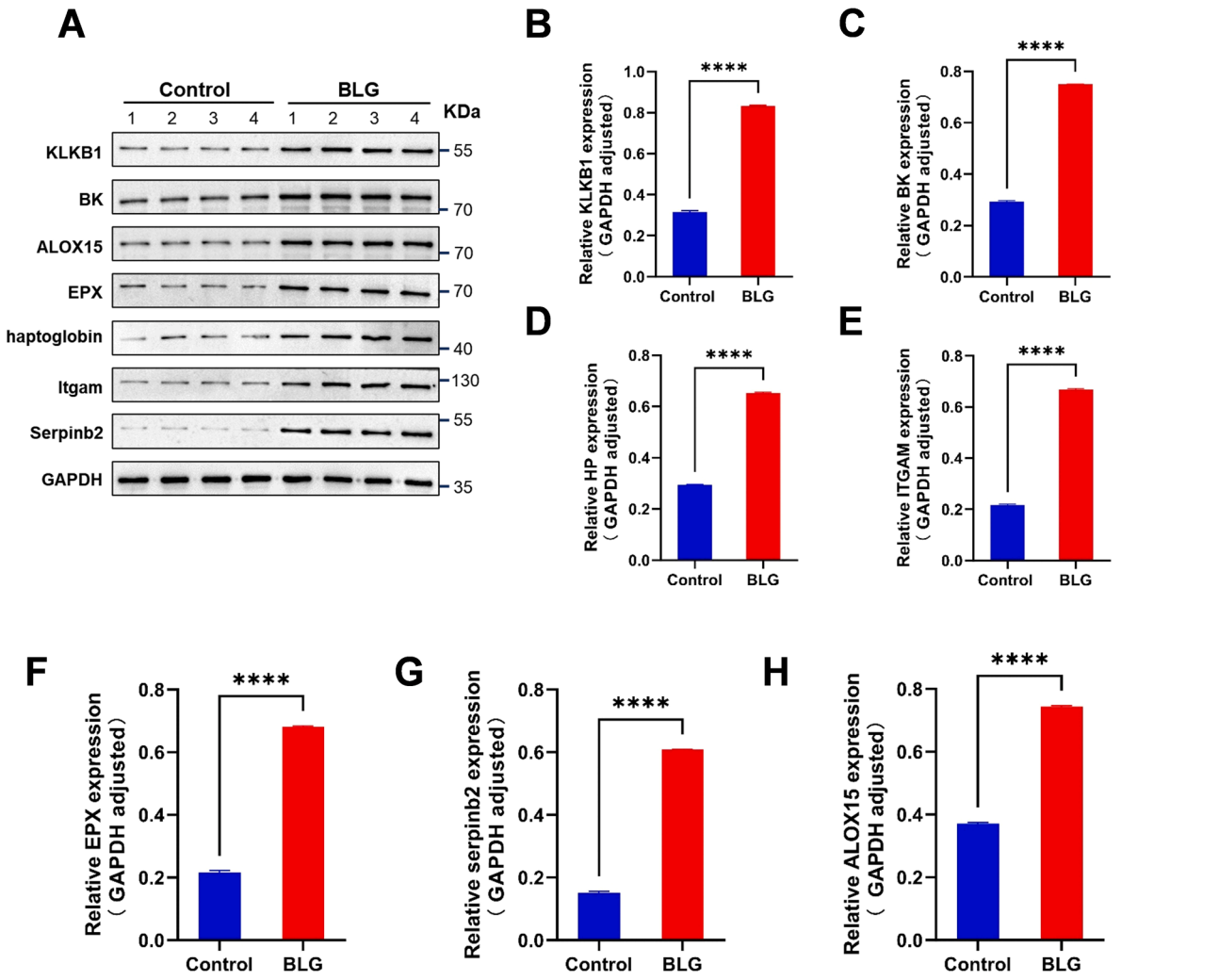

Fig. 7. Western blotting validation of proteomics sequencing results. (A) Representative Western blotting images of kallikrein B1 (KLKB1), bradykinin (BK), arachidonate 15-lipoxygenase (ALOX15), eosinophil peroxidase (EPX), haptoglobin (HP), Integrin Subunit Alpha M (Itgam), and serpin family B member 2 (serpinb2) in jejunal tissue; GAPDH was used as the internal standard; (B-H) Image J software was used to quantitatively analyze KLKB1, BK, ALOX15, EPX, HP, Itgam, and serpinb2 in the jejunal tissues of two groups. Data are expressed as mean ± SEM; n = 4 mice/group; *** $p < 0.001$; **** $p < 0.0001$.

6. Author disclosure statement

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

7. Ethics approval and consent to participate

This research protocol was approved by the Experimental Animal Management Committee of the Artificial Intelligence Research Institute of Hefei Comprehensive National Science Center (No. 2023-N(A)-017).

CRediT authorship contribution statement

Qunchao Li: Writing – review & editing, Writing – original draft, Visualization, Methodology. **Yan Deng:** Writing – review & editing, Visualization, Methodology. **Zhiwei Xu:** Validation, Investigation, Data curation. **Haoquan Zhou:** Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Data availability

Data will be made available on request.

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